Post-Graduate Degree Programme (CBCS)

in

ZOOLOGY

SEMESTER-III

ELECTIVE THEORY-I
CELL AND DEVELOPMENTAL BIOLOGY
ZET-301

SELF LEARNING MATERIAL

DIRECTORATE OFOPEN AND DISTANCE LEARNING
UNIVERSITY OF KALYANI

Kalyani, Nadia
West Bengal, India

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Director's Message

Satisfying the varied needs of distance learners, overcoming the obstacle of distance and reaching the unreached students are the threefold functions catered by Open and Distance Learning (ODL) systems. The onus lies on writers, editors, production professionals and other personnel involved in the process to overcome the challenges inherent to curriculum design and production of relevant Self Learning Materials (SLMs). At the University of Kalyani a dedicated team under the able guidance of the Hon'ble Vice-Chancellor has invested its best efforts, professionally and in keeping with the demands of Post Graduate CBCS Programmes in Distance Mode to devise a self-sufficient curriculum for each course offered by the Directorate of Open and Distance Learning (DODL), University of Kalyani.

Development of printed SLMs for students admitted to the DODL within a limited time to cater to the academic requirements of the Course as per standards set by Distance Education Bureau of the University Grants Commission, New Delhi, India under Open and Distance Mode UGC Regulations, 2017 had been our endeavour. We are happy to have achieved our goal.

Utmost care and precision have been ensured in the development of the SLMs, making them useful to the learners, besides avoiding errors as far as practicable. Further suggestions from the stakeholders in this would be welcome.

During the production-process of the SLMs, the team continuously received positive stimulations and feedback from Professor (Dr.) Sankar Kumar Ghosh, Hon'ble Vice- Chancellor, University of Kalyani, who kindly accorded directions, encouragements and suggestions, offered constructive criticism to develop it within proper requirements. We gracefully, acknowledge his inspiration and guidance.

Sincere gratitude is due to the respective chairpersons as well as each and every member of PGBOS (DODL), University of Kalyani. Heartfelt thanks is also due to the Course Writers-faculty members at the DODL, subject-experts serving at University Post Graduate departments and also to the authors and academicians whose academic contributions have enriched the SLMs. We humbly acknowledge their valuable academic contributions. I would especially like to convey gratitude to all other University dignitaries and personnel involved either at the conceptual or operational level of the DODL of University of Kalyani.

Their persistent and co-ordinated efforts have resulted in the compilation of comprehensive, learner-friendly, flexible texts that meet the curriculum requirements of the Post Graduate Programme through Distance Mode.

All the Self Learning Materials are self writing and collected from e-book, journals and websites.

Prof Manas Mohan Adhikary
Director
Directorate of Open and Distance Learning
University of Kalyani

ELECTIVE THEORY I

CELL AND DEVELOPMENTAL BIOLOGY (ZET 301)

Module	Unit	Content	Credit	Class	Time (h)	Page No.
ZET - 301 (Cell and Developmental Biology)	**I**	Separation and identification of materials i) Chromatography: Gel chromatography, Ion - exchange Chromatography, Affinity chromatography, High-performance liquid chromatography.	**2**	**1**	**1**	
	II	Electrophoresis: PAGE, SDS-PAGE, Agarose Gel Electrophoresis of double stranded DNA, Isoelectric Focusing, Immunoeleptrophoresis.		**1**	**1**	
	III	Spectroscopic methods : Colorimetry, Spectrophotometry, Atomic Absorption Spectrophotometry		**1**	**1**	
	IV	Sedimentation : Instrument for Ultra centrifugation, Zonal Centrifugation through Density Gradients		**1**	**1**	
	V	Direct observation: i) Light microscopy, Phase contrast microscopy, Interference Microscopy Polarization microscopy, Fluorescence Microscopy ii) Electron microscopy: i) Transmission ii) Scanning		**1**	**1**	
	VI	Fixation & staining: i) Solutions : Definition, Composition, Expression, Ideal & non-ideal Solution ii) Chemical & physical effects of some primary fixatives: Formalin, alcohol, picric acid, acetic acid		**1**	**1**	
	VII	Source and chemical composition of some dyes: Basic fuchsin, carmine, hematin, eosin		**1**	**1**	
	VIII	Special application: Finger printing, Southern, Northern & Western transfers		**1**	**1**	

Unit I

Separation and identification of materials i) Chromatography: Gel chromatography, Ion - exchange Chromatography, Affinity chromatography, High-performance liquid chromatography.

Objective: In this unit, you will learn about the Separation and identification of materials i) Chromatography: Gel chromatography, Ion - exchange Chromatography, Affinity chromatography, High-performance liquid chromatography.

Principle of Chromatography

PRINCIPLES OF CHROMATOGRAPHY

Distribution coefficients

The basis of all forms of chromatography is the distribution or partition coefficient (K_d), which describes the way in which a compound (the analyte) distributes between two immiscible phases. For two such phases A and B, the value for this coefficient is a constant at a given temperature and is given by the expression:

$$\frac{\text{concentration in phase A}}{\text{concentration in phase B}} = K_d \qquad (11.1)$$

Distribution coefficients

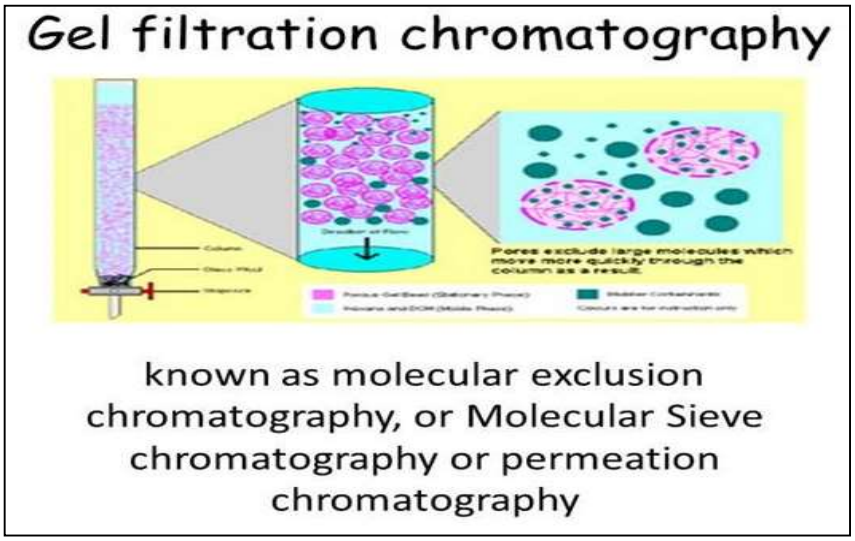
The basis of all forms of chromatography is the distribution or partition coefficient Kd), which describes the way in which a compound (the analyte) distributes between two immiscible phases. For two such phases A and B, the value for this coefficient is a constant at a given temperature and is given by the expression: concentration in phase A concentration in phase B ¼ Kd ð11:1Þ The term effective distribution. coefficient is defined as the total amount, as distinct from the concentration, of analyte present in one phase divided by the total amount present in the other phase. It is in fact the distribution coefficient multiplied by the ratio of the volumes of the two phases present. If the distribution coefficient of an analyte between two phases A and B is 1, and if this analyte is distributed between 10 cm3 of A and 1 cm3 of B, the concentration in the two phases will be the same, but the total amount of the analyte in phase A will be 10 times the amount in phase B.

Gel chromatography/ Gel filtration chromatography

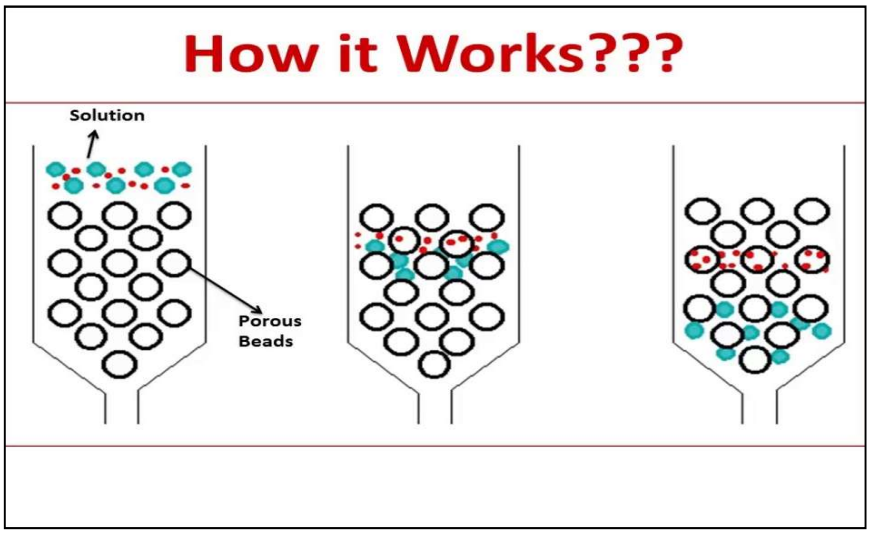
Definition

Gel chromatography, also called **Gel Filtration**, in analytical chemistry, technique for separating chemical substances by exploiting the differences in the rates at which they pass through a bed of a porous, semisolid substance.

Principle



The method is especially useful for separating enzymes, proteins, peptides, and amino acids from each other and from substances of low molecular weight. The separation of the components of a mixture by gel chromatography is based on the differences in the molecular sizes of the components. Small molecules tend to diffuse into the interior of the porous particles so that their flow is restricted, while large molecules are unable to enter the pores and tend to flow unhindered. Thus, the components of highest molecular weight leave the bed first, followed by successively smaller molecules. The bed materials most extensively used are polyacrylamide and a polymer prepared from dextran and epichlorohydrin. The dry polymers are usually suspended in suitable agents to form a homogeneous, semisolid mixture.



Method

Gel Filtration Chromatography Mechanism

In a gel filtration chromatography column, the stationary phase is composed of a porous matrix, and the mobile phase is the buffer that flows in between the matrix beads. The beads have a defined pore size range, known as the fractionation range. Molecules and complexes that are too large to enter the pores stay in the mobile phase and move through the column with the flow of the buffer. Smaller molecules and complexes that are able to move into the pores enter the stationary phase and move through the gel filtration column by a longer path through pores of the beads.

Any molecule or complex that is above the fractionation range for a particular gel filtration chromatography column will move through the column faster than any molecule that can enter the stationary phase. Therefore, any constituent in the sample that is above the fractionation range will elute first (in the void volume) before anything that is in the fractionation range. The minimum size that will remain in the mobile phase and not enter the stationary phase is known as the exclusion limit. Bio-Rad offers gel filtration chromatography media and columns with exclusion limits ranging over three orders of magnitude, from 100 Daltons to 100,000 Daltons (100 kDa).

Molecules and complexes that can enter the stationary phase will be fractionated according to their sizes. Smaller molecules will migrate deep into the pores and will be retarded more thanlarger molecules that do not so easily enter the pores, and are thus eluted from the column more quickly. This difference in pore migration leads to fractionation of components by size with the largest eluting first.

In gel filtration chromatography columns designed for desalting, buffer exchange, and the removal of small molecules such as nucleotides, the salts and small compounds readily enter the pores, are retarded, and migrate more slowly through the column than the larger proteins or nucleic acids. Therefore, the components of interest in the sample are eluted in advance of salts, nucleotides, etc. DNA cleanup kits using this mechanism often contain gel filtration spin columns.

Resolution, here defined as the sharpness of the boundaries between size fractions, is determined by bead size and a number of other factors. Smaller bead size generally yields higher resolution in a gel filtration chromatography column. Compact molecules diffuse through the stationary phase faster than linear molecules. Size exclusion, fractionation range, and elution rate are affected by buffer composition, ionic strength, and pH. For the fractionation of complex mixtures of proteins, elution times and size exclusion limits may need to be determined empirically.

Application

Gel Filtration Chromatography Applications, a type of size exclusion chromatography, can be used to either fractionate molecules and complexes in a sample into fractions with a particular size range, to remove all molecules larger than a particular size from the sample, or a combination of both operations. Gel filtration chromatography can be used to separate compounds such as small molecules, proteins, protein complexes, polysaccharides, and nucleic acids when in aqueous solution. When an organic solvent is used as the mobile phase, the process is instead referred to as gel permeation chromatography.

Gel filtration chromatography can also be used for:

- Fractionation of molecules and complexes within a predetermined size range
- Size analysis and determination
- Removal of large proteins and complexes
- Buffer exchange
- Desalting

- Removal of small molecules such as nucleotides, primers, dyes, and contaminants
- Assessment of sample purity
- Separation of bound from unbound radioisotopes

Gel filtration chromatography media for all of the above uses are available in prepacked gravity flow columns, spin columns, low-pressure and medium-pressure chromatography columns, and bottled resins.

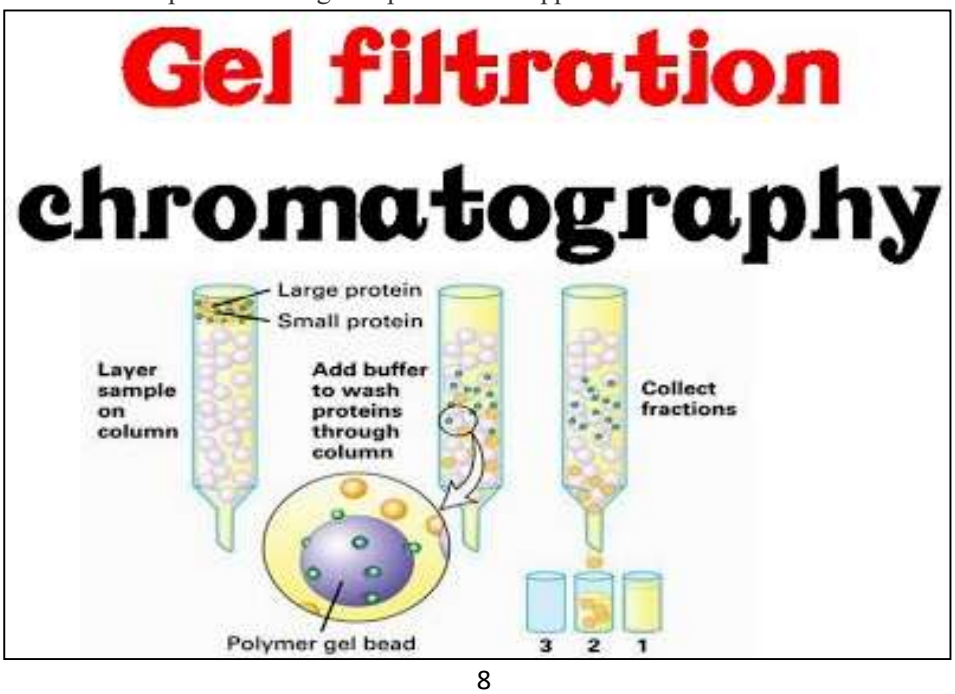
Gel Filtration Chromatography Media:

An important criterion for gel filtration chromatography media is that media is inert and that nothing in the sample or any buffer binds to the media. Another consideration is the type of gel filtration column being used and whether it is used in a pressurized chromatography system or gravity flow or spin columns. If a pressurized chromatography system is being used, both the column and the media must be able to tolerate the pressure and flow rates used.

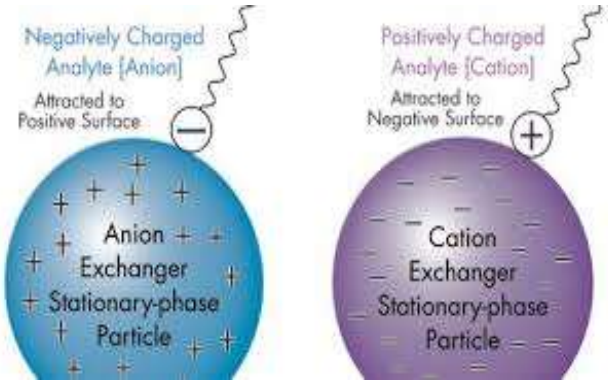
Commonly used media for gel filtration chromatography are based on agarose or polyacrylamide beads, dextrose for gravity or low-pressure systems, and polymeric resins for medium-pressure systems. The choice of media depends on the properties of the components to be separated and other experimental factors. The following are general considerations when determining the choice of gel filtration chromatography media:

- Fractionation range
- Size exclusion limit
- Operating pressure
- Flow rate
- Sample viscosity
- pH range
- Autoclavability
- Tolerance for water-miscible organic solvents; some samples may be more soluble in a water-organic mix
- Tolerance for detergents, chaotropic agents, formamide, etc.
- Operating temperature

The types of samples, choice of media, and the chromatography system setup will determine which parameters are the most important for a given purification application.



ION EXCHANGE CHROMATOGRAPHY



Definition:

Ion chromatography is a chromatography process that separates ions and polar molecules based on their affinity to the ion exchanger. It works on almost any kind of charged molecule—including large proteins, small nucleotides, and amino acids.

Metrohm 850 Ion chromatography system

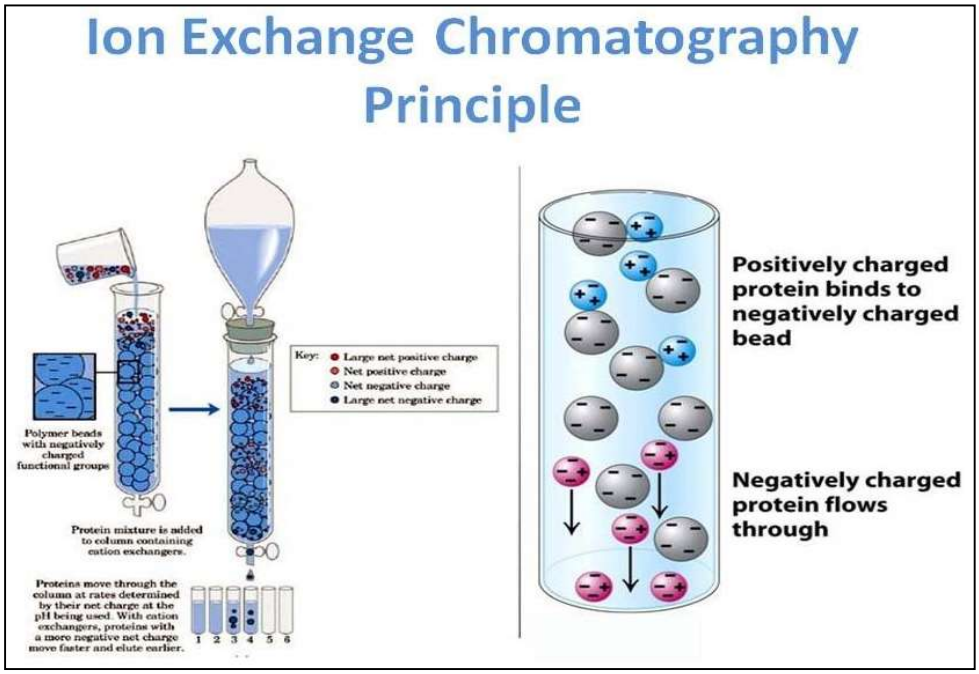
Principle:

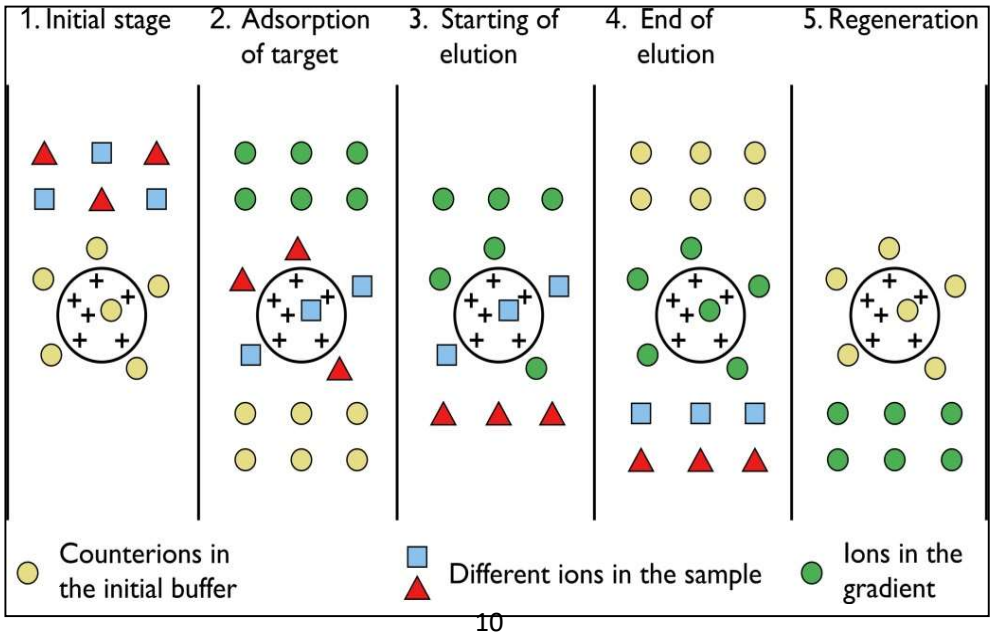
It is defined as the process of separation of the individual components of a mixture based on their relative affinities towards stationary and mobile phases. The samples are subjected to flow by mobile liquid onto or through the stable stationary phase.

It works on almost any kind of charged molecule—including large proteins, small nucleotides, and amino acids. However, ion chromatography must be done in conditions that are one unit away from the isoelectric point of a protein Exchange of ions is the basic principle in this type of Chromatography. In this process two types of exchangers i.e., cationic and anionic exchangers can be used. Cationic exchangers possess negatively charged group, and these will attract positively charged cations. These exchangers are also called "Acidic ion exchange" materials, because their negative charges result from the ionization of acidic group. The stationary phase consists of an immobile matrix that contains charged ionizable functional groups or ligands. The stationary phase surface displays ionic functional groups (R-X) that interact with analyte ions of opposite charge. To achieve electroneutrality, these inert charges couple with exchangeable counterions in the solution. Ionizable molecules that are to be purified compete with these exchangeable counterions for binding to the immobilized charges on the stationary phase. These ionizable molecules are retained or eluted based on their charge.

Procedure

A sample is introduced, either manually or with an autosampler, into a sample loop of known volume. A buffered aqueous solution known as the mobile phase carries the sample from the loop onto a column that contains some form of stationary phase material. This is typically a resin or gel matrix consisting of agarose or cellulose beads with covalently bonded charged functional groups. Equilibration of the stationary phase is needed in order to obtain the desired charge of the column. If the column is not properly equilibrated the desired molecule may not bind strongly to the column. The target analytes (anions or cations) are retained on the stationary phase but can be eluted by increasing the concentration of a similarly charged species that displaces the analyte ions from the stationary phase. For example, in cation exchange chromatography, the positively charged analyte can be displaced by adding positively charged sodium ions. The analytes of interest must then be detected by some means, typically by conductivity or UV/visible light absorbance.





Applications:

Clinical :

A use of ion chromatography can be seen in the argentation ion chromatography. Usually, silver and compounds containing acetylenic and ethylenic bonds have very weak interactions. This phenomenon has been widely tested on olefin compounds. The ion complexes the olefins make with silver ions are weak and made based on the overlapping of pi, sigma, and d orbitals and available electrons therefore cause no real changes in the double bond. This behavior was manipulated to separate lipids, mainly fatty acids from mixtures in to fractions with differing number of double bonds using silver ions. The ion resins were impregnated with silver ions, which were then exposed to various acids (silicic acid) to elute fatty acids of different characteristics.

Another clinical application of ion chromatography is in the rapid anion exchange chromatography technique used to separate creatine kinase (CK) isoenzymes from human serum and tissue sourced in autopsy material (mostly CK rich tissues were used such as cardiac muscle and brain).

Industrial applications:

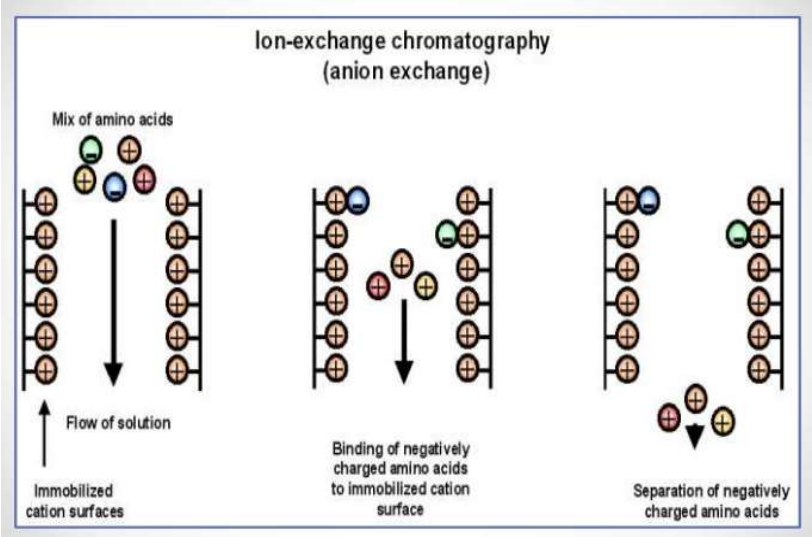
Since 1975 ion chromatography has been widely used in many branches of industry. The main beneficial advantages are reliability, very good accuracy and precision, high selectivity, high speed, high separation efficiency, and low cost of consumables.

The usage of ion exchange chromatography in pharmaceuticals has increased in recent years, and in 2006, a chapter on ion exchange chromatography was officially added to the United States Pharmacopia-National Formulary (USP-NF).

Drug development:

An ion chromatography system used to detect and measure cations such as sodium, ammonium and potassium in Expectorant Cough Formulations.
There has been a growing interest in the application of IC in the analysis of pharmaceutical drugs. IC is used in different aspects of product development and quality control testing. For example, IC is used to improve stabilities and solubility properties of pharmaceutical active drugs molecules as well as used to detect systems that have higher tolerance for organic solvents. IC has been used for the determination of analytes as a part of a dissolution test.

IC methodology also established in analysis of impurities in drug substances and products. Impurities or any components that are not part of the drug chemical entity are evaluated and they give insights about the maximum and minimum amounts of drug that should be administered in a patient per day.



AFFINITY CHROMATOGRAPHY

Definition:

Affinity chromatography is a method of separating biochemical mixtures based on a highly specific interaction between antigen and antibody, enzyme and substrate, receptor and ligand, or protein and nucleic acid.

Principle:

It is a type of chromatographic laboratory technique used for purifying biological molecules within a mixture by exploiting molecular properties. Biological macromolecules, such as enzymes and other proteins, interact with other molecules with high specificity through several different types of bonds and interaction. Such interactions including hydrogen bonding, ionic interaction, disulfide bridges, hydrophobic interaction, and more. The high selectivity of affinity chromatography is caused by allowing the desired molecule to interact with the stationary phase and be bound within the column in order to be separated from the undesired material which will not interact and elute first. The molecules no longer needed are first washed away with a buffer while the desired proteins are let go in the presence of the eluting solvent (of higher salt concentration). This process creates a competitive interaction between the desired protein and the immobilized stationary molecules, which eventually lets the now highly purified proteins be released.

Method:

Binding to the solid phase may be achieved by column chromatography whereby the solid medium is packed onto a column, the initial mixture run through the column to allow setting, a wash buffer run through the column and the elution buffer subsequently applied to the column and collected. These steps are usually done at ambient pressure. Alternatively, binding may be achieved using a batch treatment, for example, by adding the initial mixture to the solid phase in a vessel, mixing, separating the solid phase, removing the liquid phase, washing, re-centrifuging, adding the elution buffer, re-centrifuging and removing the elute.

Sometimes a hybrid method is employed such that the binding is done by the batch method, but the solid phase with the target molecule bound is packed onto a column and washing and elution are done on the column. The ligands used in affinity chromatography are obtained from both organic and inorganic sources. Examples of biological sources are serum proteins, lectins and antibodies. Inorganic sources as moronic acts, metal chelates and triazine dyes. A third method, expanded bed absorption, which combines the advantages of the two methods mentioned above, has also been developed. The solid phase particles are placed in a column where liquid phase is pumped in from the bottom and exits at the top. The gravity of the particles ensures that the solid phase does not exit the column with the liquid phase.
Affinity columns can be eluted by changing salt concentrations, pH, pI, charge and ionic strength directly or through a gradient to resolve the particles of interest.

More recently, setups employing more than one column in series have been developed. The advantage compared to single column setups is that the resin material can be fully loaded, since non-binding product is directly passed on to a consecutive column with fresh column material. These chromatographic processes are known as periodic counter-current chromatography (PCC). The resin costs per amount of produced product can thus be drastically reduced. Since one column can always be eluted and regenerated while the other column is loaded, already two columns are sufficient to make full use of the advantages. Additional columns can give additional flexibility for elution and regeneration times, at the cost of additional equipment and resin costs.

Application

Affinity chromatography can be used in a number of applications, including nucleic acid purification, protein purification from cell free extracts, and purification from blood.By using affinity chromatography, one can separate proteins that bind a certain fragment from proteins that do not bind that specific fragment. Because this technique of purification relies on the biological properties of the protein needed, it is a useful technique and proteins can be purified many folds in one step

Various affinity media:

Many different affinity media exist for a variety of possible uses. Briefly, they are (generalized):

- Activated/Functionalized – Works as a functional spacer, support matrix, and eliminates handling of toxic reagents.
- Amino Acid – Used with a variety of serum proteins, proteins, peptides, and enzymes, as well as rRNA and dsDNA.
- Avidin Biotin – Used in the purification process of biotin/avidin and their derivatives.
- Carbohydrate Bonding – Most often used with glycoproteins or any other carbohydrate-containing substance.
- Carbohydrate – Used with lectins, glycoproteins, or any other carbohydrate metabolite protein.
- Dye Ligand – This media is nonspecific, but mimics biological substrates and proteins.

Immunoaffinity:

Another use for the procedure is the affinity purification of antibodies from blood serum. If the serum is known to contain antibodies against a specific antigen (for example if the serum comes from an organism immunized against the antigen concerned) then it can be used for the affinity purification of that antigen. This is also known as Immunoaffinity Chromatography. For example, if an organism is immunised against a GST-fusion protein it will produce antibodies against the fusion-protein, and possibly antibodies against the GST tag as well. The protein can then be covalently coupled to a solid support such as agarose and used as an affinity ligand in purifications of antibody from immune serum

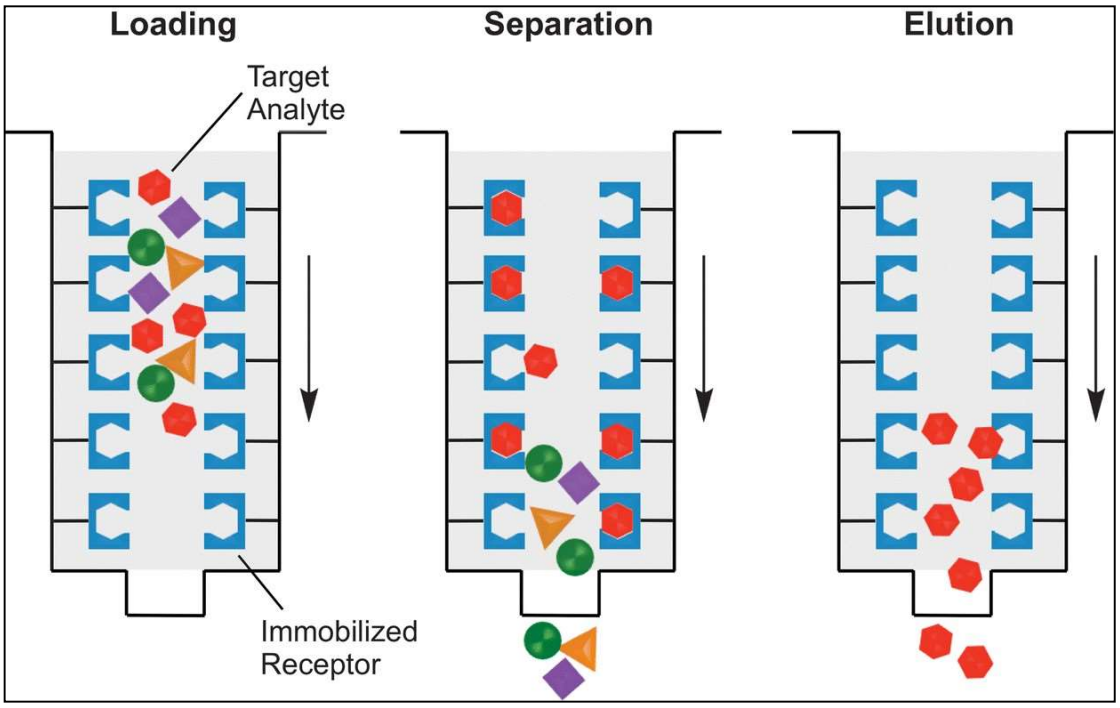
Immobilized metal ion affinity chromatography

Immobilized metal ion affinity chromatography (IMAC) is based on the specific coordinate covalent bond of amino acids, particularly histidine, to metals. This technique works by allowing proteins with an affinity for metal ions to be retained in a column containing immobilized metal ions, such as cobalt, nickel, copper for the purification of histidine-containing proteins or peptides, iron, zinc or gallium for the purification of phosphorylated proteins or peptides. Many naturally occurring proteins do not have an affinity for metal ions, therefore recombinant DNA technology can be used to introduce such a protein tag into the relevant gene. Methods used to elute the protein of interest include changing the pH, or adding a competitive molecule, such as imidazole

Recombinant proteins
Possibly the most common use of affinity chromatography is for the purification of recombinant proteins. Proteins with a known affinity are protein tagged in order to aid their purification.

Specialty
Another use for affinity chromatography is the purification of specific proteins using a gel matrix that is unique to a specific protein. For example, the purification of E. coli β-galactosidase is accomplished by affinity chromatography using p-aminobenyl-1-thio-β-D-galactopyranosyl agarose as the affinity matrix. p-aminobenyl-1-thio-β-D-galactopyranosyl agarose is used as the affinity matrix because it contains a galactopyranosyl group, which serves as a good substrate analog for E.Coli-B-Galactosidase. This property allows the enzyme to bind to the stationary phase of the affinity matrix and is eluted by adding increasing concentrations of salt to the column.

Serum albumin purification
Of many uses of affinity chromatography, one use of it is seen in affinity purification of albumin and macroglobulin contamination. This type of purification is helpful in removing excess albumin and α_2-macroglobulin contamination, when performing mass spectrometry. In affinity purification of serum albumin, the stationary used for collecting or attracting serum proteins can be Cibacron Blue-Sepharose. Then the serum proteins can be eluted from the adsorbent with a buffer containing thiocyanate



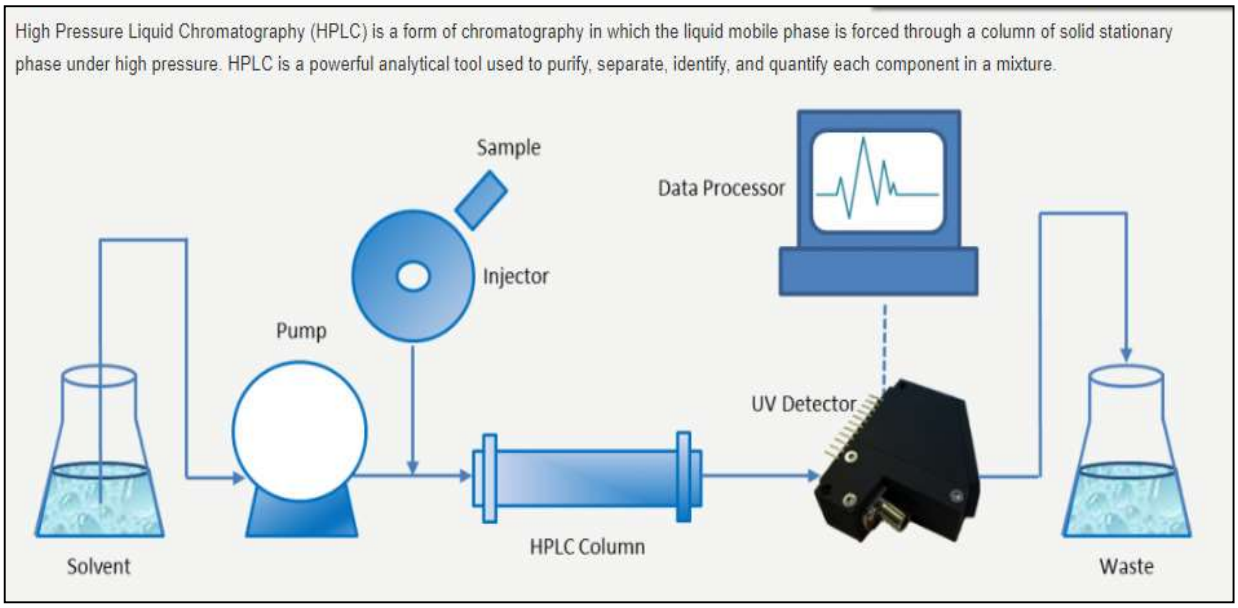
HIGH PERFIRMANCE LIQUID CHROMATOGRAPHY

Definition:

High-performance liquid chromatography (**HPLC**; formerly referred to as **high-pressure liquid chromatography**), is a technique in analytical chemistry used to separate, identify, and quantify each component in a mixture. It relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out of the column.

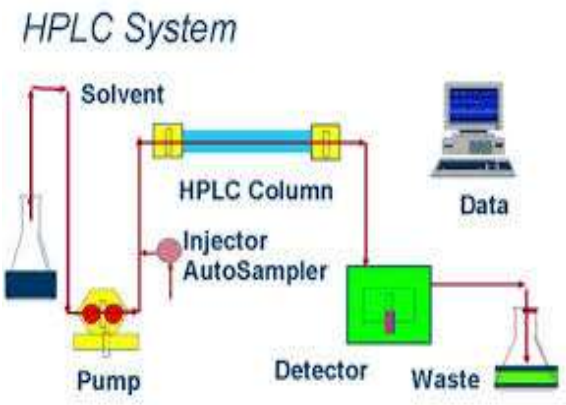
Principle:

HPLC relies on pumps to pass a pressurized liquid and a sample mixture through a column filled with adsorbent, leading to the separation of the sample components. The active component of the column, the adsorbent, is typically a granular material made of solid particles (*e.g.*, silica, polymers, etc.), 2–50 µm in size. The components of the sample mixture are separated from each other due to their different degrees of interaction with the adsorbent particles. The pressurized liquid is typically a mixture of solvents (*e.g.*, water, acetonitrile and/or methanol) and is referred to as a "mobile phase". Its composition and temperature play a major role in the separation process by influencing the interactions taking place between sample components and adsorbent. These interactions are physical in nature, such as hydrophobic.



Types

- Partition chromatography
- **Normal–phase chromatography**
- **Displacement chromatography**
- **Reversed-phase chromatography (RPC)**
- **Size-exclusion chromatography**
- **Ion-exchange chromatography**
- **Bioaffinity chromatography**
- **Aqueous normal-phase chromatography**



Applications

Manufacturing:

HPLC has many applications in both laboratory and clinical science. It is a common technique used in pharmaceutical development, as it is a dependable way to obtain and ensure product purity. While HPLC can produce extremely high quality (pure) products, it is not always the primary method used in the production of bulk drug materials.

Legal:
This technique is also used for detection of illicit drugs in urine. The most common method of drug detection is an immunoassay. This method is much more convenient. However, convenience comes at the cost of specificity and coverage of a wide range of drugs.

Research:
Similar assays can be performed for research purposes, detecting concentrations of potential clinical candidates like anti-fungal and asthma drugs

Medical
Medical use of HPLC can include drug analysis, but falls more closely under the category of nutrient analysis.

Methods:
Sample carried by a moving gas stream of Helium or Nitrogen. High PerformanceLiquid Chromatography (HPLC) is a form of column chromatography that pumps a sample mixture or analyte in a solvent (known as the mobile phase) at high pressure through a column with chromatographic packing material (stationary phase).
For all modes, a high-powered pump moves the sample and the mobile phase through the column. A typical run can take between 10-60 minutes.
The UV, VIS, and PDA detectors are categorized as absorbance detectors. They provide good sensitivity for light-absorbing compounds at ~pg level. They are easy to operate and provide good stability. UV detector is a very commonly used detector for HPLC analysis.

Complete Step by Step HPLC guide

Materials
HPLCauto sampler *vials*
I only use auto samplers since manual injection is tedious 🙂
Centrifugal filters *with 0.2 um pores*
To clean up samples
Eppendorf vials
For centrifuging
HPLC machine

Methods
In a typical HPLC procedure you can decide the following variables:

Flow rate
With fast flow peaks come out sooner but there's they're harder to resolve and tend to blend together. For more resolution, run slower.

Pressure
Affected by flow rate and solvent

Solvent Buffers
Determines signal intensity, how quickly the peaks come out, signal fidelity

Column Type
Determines the type of interaction with the sample

Detection Parameters
If using UV or FLD, you need to set the right excitation/emission wavelengths
Since HPLC is a very machine-variable technique, I can only provide general guidelines.

For sample preparation:

1. Dissolve your biopolymers or small molecules in a suitable solvent such as methanol
2. Centrifuge at 10,000 rcf in an eppendorf vial and keep the supernatant to remove any large particular matter
3. With a centrifugal filter, add 500 ul of your sample solution onto the top
4. Centrifuge at 10,000 rcf and collect the filtrate (the solution that successfully passes through the filter)
5. Load this sample into an HPLC vial

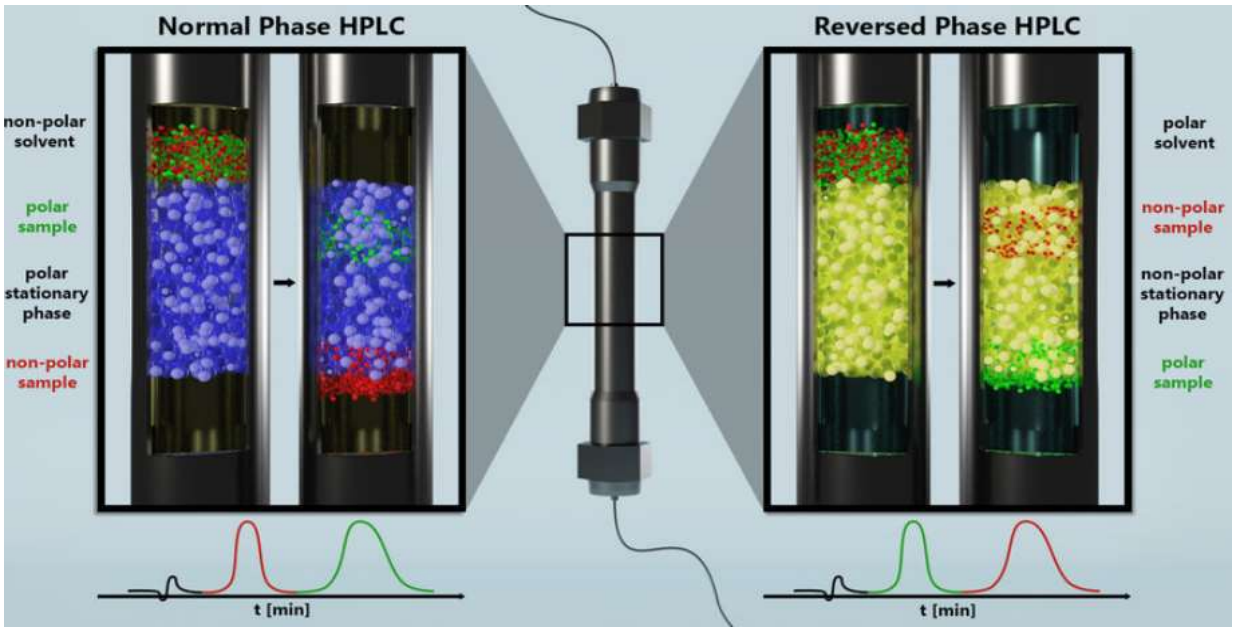
For setting up the HPLC machine:

1. Make sure you have all your buffers set up
2. Open the purge valve and purge the system for 5 minutes.
3. Add your samples into the auto sampler tray
4. Stop the purge
5. Close the purge valve
6. Run the system at a normal flow rate (1 ml/min) with your buffer to equilibrate the column for 10 minutes
7. Make sure that your pressure is stable (ie, less than 2-3 bar of fluctuation)

8. Set up your sequence and your method
9. Run a standard before your actual samples or as part of the same sequence

Example buffer system to determine Fluoresceinamine levels in samples

Sample: Add 10 ug of fluoresceinamine into 1 ml of Acetonitrile.
Buffer: Pure acetonitrile buffer on a C-18 column; this is "reverse phase".
Flow rate: 1 ml/min.
Column: 4.6 mm x 30 cm size.
Detection: Detect via a fluorescence detector set to Excitation @ 485 nm and Emission@535nm



Probable questions:

1. Define chromatography? What is the basic principle of chromatography?
2. What do you mean by gel filtration chromatography? What is HPLC?
3. Differentiate between ion exchange chromatography and gel filtration chromatography?

Suggested readings:

Wilson and Walker: Principle and technique of biochemistry and molecular biology, 7th Edition.

Unit- II

Electrophoresis: PAGE, SDS-PAGE, Agarose Gel Electrophoresis of double stranded DNA, Isoelectric Focusing, Immunoeleptrophoresis

Objective: In this unit, you will learn aboutElectrophoresis: PAGE, SDS-PAGE, Agarose Gel Electrophoresis of double stranded DNA, Isoelectric Focusing, Immunoeleptrophoresis.

1. ii) AGAROSE GEL ELECTROPHORESIS

Principles of nucleic acid separation by agarose gel electrophoresis:
Nucleic acid molecules are size separated by the aid of an electric field where negatively charged molecules migrate toward anode (positive) pole. The migration flow is determined solely by the molecular weight where small weight molecules migrate faster than larger ones (Sambrook & Russel 2001). In addition to size separation, nucleic acid fractionation using agarose gel electrophoresis can be an initial step for further purification of a band of interest. Extension of the technique includes excising the desired "band" from a stained gel viewed with a UV transilluminator (Sharp et al., 1973).

Agarose Gel Electrophoresis Uses:

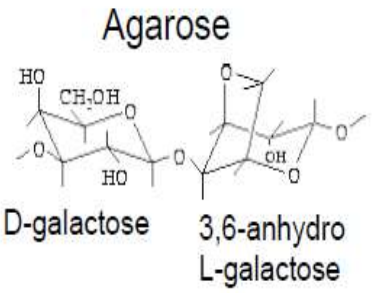
- Estimate the size of DNA molecules
- Analyse PCR products, e.g. in molecular diagnosis or genotyping
- Determine the quality or quantity of DNA
- Purification of DNA

The agarose gel electrophoresis protocol can be divided into three stages:
1. A gel with a DNA dye is prepared with an agarose concentration appropriate for the size of DNA fragments to be separated.
2. The DNA samples are loaded into the sample wells of the gel, and the gel is run at a voltage and time that will achieve optimal separation.
3. The gel is visualized and the image is recorded.

Electrophoresis buffer

Various buffers are used for agarose electrophoresis. The two most common buffers for nucleic acids are Tris/Acetate/EDTA (TAE) and Tris/Borate/EDTA (TBE). DNA fragments migrate with different rates in these two buffers due to differences in ionic strength. Buffers not only establish an ideal pH, but provide ions to support conductivity. In general, the **ideal buffer should produce less heat, have a long life and a good conductivity.**

Agarose concentration
Agarose gel electrophoresis can be used for the separation of DNA fragments ranging from 50 base pair to several mega bases (Mb) using specialized apparatus. In the gel, the distance between DNA bands of a given length is determined by the percent agarose. Higher **concentrations have the disadvantage of long run times.**

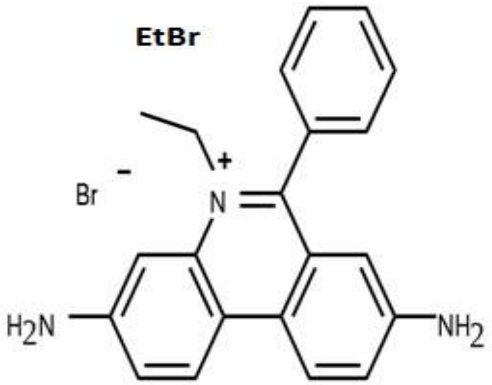


Voltage

Migration of fragments in an agarose gel depends on the difference in electric current. Different optimal voltages are required for different fragment sizes. For instance, the best resolution for fragments larger than 2 kb could be obtained by applying no more than 5 **volts per cm to the gel**

Visualization

Ethidium bromide (EtBr) is the common dye for nucleic acid visualization. The early protocol that describes the usage of Ethidium bromide (2,7-diamino-10-ethyl-9-phenylphenanthridiniumbromide-) for staining DNA and RNA in agarose gels. Although the with a lower efficiency compare to the double- stranded DNA, EtBr is also used to stain single- stranded DNA or RNA. Under UV illumination, the maximum excitation and fluorescence emission of EtBr can be obtained from 500- 590 nm. Exposing DNA to UV fluorescence should be performed rapidly because nucleic acids degrade by long exposures and thus, the sharpness of the bands would be negatively affected.



Loading buffer:

Since EtBr stained DNA is not visible in natural light, negatively charged loading buffers are commonly added to DNA prior to loading to the gel. Loading buffers are particularly useful because they are visible in natural light and they co-sediment with DNA. Xylene cyanol and Bromophenol blue are the two common dyes used as loading buffers and they run about the same speed as DNA fragments that are 5000 bp and 300 bp respectively. The other less frequently used progress markers are Cresol Red and Orange G which run at about 125 bp and 50 bp, respectively.

Effect of ethidium bromide

Ethidium bromide is a fluorescent dye and it intercalates between nucleic acids bases and provides opportunity to easily detect nucleic acid fragments in gels. The gel subsequently is being illuminated with an ultraviolet lamp usually by placing it on a light box. An apparatus integrated with the illumination system is used to take images of the gel with the presence of UV illumination. The gel can be subsequently photographed usually with a digital camera and images are usually shown in black and white, despite the fact that the stained nucleic acid fluoresces reddish-orange.

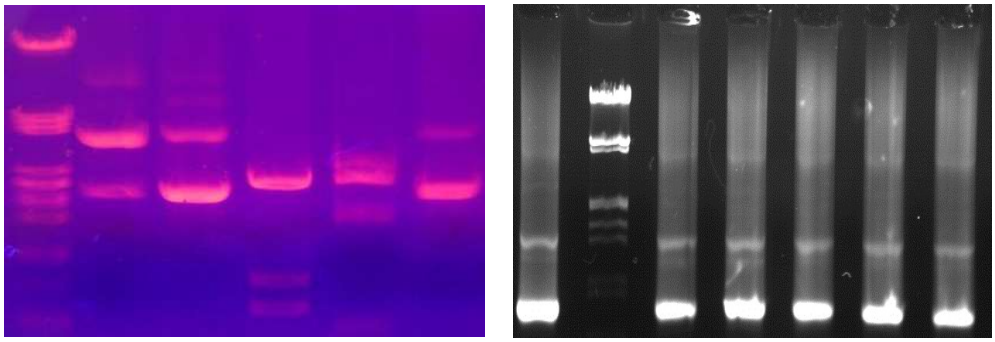


Fig: Gel electrophoresis based image analysis**.** Agarose gels, stained by Ethidium bromide and seen under UV lamp and another gel photographed in black and white

Materials

Nucleic Acids and Oligonucleotides; DNA samples**,** DNA size standards and PCR product

Buffers and Solutions; Agarose solutions, Electrophoresis buffer**,** DNA staining solution and 6x Gel-loading buffer

DNA Staining Solution; Ethidium bromide (10 mg/ml) or SYBR Green.

Ethidium Bromide: Add 1 g of ethidium bromide to 100 ml of H2O. Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved. Wrap the container in aluminum foil or transfer the %1 (10 mg/ml) solution to a dark bottle and store at room **temperature. Ethidium bromide is a powerful mutagen and toxic.**

Electrophoresis Buffer; TAE, TPE and TBE **TAE;**

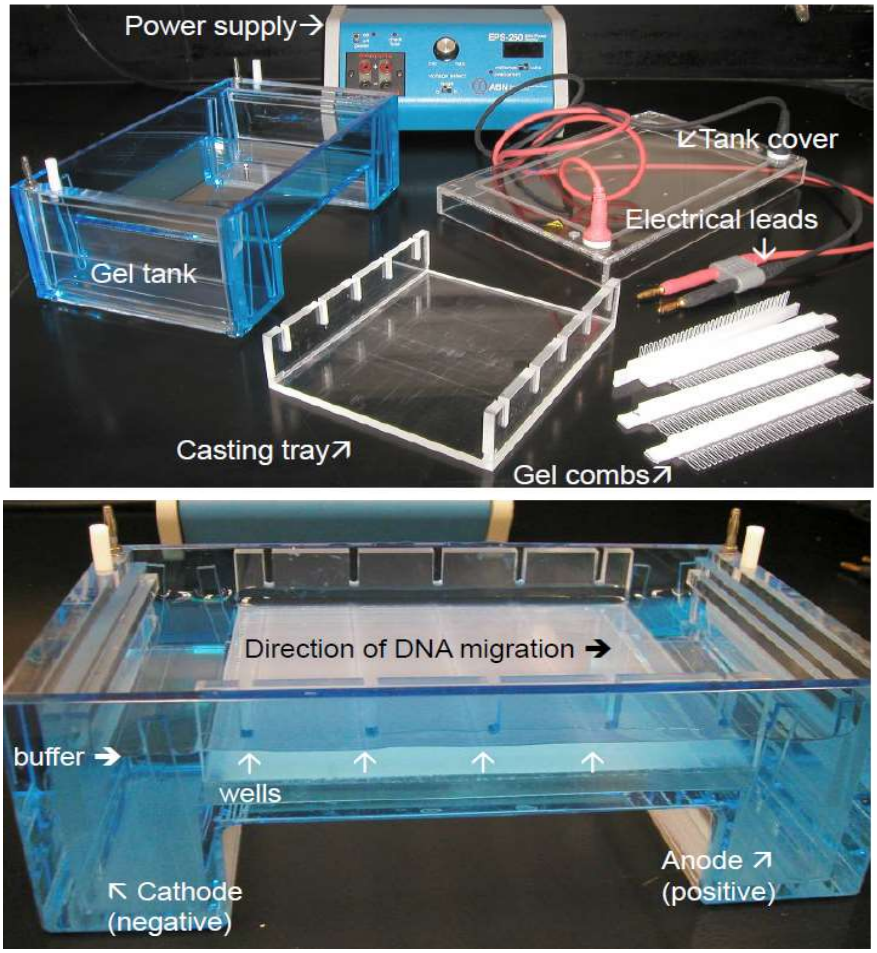
- 10x stock solution was prepared in 1 liter of H_2O
- 48.4 g Tris base [tris(hydroxymethyl)aminomethane]
- 11.4 ml glacial acetic acid (17.4 M)
- 20 ml of 0.5 M EDTA or 3.7 g EDTA, disodium salt.
- All the above were dissolved in 800 ml deionized water and mass up to 1 liter, stored in room temperature and the solution was diluted to 1X prior to use [100 ml (10 x stock) up to 1 liter deionized **water].**

6x Gel-loading Buffer I
0.25% (w/v) bromophenol blue
0.25% (w/v) xylene cyanol FF
40% (w/v) sucrose in H2O

Method
1. Prepare a solution of agarose in electrophoresis buffer at a concentration appropriate for separating the particular size fragments expected in the DNA sample(s).
2. If using a glass bottle, loose the cap. Heat the mixture in a boiling-water bath or a microwave oven until the agarose dissolves.
3. Use insulated gloves to transfer the flask into a water bath at 55°C. When the melted gel has cooled, add ethidium bromide to a final concentration of 0.5 μg/ml. Mix the gel solution thoroughly by gentle swirling.
4. While the agarose solution is cooling, choose an appropriate comb for forming the sample slots in the gel. Position the comb 0.5-1.0 mm above the plate so that a complete well is formed when the agarose is added to the mold.
5. Pour the warm agarose solution into the mold.
6. Allow the gel to polymerize completely (20-45 minutes at room temperature), then pour a small amount of electrophoresis buffer on the top of the gel, and carefully remove the comb. Pour off the electrophoresis buffer and carefully remove the tape. Mount the gel in the electrophoresis tank.
7. Place the gel into the electrophoresis device and enough electrophoresis buffers to cover the gel to a depth of approx. 1 mm.
8. Mix the sample by loading dye with a ration 1:5 or 1:10.
9. Slowly load the sample mixture into the slots of the submerged gel using a disposable micropipette, an automatic micropipettor, or a drawn-out Pasteur pipette or glass capillary tube. Load size standards into slots on both the right and left sides of the gel.
10. Close the lid of the gel tank and attach the electrical leads so that the DNA will migrate toward the positive anode (red lead). Apply a voltage of 1-5 V/cm. If the leads have been attached correctly, bubbles should be generated at the anode and cathode, and within a few minutes, the bromophenol blue should

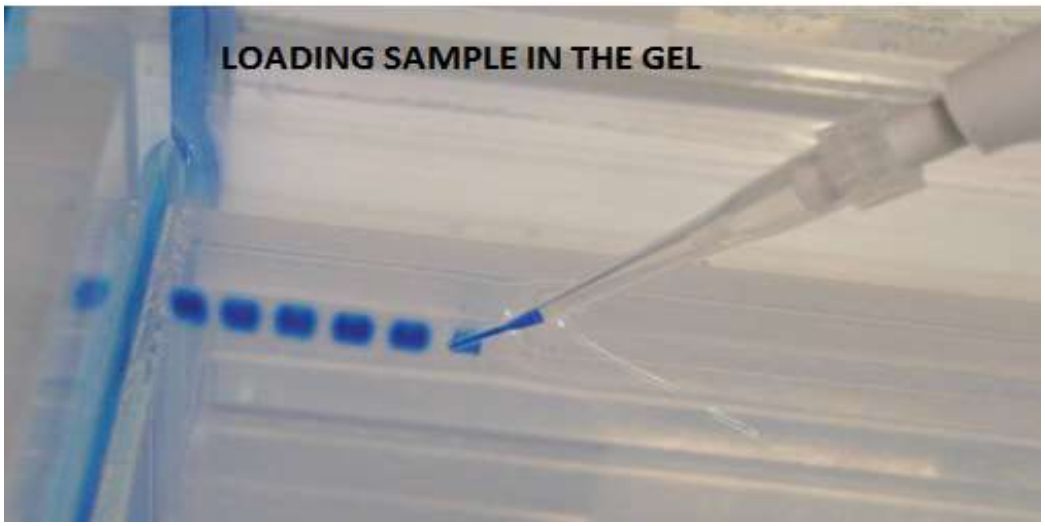
migrate from the wells into the body of the gel. Run the gel until the bromophenol blue and xylene cyanol FF have migrated for distance through the often to the last third og the gel.
11. When the DNA samples or dyes have migrated for a sufficient distance through the gel, turn off the electric current and remove the leads and lid from the gel tank. Otherwise, stain the gel by immersing it in electrophoresis buffer or H2O containing ethidium bromide (0.5 µg/ml) for 20-45 minutes at room temperature or by soaking in a 1:10,000- fold dilution of SYBR Green stock solution in electrophoresis buffer.

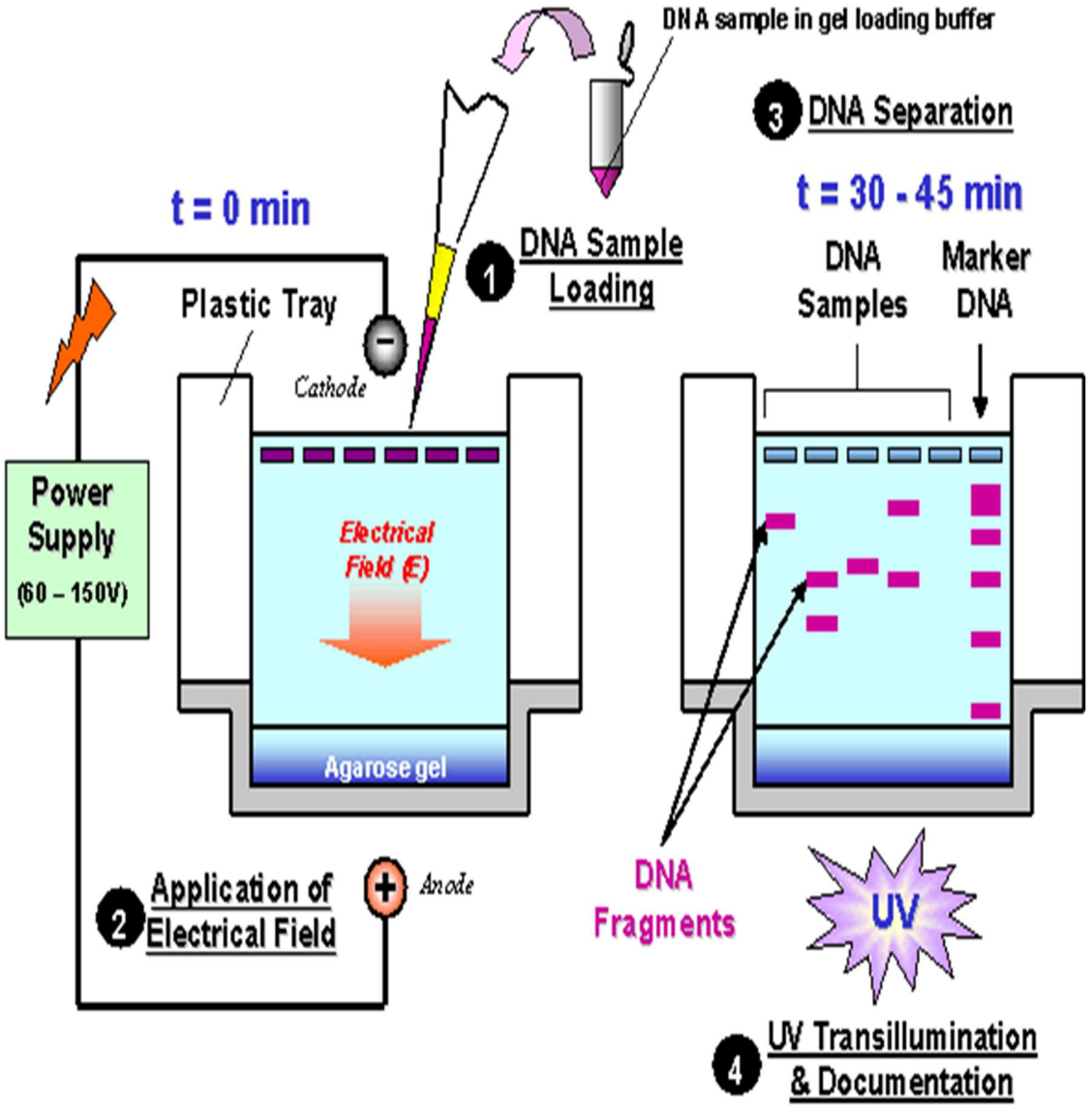
Electrophoresis equipment



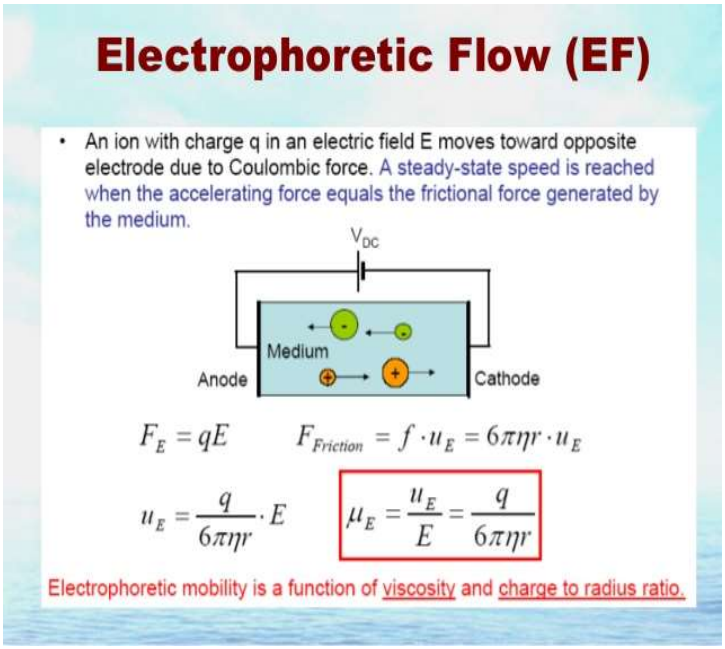
3. Detection of DNA in agarose gels

Nucleic acids running on an electrophoresis can be detected by staining with a dye and visualized under 300-nm UV light. Staining and visualization of DNA are conducted by using either ethidium bromide or SYBR Green. The most convenient and commonly used method to visualize DNA in agarose a gel is ethidium bromide. Ethidium bromide can be used to detect both single- and double-stranded nucleic acids (both DNA and RNA). However, the resolution of single-stranded nucleic acid is relatively low and the fluorescent yield is poor compared to the SYBR Green. In fact, most fluorescence associated with staining single-stranded DNA or RNA is attributable to binding of the dye to short intrastrand duplexes in the molecules (Sambrook &Russel 2001). The banding pattern of DNA resolved through the gel by recorded images. Images of ethidium bromide stained gels may be captured by using transmitted or incident UV light.


LOADING SAMPLE IN THE GEL


DNA sample in gel loading buffer
3 DNA Separation
t = 0 min
t = 30 - 45 min
1 DNA Sample Loading
DNA Samples
Marker DNA
Plastic Tray
Cathode
Power Supply
(60 – 150V)
Electrical Field (E)
Agarose gel
2 Application of Electrical Field
Anode
DNA Fragments
UV
4 UV Transillumination & Documentation
Graphics©E Schmid/2001

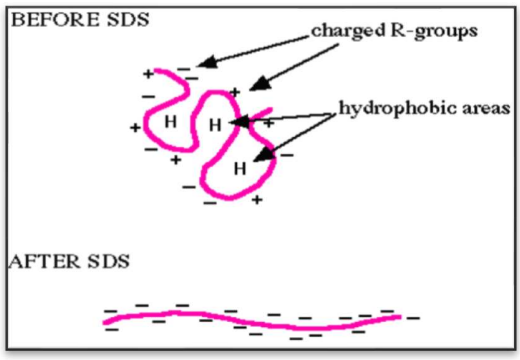
POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)



Polyacrylamide gel electrophoresis (PAGE) is a technique widely used in biochemistry, forensic chemistry, genetics, molecular biology and biotechnology to separate biological macromolecules, usually proteins or nucleic acids, according to their electrophoretic mobility. Electrophoretic mobility is a function of the length, conformation and charge of the molecule. Polyacrylamide gel electrophoresis is a powerful tool used to analyze RNA samples. When polyacrylamide gel is denatured after electrophoresis, it provides information on the sample composition of the RNA species.
Hydration of acrylonitrile results in formation of acrylamide molecules (C_3H_5NO) by nitrile hydratase. Acrylamide monomer is in a powder state before addition of water. Acrylamide is toxic to the human nervous system, therefore all safety measures must be followed when working with it. Acrylamide is soluble in water and upon addition of water it polymerizes resulting in formation of polyacrylamide. It is useful to make polyacrylamide gel via acrylmide hydration because pore size can be regulated. Increased concentrations of acrylamide result in decreased pore size after polymerization. Polyacrylamide gel with small pores helps to examine smaller molecules better since the small molecules can enter the pores and travel through the gel while large molecules get trapped at the pole openings.
As with all forms of gel electrophoresis, molecules may be run in their native state, preserving the molecules' higher-order structure. This method is called native-PAGE. Alternatively, a chemical denaturant may be added to remove this structure and turn the molecule into an unstructured molecule whose mobility depends only on its length (because the protein-SDS complexes all have a similar mass-to-charge ratio). This procedure is called SDS-PAGE. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a method of separating molecules based on the difference of their molecular weight.

SDS-PAGE SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS

SDS-PAGE, is a technique widely used in biochemistry ,forensics, genetics and molecular biology to separate and identify proteins according to their molecular weight.



Principle:
-Sodium Dodecyl Sulfate [SDS]: is a detergent which denature proteins by binding to the hydrophobic regions, all non-covalent bonds will disrupted and the proteins acquire a negative net charge.
-So, the proteins samples are having uniformed structure and charge → the separation will depend on their molecular

weight only. Small proteins migrate faster through the gel under the influence of the applied electric field. The number of SDS molecules that bind is proportional to the size of the protein,

Thereby in the electrical field, protein molecules move towards the anode (+) and separated only according to their molecular weight.

-the proteins samples are having uniformed structure and charge → the separation will depend on their molecular weight only.
-SDS-treated proteins have very similar charge-to-mass ratios, and similar shapes. During PAGE, the rate of migration of SDS-treated proteins is effectively determined by molecular weight.
-Small proteins migrate faster through the gel under the influence of the applied electric field, whereas large proteins are successively retarded, due to the sieving effect of the gels.
- The polyacrylamide gel is formed by co-polymerization of acrylamide and a cross-linking
By N,N'-methylene-bis-acrylamide " bis-acrylamide ".
To polymerize the gel a system, consisting of ammonium persilfate (initiator) and tetramethylene ethylene diamin (TEMED) is added[catalyst].

Acrylamide stock should be prepared first :
Cross-linked polyacrylamide gels are formed from the polymerisation of acrylamide monomer in the presence of smaller amounts of N,N'-methylene-bisacrylamide (normally referred to as 'bis'-acrylamide).

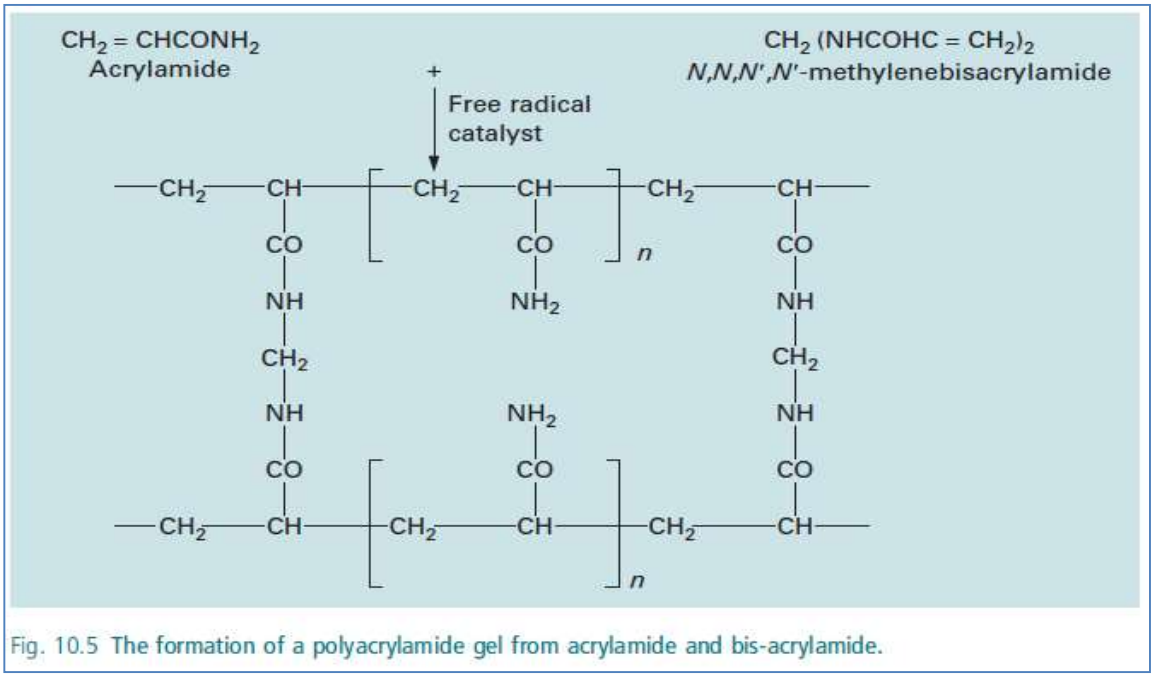
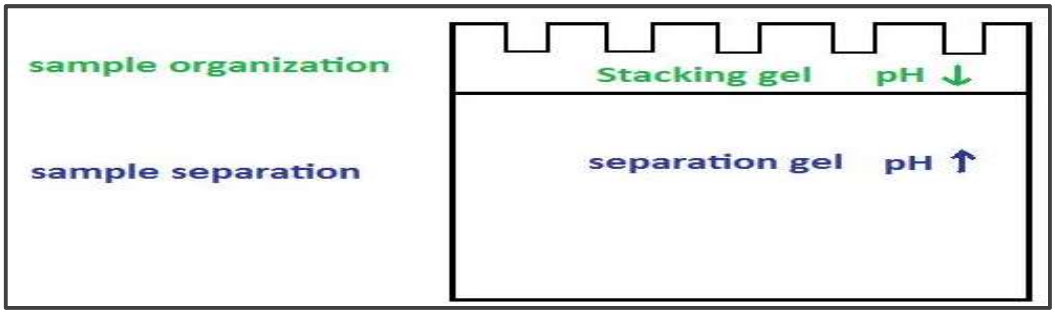


Fig. 10.5 The formation of a polyacrylamide gel from acrylamide and bis-acrylamide.



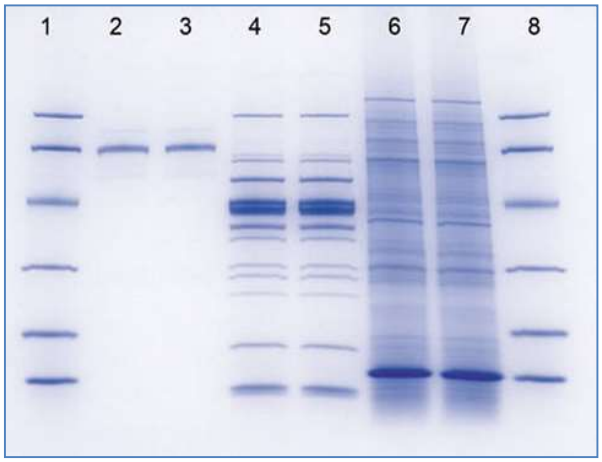
Separation gel preparation: B-Stacking gel preparation:

Stock solutions	Volume of stock solution required to make 12% polyacrylamide gel
0.5M Tris/HCl, pH6.8	1.0 ml
Acrylamide stock	1.0 ml
Water	3.0 ml
10% SDS	80 µl
10% Ammonium persulphate (fresh)	100 µl
TEMED	20 µl

Stock solutions	Volume of stock solution required to make 12% polyacrylamide gel
1.5 M Tris/HCl, pH 8.8	2.0 ml
Acrylamide stock	3.2 ml
Water	2.8 ml
10% SDS	80 µl
10% Ammonium persulphate (fresh)	100 µl
TEMED	20 µl

3-Running the gel using , Running buffer 1x pH 8.4:
It is contain:
-Tris-HCl.
-Glycine.
-SDS.

4- Stain the gel using staining buffer :
It is contain:
-Glacial acetic acid
-Methanol
-Coomassie brilliant blue 250-R



5- De-stain the gel using De-staining buffer:
It is contain:
-Glacial acetic acid
-Methanol

Application:

- Determine protein size
- Identify protein
- Determine sample purity
- Identify existence of disulfide bonds
- Quantify amounts of protein

ISOELECTRIC FOCUSING

Isoelectric focusing (IEF), also known as electrofocusing, is a technique for separating different molecules by differences in their isoelectric point (pI). It is a type of zone electrophoresis, usually performed on proteins in a gel, that takes advantage of the fact that overall charge on the molecule of interest is a function of the pH of its surroundings. Electrophoretic method is ideal for separation of amphoteric substances Seperation is achieved by applying a potential difference across a gel that contain a pH gradient Isoelectric focusing requires solid support such as agarose gel and polyacrylamide gel. It gives good separation with a high resolution compared to any other method. Their resolution depends on

- The pH gradient,
- The thickness of the gel
- Time of electrophoresis,
- The applied voltage,
- Diffusion of the protein into the gel.

PREPARATION

IEF involves adding an ampholyte solution into immobilized pH gradient (IPG) gels. IPGs are the acrylamide gel matrix co-polymerized with the pH gradient, which result in completely stable gradients except the most alkaline (>12) pH values. The immobilized pH gradient is obtained by the continuous change in the ratio of immobilines. An immobiline is a weak acid or base defined by its pK value.

A protein that is in a pH region below its isoelectric point (pI) will be positively charged and so will migrate towards the cathode (negatively charged electrode). As it migrates through a gradient of increasing pH, however, the protein's overall charge will decrease until the protein reaches the pH region that corresponds to its pI. At this point it has no net charge and so migration ceases (as there is no electrical attraction towards either electrode). As a result, the proteins become focused into sharp stationary bands with each protein positioned at a point in the pH gradient corresponding to its pI. The technique is capable of extremely high resolution with proteins differing by a single charge being fractionated into separate bands.

Molecules to be focused are distributed over a medium that has a pH gradient (usually created by aliphatic ampholytes). An electric current is passed through the medium, creating a "positive" anode and "negative" cathode end. Negatively charged molecules migrate through the pH gradient in the medium toward the "positive" end while positively charged molecules move toward the "negative" end. As a particle moves towards the pole opposite of its charge it moves through the changing pH gradient until it reaches a point in which the pH of that molecules isoelectric point is reached. At this point the molecule no longer has a net electric charge (due to the protonation or deprotonation of the associated functional groups) and as such will not proceed any further within the gel. The gradient is established before adding the particles of interest by first subjecting a solution of small molecules such as polyampholytes with varying pI values to electrophoresis.

The method is applied particularly often in the study of proteins, which separate based on their relative content of acidic and basic residues, whose value is represented by the pI. Proteins are introduced into an Immobilized pH gradient gel composed of polyacrylamide, starch, or agarose where a pH gradient has been established. Gels with large pores are usually used in this process to eliminate any "sieving" effects, or artifacts in the pI caused by differing migration rates for proteins of differing sizes. Isoelectric focusing can resolve proteins that differ in pI value by as little as 0.01. Isoelectric focusing is the first step in two-dimensional gel electrophoresis, in which proteins are first separated by their pI and then further separated by molecular weight through SDS-PAGE.

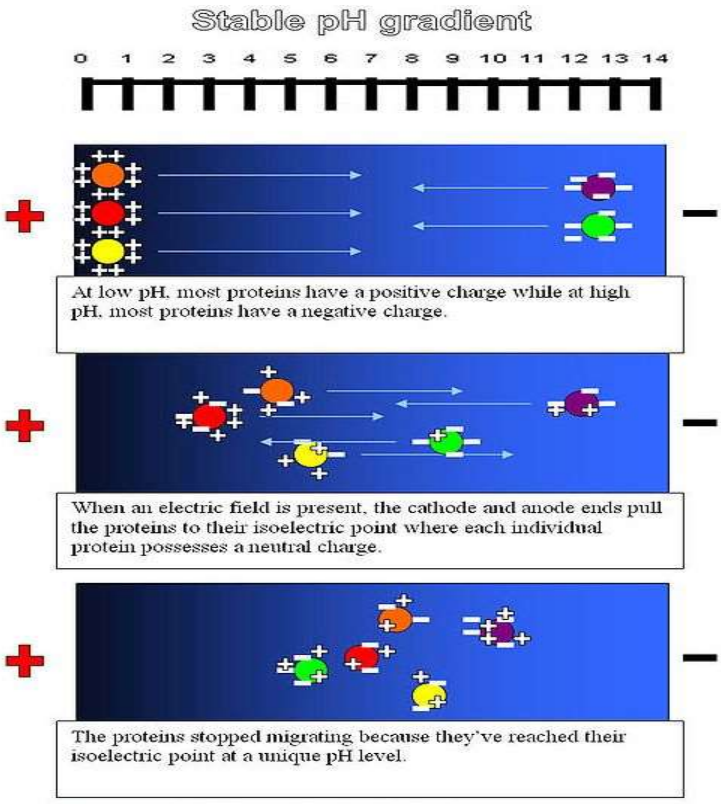
PREPARATION OF IEF GEL

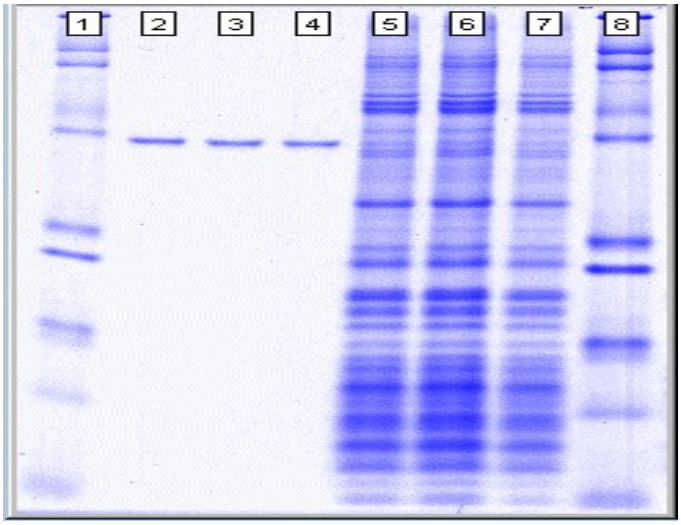
- Carrier ampholytes (suitable pH) and riboflavin mixed with acrylamide solution
- Mixture is poured over a glass plate which contain spacer
- Second glass plate is placed on first
- Gel is polymerised

- This takes 2-3 hr
- After the gel has set glass plates are prised apart
- Electrode wicks are laid along the long length of each side of the gel
- Potential difference is applied
- Ampholytes form a pH gradient between anode and cathode

- The power is then turned off
- Samples applied by laying on gel filter paper soaked in the sample
- Voltage is again applied for 30 min
- Proteins having positive charge will migrates towards the cathode. negatively charged protein will migrates towards anode
- Become stationary when they reaches isoelectric point

- The gel is washed with trichloroacetic acid
- This precipitaes the proteins and allows smaller ampholytes to be washed out
- Gel is stained with Coomasie Brilliant Blue
- Destained





TYPICAL ISOELECTRIC FOCUSING GEL

IMMUNOELECTROPHORESIS

This technique combines electrophoresis with double immunodiffusion.

In Immunoelectrophoresis, the antigenic mixture is first electrophoresed to separate its components by charge in an given electric field.

In second step, troughs are then cut into the agar gel parallel to the direction of electric field and then antiserum is added to the troughs to ditect antigen of interest in given sample.

Then antigen and antibody diffuse toward each other produce lines of precipitation (precipitin arcs)where they meet in appropriate proportions.

This technique is useful for determination of presence or absence of serum proteins(albumin, immunoglobulin) and detection of unusual proteins such as human myeloma protein. However, it is strictly a qualitative technique that can detect relatively high antibody concentrations.

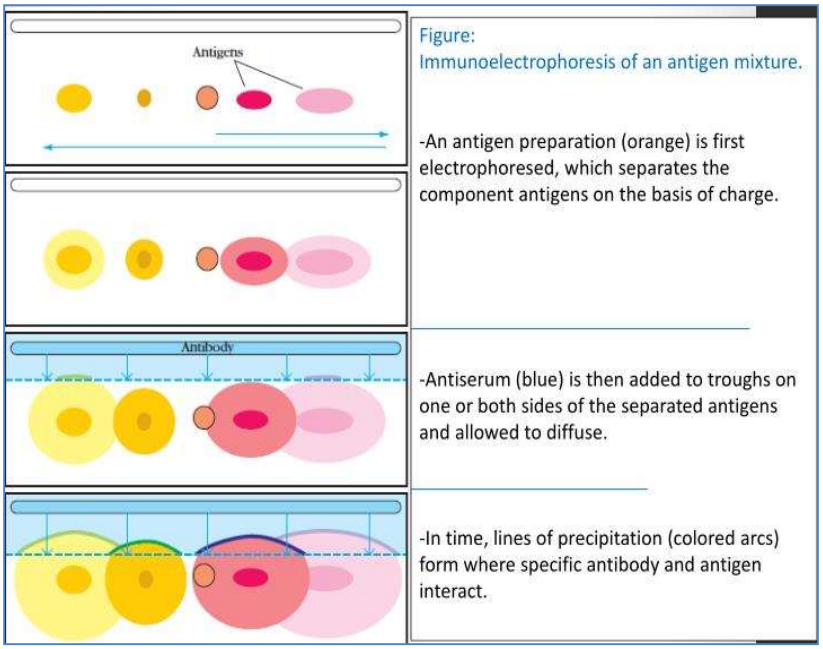
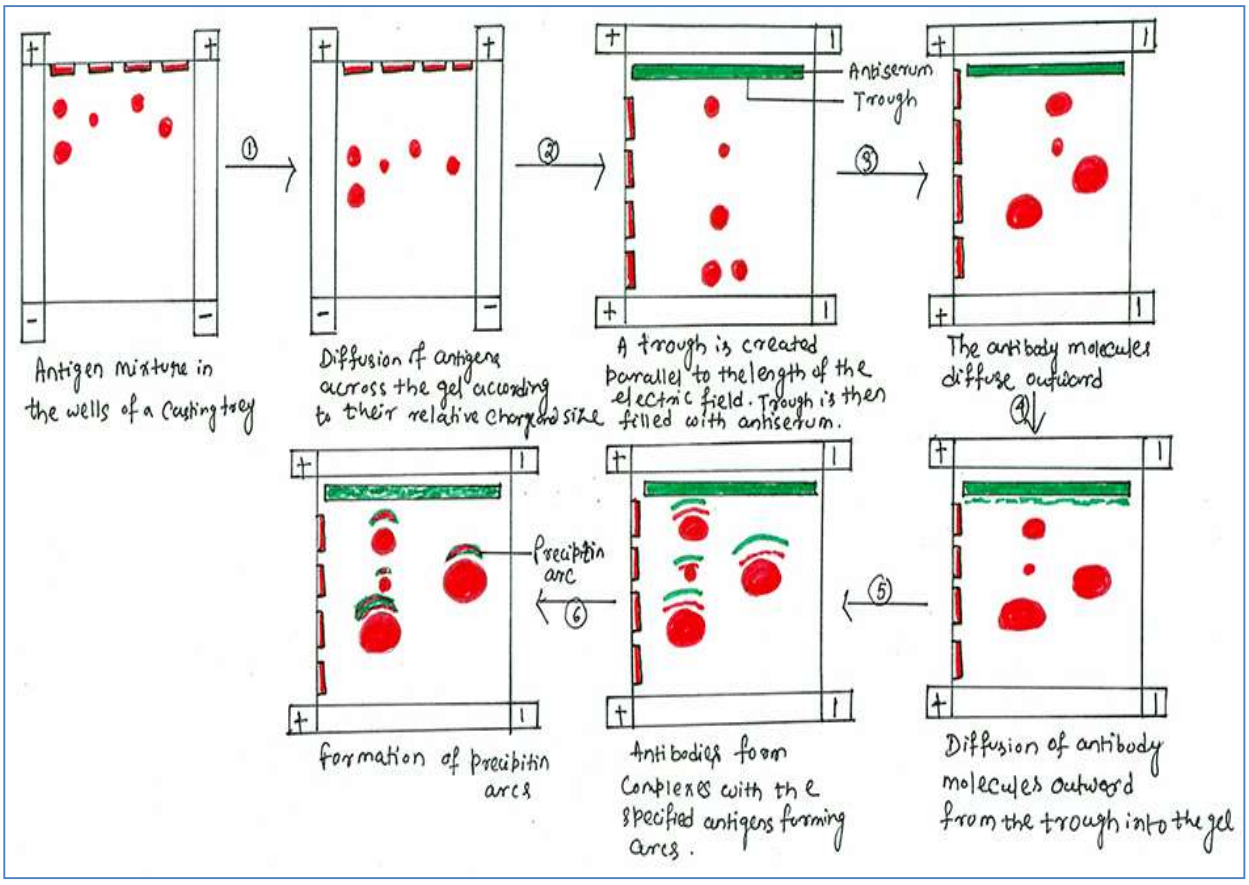


Figure:
Immunoelectrophoresis of an antigen mixture.

-An antigen preparation (orange) is first electrophoresed, which separates the component antigens on the basis of charge.

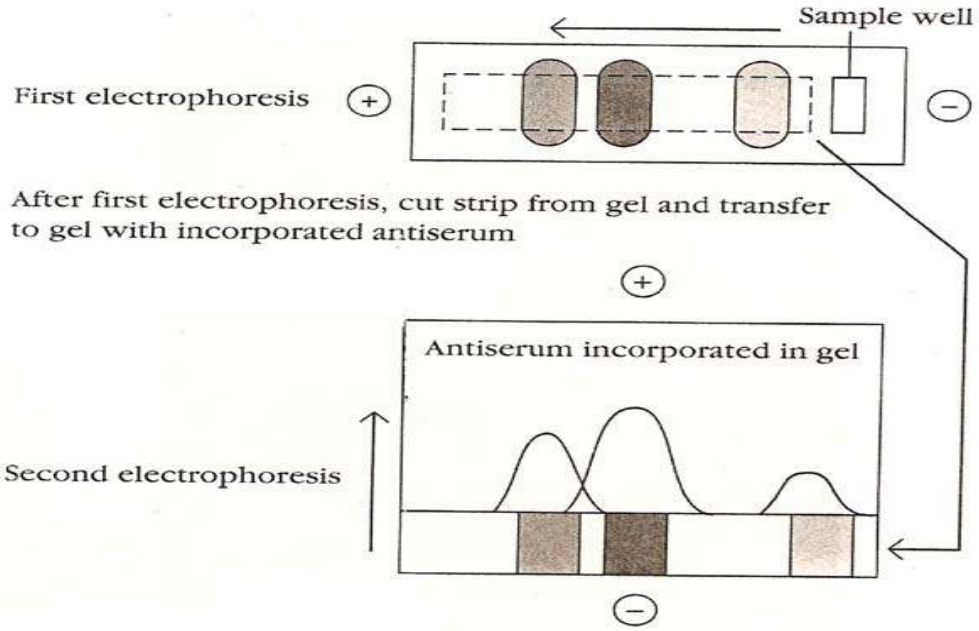
-Antiserum (blue) is then added to troughs on one or both sides of the separated antigens and allowed to diffuse.

-In time, lines of precipitation (colored arcs) form where specific antibody and antigen interact.

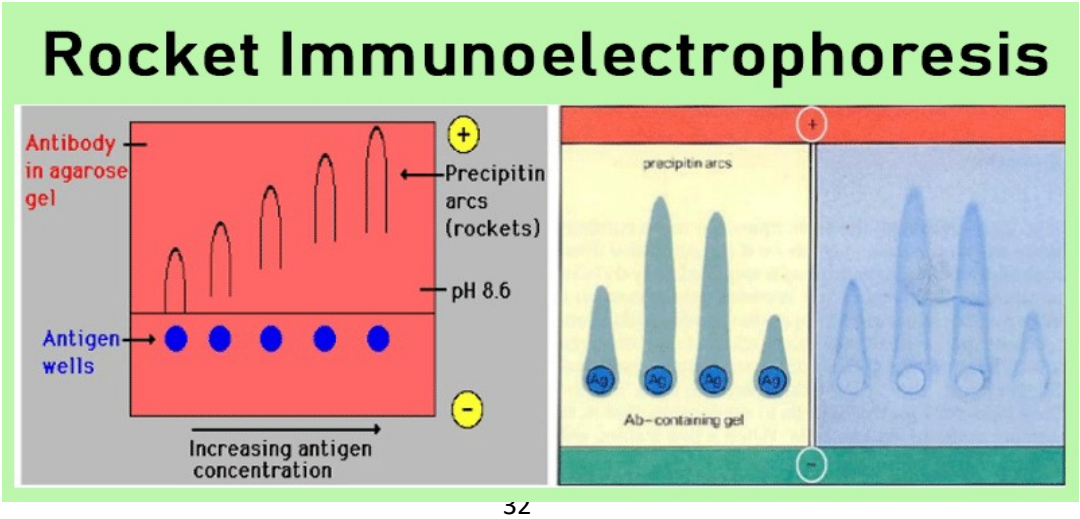


Crossed immunoelectrophoresis: It is also called two-dimensional quantitative immune-electrophoresis . In this method the proteins are first separated during the first dimension electrophoresis, then instead of the diffusion towards the antibodies, the proteins are electrophoresed into an antibody-containing gel in the second dimension. Immunoprecipitation will take place during the second dimension electrophoresis and the immunoprecipitates have a characteristic bell-shape, each precipitate representing one antigen, the position of the precipitate being dependent on the amount of protein as well as the amount of specific antibody in the gel, so relative quantification can be performed. The sensitivity and resolving power of crossed immune-electrophoresis is than that of the classical immunoelectrophoretic analysis and there are multiple variations of the technique useful for various purposes. Crossed immune-electrophoresis has been used for studies of proteins in biological fluids, particularly human serum, and biological extracts.

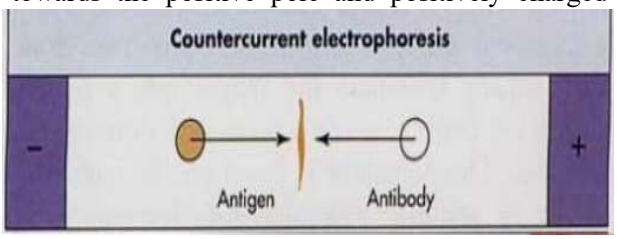
Rocket



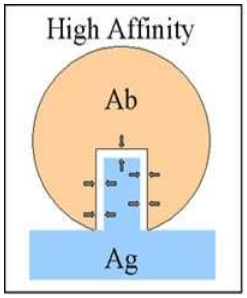
electrophoresis- A related quantitative technique , does permit measurement of antigen levels. At first, a negatively charged antigen is electrophoresed in a gel containing antibody. The precipitate formed between antigen and antibody has the shape of a rocket , the height of which is proportional to the concentration of antigen in the well. One limitation of rocket electrophoresis is need for the antigen to be negatively charged for electrophoretic movement.

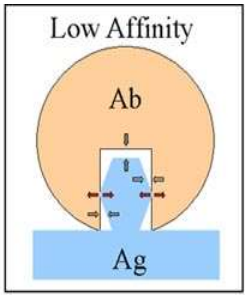


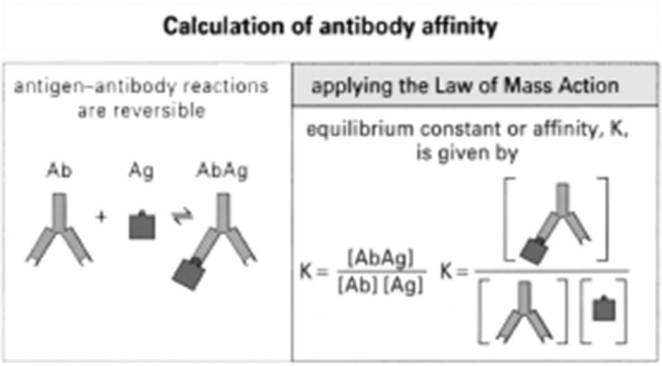
Counter current immune-electrophoresis- It relies on movement of antigen and antibody in opposite direction. Antigen carrying negative charge migrates towards the positive pole and positively charged antibody move toward the negative pole. Antigen and homologous antibody are placed in separate cells in agar gel through which electric current is passed. Line of precipitin appear at a point where the two lines meet in optimal proportions. This test is more faster and more sensitive than double diffusion technique. It is useful in detection of hepatitis B antigen and antibodies.



Affinity immune-electrophoresis is based on changes in the electrophoretic pattern of proteins through specific interaction or complex formation with other macromolecules or ligands. Affinity immune-electrophoresis has been used for estimation of binding constants, as for instance with lectins or for characterization of proteins with specific features like glycan content or ligand binding. Some variants of affinity immune-electrophoresis are similar to affinity chromatography by use of immobilized ligands.







Two factors determine that immunoelectrophoretic methods are not widely used. First they are rather work intensive and require some manual expertise. Second they require rather large amounts of polyclonal antibodies. Today gel electrophoresis followed by electroblotting is the preferred method for protein characterization because its ease of operation, its high sensitivity, and its low requirement for specific antibodies. In addition proteins are separated by gel electrophoresis on the basis of their apparent molecular weight, which is not accomplished by immune-electrophoresis, but nevertheless immunoelectrophoretic methods are still useful when non-reducing conditions are needed.

Probable questions:

1. Describe the principle and methodology of agarose gel electrophoresis.
2. What is isoelectric focusing?
3. State the role of SDS in electrophoresis.
4. What is rocket electrophoresis?
5. State the procedure of affinity electrophoresis.

Suggested readings:

1. Sambrook J&Russel DW(2001). Molecular Cloning: A Laboratory Manual 3rd Ed. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY.
2. Sharp P.A., Sugden B. & Sambrook J. (1973). Detection of two restriction endonuclease activities in *Haemophilus parainfluenzae*using analytical agarose-ethidium bromide electrophoresis. Biochemistry. 12:3055-3063.

Unit- III

Spectroscopic methods : Colorimetry, Spectrophotometry, Atomic Absorption Spectrophotometry

Objective: In this unit, you will learn about Spectroscopic methods : Colorimetry, Spectrophotometry, Atomic Absorption Spectrophotometry.

Principle for all spectroscopic methods:

Quantification of light absorption: The chance for a photon to be absorbed by matter is given by an extinction coefficient which itself is dependent on the wavelength l of the photon. If light with the intensity I0 passes through a sample with appropriate transparency and the path length (thickness) d, the intensity I drops along the pathway in an exponential manner. The characteristic absorption parameter for the sample is the extinction coefficient a, yielding the correlation $I= I_0 e^{-\alpha d}$ .The ratio $T= I/I_0$ is called transmission. Biochemical samples usually comprise aqueous solutions, where the substance of interest is present at a molar concentration c. Algebraic transformation of the exponential correlation into an expression based on the decadic logarithm yields the law of Beer–Lambert.

The **Beer-Lambert law (or Beer's law)** is the linear relationship between absorbance and concentration of an absorbing species. The general Beer-Lambert law is usually written as:
$A = a(\lambda) * b * c$
where A is the measured absorbance, $a(\lambda)$ is a wavelength-dependent absorptivity coefficient, **b** is the path length, and **c** is the analyte concentration. When working in concentration units of molarity, the *Beer-*

$$\lg \frac{I_0}{I} = \lg \frac{1}{T} = \varepsilon \times c \times d = A \qquad (12.2)$$

where $[d] = 1$ cm, $[c] = 1$ mol dm^{-3}, and $[\varepsilon] = 1$ dm^3 mol^{-1} cm^{-1}. ε is the molar absorption coefficient (also molar extinction coefficient) ($\alpha = 2.303 \times c \times \varepsilon$). A is the absorbance of the sample, which is displayed on the spectrophotometer.

***Lambert law*is written as:**

$A = \varepsilon * b * c$
Where, ε is the wavelength-dependent molar absorptivity coefficient with units of $M^{-1} cm^{-1}$. Data are frequently reported in percent transmission ($I/I_0 * 100$) or in absorbance [$A = \log (I/I_0)$]. The latter is particularly convenient. [common coefficients of near-ultraviolet absorption bands of some amino acids and nucleotides]

Sometimes the extinction coefficient is given in other units; for example,
$A = E^{1\%} * b * c$
where the concentration C is in gram per 100 ml of solution. This useful when the molecular weight of the solute is unknown or uncertain.

The Beer–Lambert law is valid for low concentrations only. Higher concentrations might lead to association of molecules and therefore cause deviations from the ideal behaviour. Absorbance and extinction coefficients

are additive parameters, which complicates determination of concentrations in samples with more than one absorbing species. Note that in dispersive samples or suspensions scattering effects increase the absorbance, since the scattered light is not reaching the detector for readout. The absorbance recorded by the spectrophotometer is thus overestimated and needs to be corrected.

Deviations from the Beer–Lambert law

According to the Beer–Lambert law, absorbance is linearly proportional to the concentration of chromophores. This might not be the case any more in samples with high absorbance. Every spectrophotometer has a certain amount of stray light, which is light received at the detector but not anticipated in the spectral band isolated by the monochromator. In order to obtain reasonable signal-to-noise ratios, the intensity of light at the chosen wavelength (Il) should be 10 times higher than the intensity of the stray light (Istray). If the stray light gains in intensity, the effects measured at the detector have nothing or little to do with chromophore concentration. Secondly, molecular events might lead to deviations from the Beer–Lambert law. For instance, chromophores might dimerize at high concentrations and, as a result, might possess different spectroscopic parameters.

COLORIMETRY

Definition: In physical and analytical chemistry, **colorimetry** or**colourimetry** is a technique "used to determine the concentration of colored compounds in solution." ... The concentration of a sample can be calculated from the intensity of light before and after it .The **colorimeter** is based on Beer-Lambert's law,

Principle: A **colorimeter** is a light-sensitive device used for measuring the transmittance and absorbance of light passing through a liquid sample. The device measures the intensity or concentration of the color that develops upon introducing a specific reagent into a solution.

The colorimeter is based on Beer-Lambert's law, according to which the absorption of light transmitted through the medium is directly proportional to the medium concentration.

In a colorimeter, a beam of light with a specific wavelength is passed through a solution via a series of lenses, which navigate the colored light to the measuring device. This analyzes the color compared to an existing standard. A microprocessor then calculates the absorbance or percent transmittance. If the concentration of the solution is greater, more light will be absorbed, which can be identified by measuring the difference between the amount of light at its origin and that after passing the solution.

To determine the concentration of an unknown sample, several sample solutions of a known concentration are first prepared and tested. The concentrations are then plotted on a graph against absorbance, thereby generating a calibration curve. The results of the unknown sample are compared to that of the known sample on the curve to measure the concentration.

Operating instructions for a typical colorimeter:

- Switch on the instrument at least 5 minutes before use to allow it to stabilize.
- Select the most appropriate filter for the analysis and insert it in the light path (or dial it in with the selector)
- Place the reagent blank solution (or water) in the cuvette and zero the instrument (consult your manufacturers instruction about how to do this.) Make sure the clear faces of the cuvette are in the light path

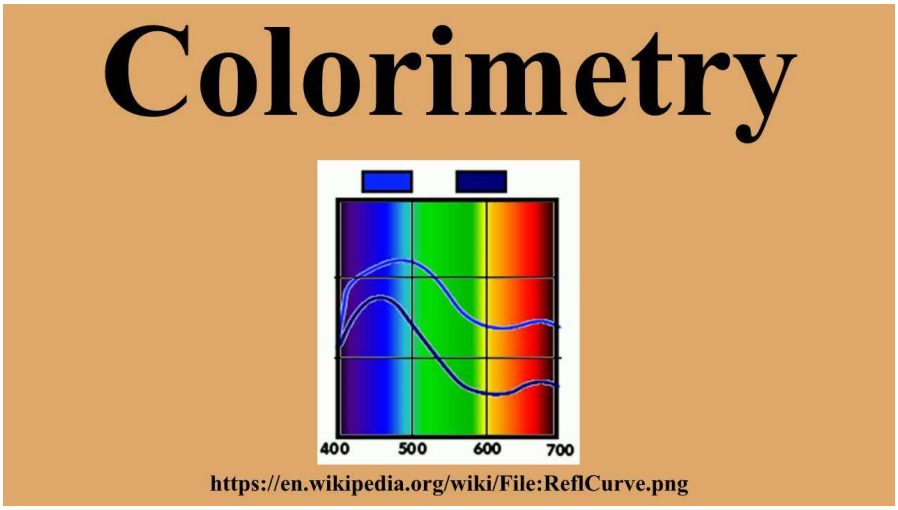
- Place the sample in the colorimeter and read the absorbance of the solution. If the absorbance is "over range" (usually > 2.0) then the sample must be diluted to yield a value within the limits of the instrument.
- At intervals, recheck the reagent blank to ensure that there is no drift in the zero value.

Notes

- colour choice.

Why red on a blue solution: (an example of why a red filter is used on a blue solution but can be developed for any other colour). The blue solution appears blue as all other colours have been *preferentially absorbed*. The Red is the most strongly absorbed. Remember: that if you want to see how the changes in the solution are progressing you need to study a colour that changes. IF the blue is always passing unhindered then red must be used. This is very*counter intuitive*. However if you want to convince yourself: shine a bright white light through a tank of water with milk. It appears blue. If you go to the end and look at the light source it will be reddish in tinge.

- Your machine must be re-zeroed (step 3) if a new filter is chosen.
- The sample cuvettes must be at least two-thirds



Spectrophotometry

Definition: In chemistry, spectrophotometry is the quantitative measurement of the reflection or transmission properties of a material as a function of wavelength. It is more specific than the general term electromagnetic spectroscopy in that spectrophotometry deals with visible light, near-ultraviolet, and near-infrared, but does not cover time-resolved spectroscopic techniques.

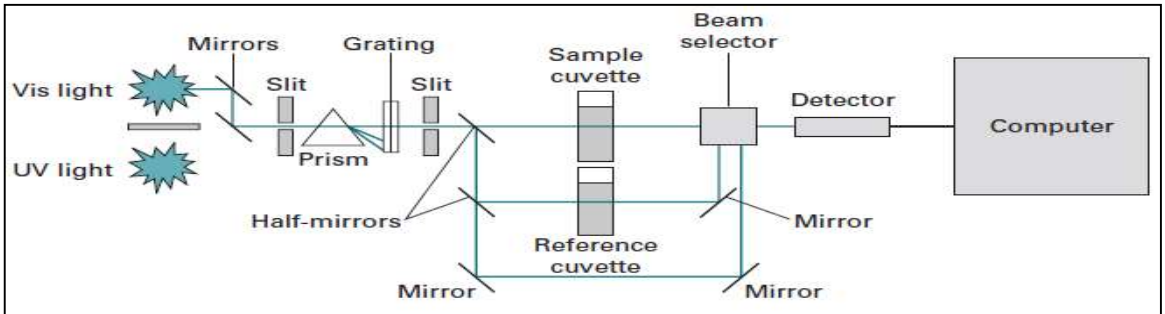


Fig. 12.6 Optical arrangements in a dual-beam spectrophotometer. Either a prism or a grating constitutes the monochromator of the instrument. Optical paths are shown as green lines.

Principle: **Spectrophotometry** is a method to measure how much a chemical substance absorbs light by measuring the intensity of light as a beam of light passes through sample solution. The basic **principle** is that each compound absorbs or transmits light over a certain range of wavelength following Beer Lambart Law.

Procedure:

Materials Required:

1. Spectrophotometer
2. Cuvette
3. Blank solution

Reagents:

1. Cobalt (II) chloride
2. Hexaaquacobalt (II) ion
3. Ferrocene
4. Crystal violet
5. Rose bengal
6. Coumarin

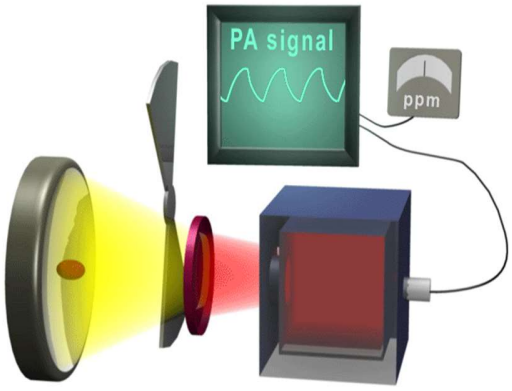
Procedure:

Determination of Molar Absorption Coefficient:

1. Select a blank cuvette and place it in the spectrophotometer. Close the lid.
2. Click on 0 ABS 100%T button, the instrument now reads 0.00000 A.
3. Choose a solution with known concentration and measure the absorbance between the wavelengths 350 nm to 700 nm.
4. Record the wavelength at the maximum absorbance value.
5. Calculate the value of molar absorption coefficient , using the equation $\epsilon = A / cl$.

Determination of Unknown Concentration:

1. Set the wavelength to the value corresponding to maximum absorbance (recorded above).
2. Place the cuvette with same solution but at an unknown concentration.
3. Read the absorbance for this wavelength.
4. Calculate the concentration with the help of the equation, molarity $= A / \epsilon l$
5. Enter the calculated concentration value in the given box. (Note : Should enter the value correct to four decimal places)
6. Repeat the same procedure for a second solution.



Atomic absorption spectrophotometry

Definition: **Atomic absorption spectroscopy** (**AAS**) is a spectro analytical procedure for the quantitative determination of chemical elements using the absorption of optical radiation (light) by free atoms in the gaseous state.

In analytical chemistry the technique is used for determining the concentration of a particular element (the analyte) in a sample to be analyzed. AAS can be used to determine over 70 different elements in solution, or directly in solid samples via electro thermal vaporization and is used in pharmacology, biophysics and toxicology research.

Principle: The technique makes use of absorption spectroscopy to assess the concentration of an analyte in a sample. It requires standards with known analyte content to establish the relation between the measured absorbance and the analyte concentration and relies therefore on the Beer-Lambert Law.

Principles of electron discharge: In a spectrum of an element, the absorption or emission wavelengths are associated with transitions that require a minimum of energy change. In order for energy changes to be minimal, transitions tend to occur between orbitals close together in energy terms. For example, excitation of a sodium atom and its subsequent relaxation gives rise to emission of orange light ('D-line') due to the transition of an electron from the 3s to the 3p orbital and return. Electron transitions in an atom are limited by the availability of empty orbitals. Filling orbitals with electrons is subject to two major rules:

• one orbital can be occupied with a maximum of two electrons; and

• the spins of electrons in one orbital need to be paired in an antiparallel fashion (Pauli principle).

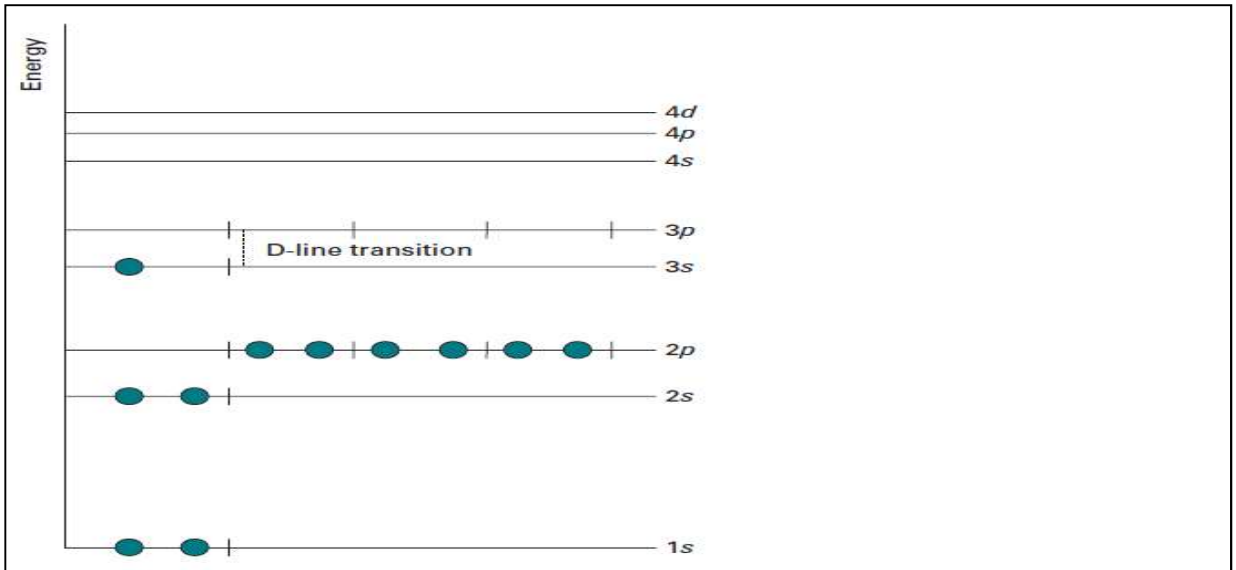


Fig. 12.19 Energy levels of atomic orbitals in the sodium atom. Each atomic orbital can be occupied by electrons following the rules of quantum chemistry until the total number of electrons for that element is reached (in case of sodium: 11 electrons). The energy gap between the 3*s* and the 3*p* orbitals in the sodium atom is such that it can be overcome by absorption of orange light.

Together, these limitations mean that emission and absorption lines are characteristic for an individual element. In short, the electrons of the atoms in the atomizer can be promoted to higher orbitals (excited state) for a short period of time (nanoseconds) by absorbing a defined quantity of energy (radiation of a given wavelength). This amount of energy, i.e., wavelength, is specific to a particular electron transition in a particular element. In general, each wavelength corresponds to only one element, and the width of an

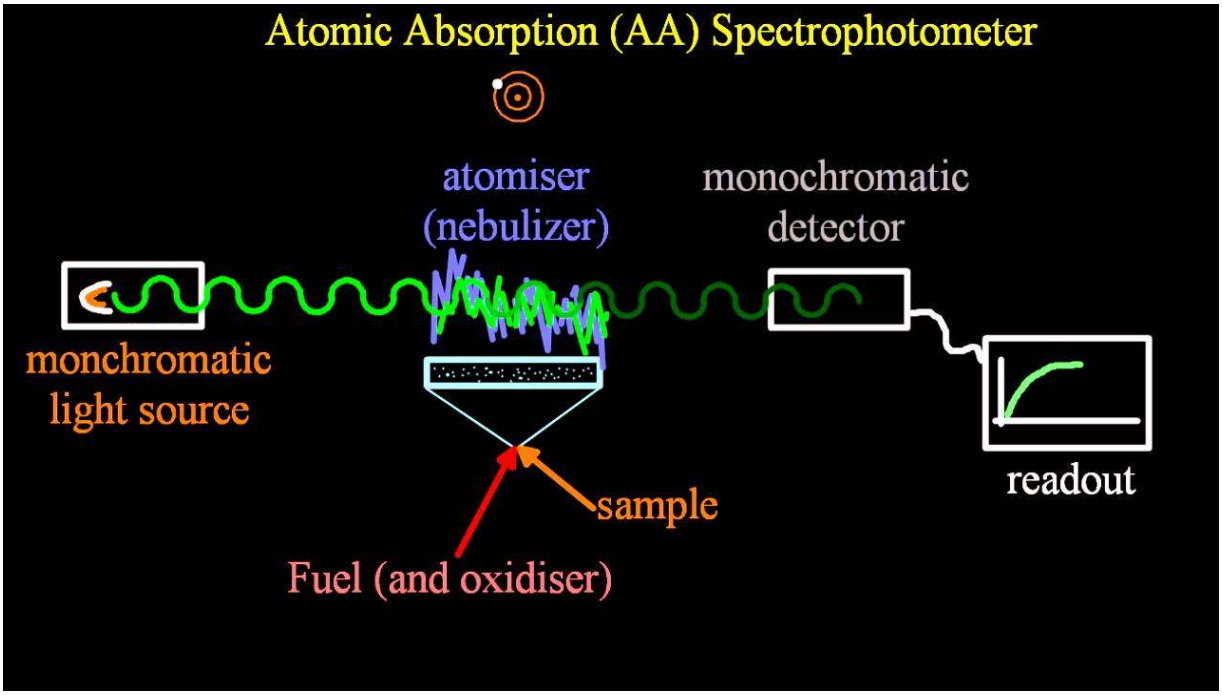
absorption line is only of the order of a few picometers (pm), which gives the technique its elemental selectivity. The radiation flux without a sample and with a sample in the atomizer is measured using a detector, and the ratio between the two values (the absorbance) is converted to analyte concentration or mass using the Beer-Lambert Law.

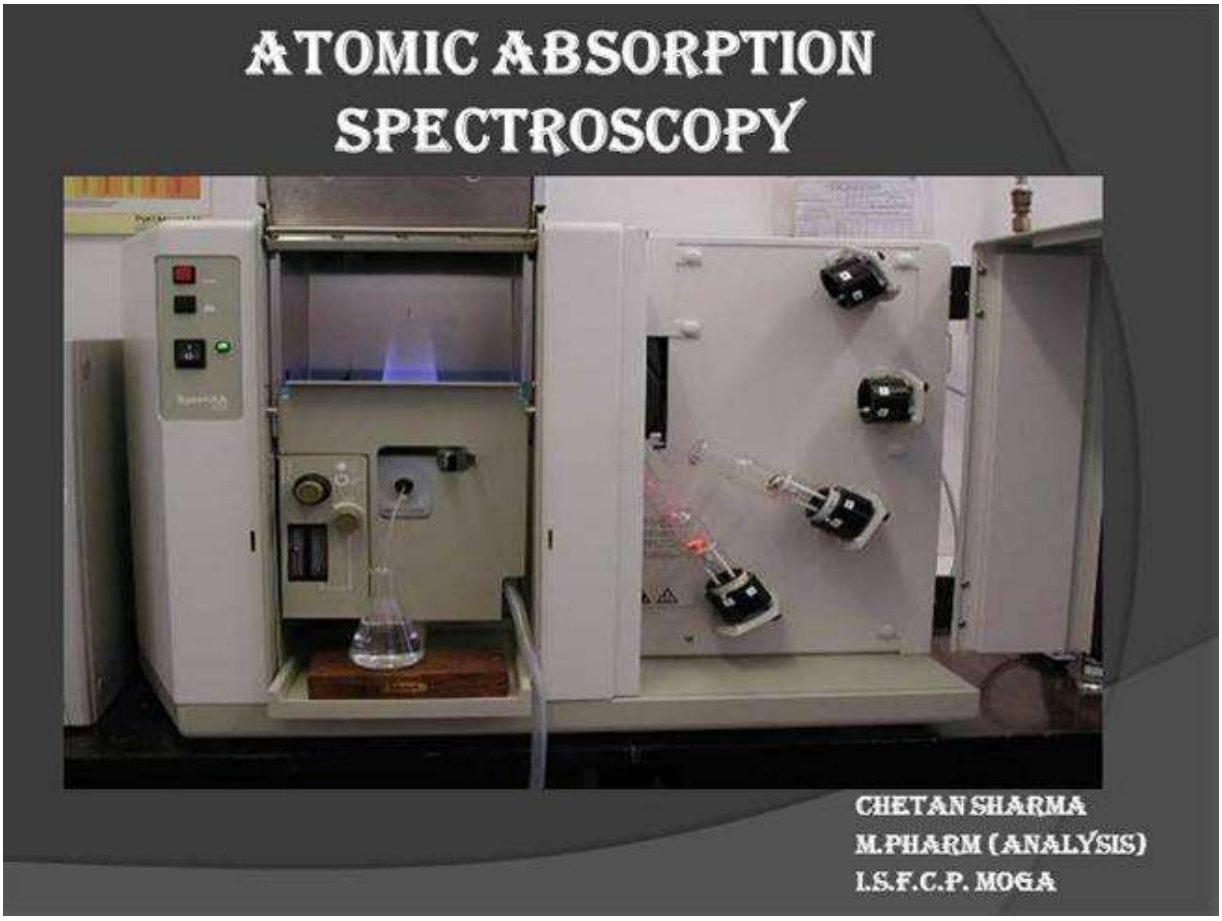
Procedure:

The process of atomic absorption spectroscopy (AAS) involves two steps:

1. Atomization of the sample
2. The absorption of radiation from a light source by the free atoms
3. The sample, either a liquid or a solid, is atomized in either a flame or a graphite furnace. Upon the absorption of ultraviolet or visible light, the free atoms undergo electronic transitions from the ground state to excited electronic states.

To obtain the best results in AA, the instrumental and chemical parameters of the system must be geared toward the production of neutral ground state atoms of the element of interest. A common method is to introduce a liquid sample into a flame. Upon introduction, the sample solution is dispersed into a fine spray, the spray is then desolvated into salt particles in the flame and the particles are subsequently vaporized into neutral atoms, ionic species and molecular species. All of these conversion processes occur in geometrically definable regions in the flame. It is therefore important to set the instrument parameters such that the light from the source (typically a hollow-cathode lamp) is directed through the region of the flame that contains the maximum number of neutral atoms. The light produced by the hollow-cathode lamp is emitted from excited atoms of the same element which is to be determined. Therefore the radiant energy corresponds directly to the wavelength which is absorbable by the atomized sample. This method provides both sensitivity and selectivity since other elements in the sample will not generally absorb the chosen wavelength and thus, will not interfere with the measurement. To reduce background interference, the wavelength of interest is isolated by a monochromator placed between the sample and the detector.





In atomic absorption (see schematic of an atomic-absorption experiment), there are two methods of adding thermal energy to a sample. A graphite furnace AAS uses a graphite tube with a strong electric current to heat the sample. In flame AAS (see photo above), we aspirate a sample into a flame using a nebulizer. The flame is lined up in a beam of light of the appropriate wavelength. The flame (thermal energy) causes the atom to undergo a transition from the ground state to the first excited state. When the atoms make their transition, they absorb some of the light from the beam. **The more concentrated the solution, the more light energy is absorbed!**

Probable questions:

1. What is the basic principle of spectroscopy?
2. What is Lambert- Beer's law?
3. Describe the basic principle of colorimetry?
4. What is atomic absorption spectroscopy?

Suggested readings:

1. Wilson and Walker: Principle and technique of biochemistry and molecular biology, 7th Ed

Unit- IV

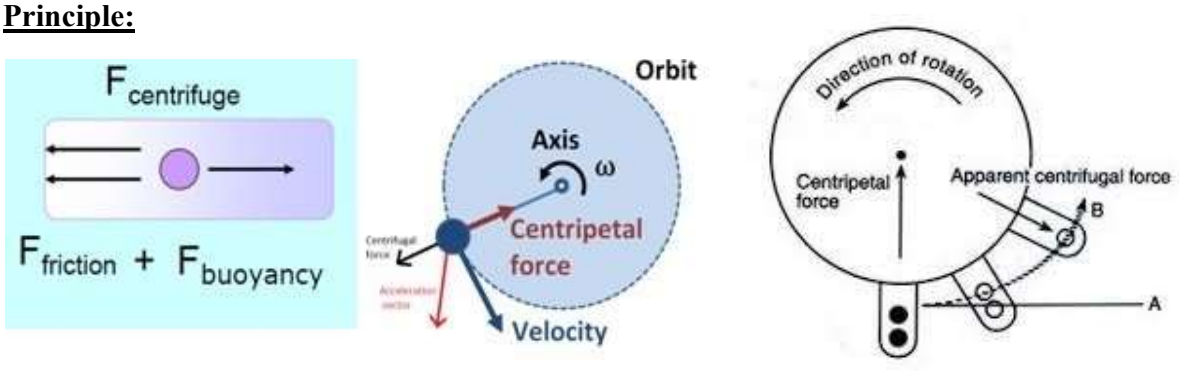
Sedimentation: Instrument for Ultra centrifugation, Zonal Centrifugation through Density Gradients

Objective: In this unit, you will learn about Sedimentation: Instrument for Ultra centrifugation, Zonal Centrifugation through Density Gradients.

SEDIMENTATION

Instrument for ultra centrifugation: The **ultracentrifuge** is a centrifuge optimized for spinning a rotor at very high speeds, capable of generating acceleration as high as1 000 000 g (approx. 9 800 km/s²). There are two kinds of ultracentrifuges, the preparative and the analytical ultracentrifuge. Both classes of instruments find important uses in molecular biology, biochemistry, and polymer science.

Principle:



From everyday experience, the effect of sedimentation due to the influence of the Earth's gravitational field (g¼981 cm s–2) versus the increased rate of sedimentation in a centrifugal field (g>981 cm s–2) is apparent. To give a simple but illustrative example, crude sand particles added to a bucket of water travel slowly to the bottom of the bucket by gravitation, but sediment much faster when the bucket is swung around in a circle. Similarly, biological structures exhibit a drastic increase in sedimentation when they undergo acceleration in a centrifugal field. The relative centrifugal field is usually expressed as a multiple of the acceleration due to gravity. Below is a short description of equations used in practical centrifugation classes.

When designing a centrifugation protocol, it is important to keep in mind that:

• the more dense a biological structure is, the faster it sediments in a centrifugal field;

• the more massive a biological particle is, the faster it moves in a centrifugal field;

• the denser the biological buffer system is, the slower the particle will move in a centrifugal field;

• the greater the frictional coefficient is, the slower a particle will move;

• the greater the centrifugal force is, the faster the particle sediments;

• the sedimentation rate of a given particle will be zero when the density of the particle and the surrounding medium are equal.

Biological particles moving through a viscous medium experience a frictional drag, whereby the frictional force acts in the opposite direction to sedimentation and equals the velocity of the particle multiplied by the frictional coefficient. The frictional coefficient depends on the size and shape of the biological particle. As the sample moves towards the bottom of a centrifuge tube in swing-out or fixed-angle rotors, its velocity will increase due to the increase in radial distance. At the same time the particles also encounter a frictional drag that is proportional to their velocity. The frictional force of a particle moving through a viscous fluid is the product of its velocity and its frictional coefficient, and acts in the opposite direction to sedimentation. From the equation (3.1) for the calculation of the relative centrifugal field it becomes apparent that when the conditions for the centrifugal separation of a biological particle are described, a detailed listing of rotor speed, radial dimensions and duration of centrifugation has to be provided. Essentially, the rate of sedimentation is dependent upon the applied centrifugal field (cm s2), G, that is determined by the radial distance, r, of the particle from the axis of rotation (in cm) and the square of the angular velocity, $\boldsymbol{\omega}$, of the rotor (in radians per second):

$$\mathbf{G = \omega^2 r}$$

The average angular velocity of a rigid body that rotates about a fixed axis is defined as the ratio of the angular displacement in a given time interval. One radian, usually abbreviated as 1 rad, represents the angle subtended at the centre of a circle by an arc with a length equal to the radius of the circle. Since 360° equals $2\boldsymbol{\pi}$ radians, one revolution of the rotor can be expressed as $2\boldsymbol{\pi}$ radians. Accordingly, the angular velocity

in rads per second of the rotor can be expressed in terms of rotor speed s as:

$$\boldsymbol{\omega = \frac{2\pi s}{60}}$$

and therefore the centrifugal field can be expressed as:

$$G = \frac{4\pi^2 (\text{rev min}^{-1})^2 r}{3600} = \frac{4\pi^2 s^2 r}{3600} \qquad (3.3)$$

The centrifugal field is generally expressed in multiples of the gravitational field, g (981cms–2). The relative centrifugal field (g), RCF, which is the ratio of the centrifugal acceleration at a specified radius and the speed to the standard acceleration of gravity, can be calculated from the following equation:

RCF units are therefore dimensionless (denoting multiples of g) and revolutions per minute are usually abbreviated as r.p.m.: RCF=1.12x10 $^{-5}$ r.p.m.^{2} r. Although the relative centrifugal force can easily be calculated, centrifugation manuals usually contain a nomograph for the convenient conversion between

$$\text{RCF} = \frac{4\pi^2 (\text{rev min}^{-1})^2 r}{3600 \times 981} = \frac{G}{g} \qquad (3.4)$$

relative centrifugal force and speed of the centrifuge at different radii of the centrifugation spindle to a point

along the centrifuge tube. A nomograph consists of three columns representing the radial distance (in mm), the relative centrifugal field and the rotor speed (in r.p.m.). For the conversion between relative centrifugal force and speed of the centrifuge spindle in r.p.m. at different radii, a straight-edge is aligned through known values in two columns, then the desired figure is read where the straight-edge intersects the third column. See Figure 3.1 for an illustration of the usage of a nomograph.

In a suspension of biological particles, the rate of sedimentation is dependent not only upon the applied centrifugal field, but also on the nature of the particle, i.e. its density and radius, and also the viscosity of the surrounding medium. Stokes' Law describes these relationships for the sedimentation of a rigid spherical particle:

$$\nu = \frac{2}{9}\frac{r^2(\rho_p - \rho_m)}{\eta} \times g \qquad (3.5)$$

where *V* is the sedimentation rate of the sphere, 2/9 is the shape factor constant for a sphere, r is the radius of particle, ρp is the density of particle, ρm is the density of medium, g is the gravitational acceleration and Z is the viscosity of the medium.
Accordingly a mixture of biological particles exhibiting an approximately spherical shape can be separated in a centrifugal field based on their density and/or their size. The time of sedimentation (in seconds) for a spherical particle is:

$$t = \frac{9}{2}\frac{\eta}{\omega^2 r_p^2(\rho_p - \rho_m)} \times \ln\frac{r_b}{r_t} \qquad (3.6)$$

where t is the sedimentation time, η is the viscosity of medium, $r_p$is the radius of particle, r_b is the radial distance from the centre of rotation to bottom of tube, r_t is the radial distance from the centre of rotation to liquid meniscus, ρp is the density of the particle, ρm is the density of the medium and ω is the angular velocity of rotor.

The sedimentation rate or velocity of a biological particle can also be expressed as its sedimentation coefficient (s), whereby:

$$\boldsymbol{s = V/\omega^2 r} \qquad \textbf{(3.7)}$$

Since the sedimentation rate per unit centrifugal field can be determined at different temperatures and with various media, experimental values of the sedimentation coefficient are corrected to a sedimentation constant theoretically obtainable in water at 20 _C, yielding the S20,W value. The sedimentation coefficients of biological macromolecules are relatively small, and are usually expressed as Svedberg units, S. One Svedberg unit equals 10^{-13} s**.**

Analytical ultracentrifuge:

In an analytical ultracentrifuge, a sample being spun can be monitored in real time through an optical detection system, using ultraviolet light absorption and/or interference optical refractive index sensitive system. This allows the operator to observe the evolution of the sample concentration versus the axis of rotation profile as a result of the applied centrifugal field. With modern instrumentation, these observations are electronically digitized and stored for further mathematical analysis. Two kinds of experiments are commonly performed on these instruments: sedimentation velocity experiments and sedimentation

equilibrium experiments. This instrument has many applications in the fundamental studies of macromolecules showing the molecular weight, purity and shape of the material. It runs at a speed of about 70-80,000 r.p.m. with about 500,000 g and consists of a specially designed rotor in a special rotor chamber which remains under vacuum at low temperature.

There is an arrangement of a special optical system to determine the concentration distributions within the sample during centrifugation.

There are two special optical cells on the rotor, known as the Analytical cell and the Counterpoise cell (Fig. 7.2). There are two holes (Reference holes) in the counterpoise cell for the calibration of distances in the analytical cell. The rotor chamber has an upper and lower lens and the upper lens is joined with a camera lens which emits lights on the photographic plate. Light from the light source comes through the bottom.

Preparative ultracentrifuge:

Preparative ultracentrifuges are available with a wide variety of rotors suitable for a great range of experiments. Most rotors are designed to hold tubes that contain the samples. *Swinging bucket rotors* allow the tubes to hang on hinges so the tubes reorient to the horizontal as the rotor initially accelerates. *Fixed angle rotors* are made of a single block of material and hold the tubes in cavities bored at a predetermined angle. *Zonal rotors* are designed to contain a large volume of sample in a single central cavity rather than in tubes. Some zonal rotors are capable of dynamic loading and unloading of samples while the rotor is spinning at high speed .The whole system is sophisticated with continuously monitoring system of the rotor temperature (temperature sensor). There is also one over speed disk system which checks the rotor so that it does not exceed its maximum allowable speed.

Zonal centrifugation through density gradients

Definition: A method where the components of a sample are separated on the basis of their **density**, in a dense medium or **density gradient**, in a **centrifuge**, according to the centrifugal force they experience. Density gradient centrifugation, developed by Brakke (1951, 1960), is a method that can be used for both isolation and assay of plant viruses. It has proved to be a highly versatile technique and has been widely used in the fields of virology and molecular biology. A centrifuge tube is partially filled with a solution having a decreasing density from the bottom to the top of the tube. For plant viruses, sucrose is commonly used to form the gradient, and the virus solution is layered on top of the gradient. With gradients formed with cesium salts, the virus particles may be distributed throughout the solution at the start of the centrifugation or they may be layered on top of the density gradient.

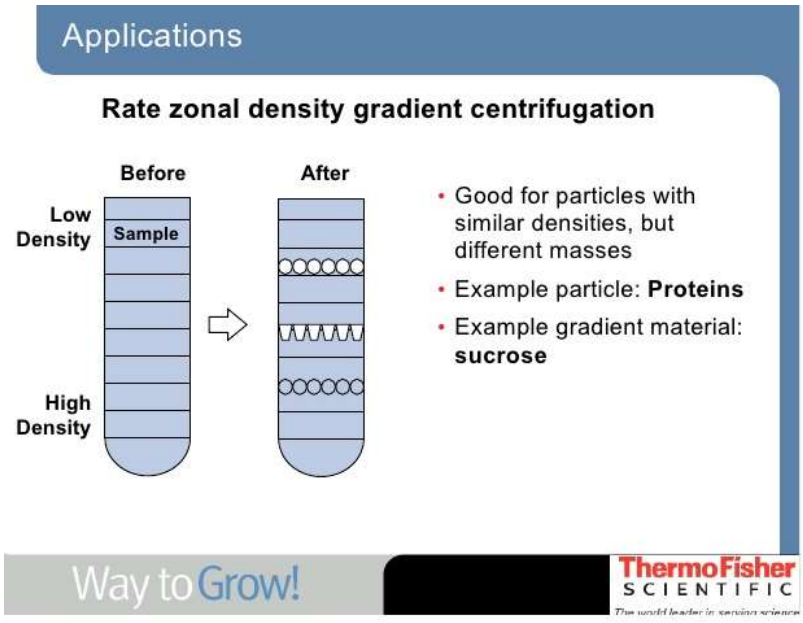
Principle:

Zonal separations in the centrifuge have been obtained by floating a layer of a solution of particles on a second solution which has a density gradient and in which the particles are soluble. During subsequent centrifugation all those particles of the same size, shape, and density sedimented as a zone. The method has been applied to potato yellow dwarf virus (*Aureogenusvastans*) using sucrose solutions for the density gradient. Using crude preparations of virus, infectivity assays showed that 90–95% of the virus was in the visible zone after horizontal centrifugation. Less than 50% of the virus was in the visible zone after angle centrifugation. The virus was not in a density-equilibrium position in either case. Except for a somewhat

greater zone width, results obtained were those to be expected if the virus particles sedimented as individuals and not as aggregates. It has been shown that density gradient centrifugation followed by photometric scanning can be used as a method of analysis. As little as 0.05 mg. virus could be determined in this manner. A zone corresponding to 0.001 mg. was visible to the unaided eye.
Reference : Archives of bio physics and bio chemistry

Method

The theories of density gradient centrifugation are complex. In practice, this technique is a simple and elegant method that has found widespread use in plant virology.
A high-speed preparative ultracentrifuge and appropriate swing-out or angle rotors are required. Following centrifugation, virus bands may be visualized due to their light scattering. The contents of the tube are removed in some suitable way prior to assay. The bottom of the tube can be punctured and the contents allowed to drip into a series of sample tubes. Fractionating devices based on upward displacement of the contents of the tube with a dense sucrose solution are available commercially. The UV absorption of the liquid column is measured and recorded, and fractions of various sizes can be collected as required. Figure 13.5 illustrates the sensitivity of this procedure.
Since successive fractions from a gradient can be collected, a variety of procedures can be used to identify the virus, non-infectious virus-like components, and host materials. These include infectivity, UV absorption spectra, and examination in the electron microscope. Using appropriate procedures, very small differences in sedimentation rate can be detected (Matthews and Witz, 1985).
With rate zonal sedimentation, if the sedimentation coefficients of some components in a mixture are known, approximate values for other components can be estimated. If antisera are available, serological tests can be applied to the fractions, or antiserum can be mixed with the sample before application to the gradient. Components reacting with the antiserum will disappear from the sedimentation pattern.



Probable questions:

1. Define centrifugation.
2. Describe the basic principle of density gradient centrifugation.
3. What is rate zonal centrifugation?
4. State the methodology of ultracentrifugation.

Suggested readings:

Wilson and Walker: Principle and technique of biochemistry and molecular biology, 7th Ed

Science direct

Unit-V

i) Light microscopy, Phase contrast microscopy, Interference Microscopy Polarization microscopy, Fluorescence Microscopy ii) Electron microscopy: a) Transmission b) Scanning

Objective: In this unit, you will learn about Direct observation: i) Light microscopy, Phase contrast microscopy, Interference Microscopy Polarization microscopy, Fluorescence Microscopy ii) Electron microscopy: a) Transmission b) Scanning

Direct observation:

Light microscopy

A light microscope (LM) is an instrument that uses visible light and magnifying lenses to examine small objects not visible to the naked eye, or in finer detail than the naked eye allows. Magnification, however, is not the most important issue in microscopy. Mere magnification without added detail is scientifically useless, just as endlessly enlarging a small photograph may not reveal any more detail, but only larger blurs. The usefulness of any microscope is that it produces better resolution than the eye. Resolution is the ability to distinguish two objects as separate entities, rather than seeing them blurred together as a single smudge. The history of microscopy has revolved largely around technological advances that have produced better resolution.

History of the Light Microscope

Light microscopes date at least to 1595, when Zacharias Jansen (1580–1638) of Holland invented a compound light microscope, one that used two lenses, with the second lens further magnifying the image produced by the first. His microscopes were collapsing tubes used like a telescope in reverse, and produced magnifications up to nine times (9x).

Antony van Leeuwenhoek (1632–1723) invented a simple (one-lens) microscope around 1670 that magnified up to 200x and achieved twice the resolution of the best compound microscopes of his day, mainly because he crafted better lenses. While others were making lenses by such methods as squashing molten glass between pieces of wood, Leeuwenhoek made them by carefully grinding and polishing solid glass. He thus became the first to see individual cells, including bacteria, protozoans, muscle cells, and sperm.

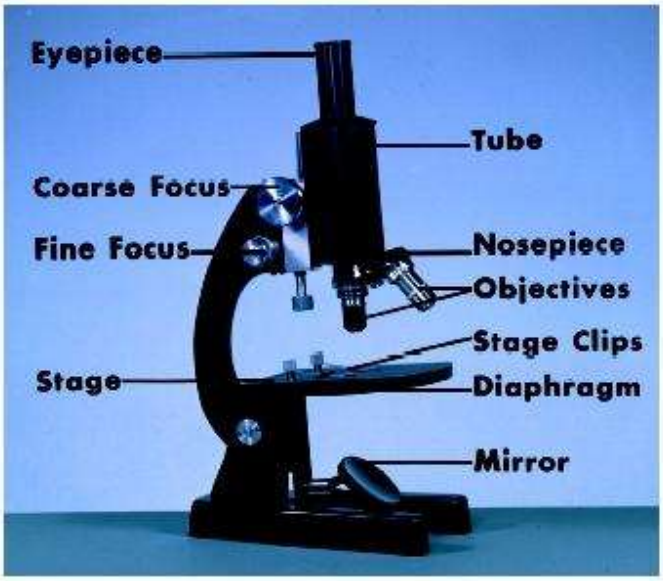
Englishman Robert Hooke (1635–1703) further refined the compound microscope, adding such features as a stage to hold the specimen, an illuminator, and coarse and fine focus controls. Until 1800, compound microscopes designed by Hooke and others were limited to magnifications of 30x to 50x, and their images exhibited blurry edges (spherical aberration) and rainbowlike distortions (chromatic aberration). The most significant improvement in microscope optics was achieved in the nineteenth century, when business partners Carl Zeiss (1816–1888) and Ernst Abbe (1840–1905) added the substage condenser and developed superior lenses that greatly reduced chromatic and spherical aberration, while permitting vastly improved resolution and higher magnification.

Tissue Preparation

The advancement of light microscopy also required methods for preserving plant and animal tissues and making their cellular details more visible, methods collectively called histotechnique (from *histo,* meaning "tissue"). In brief, classical histotechnique involves preserving a specimen in a fixative, such as formalin, to prevent decay; embedding it in a block of paraffin and slicing it very thinly with an instrument called a microtome; removing the paraffin with a solvent; and then staining the tissue, usually with two or more dyes. The slices of tissue, called histological sections, are typically thinner than a single cell. The colors of a prepared tissue are not natural colors, but they make the tissue's structural details more visible. A widely

used stain combination called hematoxylin and eosin, for example, typically colors cell nuclei violet and the cytoplasm pink.

Other methods of histotechnique have been developed for special purposes. One variation is to embed the tissue in special plastics (resins),allowing for thinner sectioning. Another is the frozen section method, in which a tissue is frozen with compressed carbon dioxide and sectioned with a special cold microtome, eliminating the time-consuming process of paraffin embedding. Some prefer this method for its relative simplicity, and its speed is an asset in hospitals, where a biopsied tissue may need to be examined rapidly and the diagnosis reported to the surgeon while the patient is in the operating room.



A compound light microscope.

Varieties of Light Microscopes

Most compound microscopes today have an illuminator built into the base. A condenser located below the stage has lenses that focus the light on the specimen and a diaphragm that regulates contrast. After passing through the specimen on the stage, the light enters an objective lens. Most light microscopes have three or four objective lenses on a rotating turret. These lenses magnify the image by 4x to 100x. The light then passes up the body tube to an ocular lens that magnifies the image another 10x to 15x. Research-grade microscopes and the better student microscopes have a pair of ocular lenses so that one can view the specimen with both eyes at once.

There are many varieties of compound light microscopes for special purposes. For viewing tissue cultures covered with liquid media, biologists can use an inverted light microscope in which the culture is illuminated from above and the objective lenses are positioned below the specimen. The phase contrast microscope can be used to enhance contrast in living specimens, thus avoiding the use of lethal fixatives and stains. The polarizing light microscope is used for analyzing crystals and minerals , among other things. The fluorescence microscope is used to examine structures that bind special fluorescent dyes. It can be used, for example, to identify where a dye tagged hormone binds to its target cell.

Compound light microscopes achieve useful magnifications up to 1200x and resolutions down to about 0.25 micrometers. That is, two objects in a cell can be as close as 0.25 micrometers and still detected as separate entities. Such resolution is good enough to see most bacteria and some mitochondria and microvilli.

These microscopes generally require thin, transparent, relatively small specimens. They also require that the user adjust to the phenomenon of optical inversion; if a specimen is moved to the left, it appears under the microscope to move right; when moved up, it appears to move down; and vice versa. The stereomicroscope works at much lower magnification and resolution, but has several advantages:

(1) it has two lens systems that view the specimen from slightly different angles, thus giving the specimen a stereoscopic (three-dimensional) appearance;

(2) it can use either transmitted or reflected light; and with reflected light, it can be used to view opaque specimens such as rocks, fossils, insects, electronic circuit boards, and so forth;

(3) it has a much greater working distance between the specimen and objective lens, allowing for the examination of relatively large objects and for easier manipulation of objects under the microscope;

(4) the working distance enables relatively easy dissection of specimens such as insects, allowing hands and instruments to reach the working space while one looks through the microscope;

(5) it does not produce optical inversion; that is, movements to the right appear to go to the right, making dissection and other manipulations much easier.

The utility of light microscopy is governed by its use of visible light, which limits resolution. The shorter the wavelength of the illumination, the better the resolution. Electron beams have shorter wavelengths than photons. The invention of the electron microscope in the late 1930s and its refinement over the next half century permitted vastly improved visualization of cell and tissue fine structure.

Phase-contrast microscopy

Phase-contrast microscopy is an optical microscopy technique that converts phase shifts in light passing through a transparent specimen to brightness changes in the image. Phase shifts themselves are invisible, but become visible when shown as brightness variations.

When light waves travel through a medium other than vacuum, interaction with the medium causes the wave amplitude and phase to change in a manner dependent on properties of the medium. Changes in amplitude (brightness) arise from the scattering and absorption of light, which is often wavelength-dependent and may give rise to colors. Photographic equipment and the human eye are only sensitive to amplitude variations. Without special arrangements, phase changes are therefore invisible. Yet, phase changes often carry important information.

The same cells imaged with traditional bright-field microscopy (left) and with phase-contrast microscopy (right)

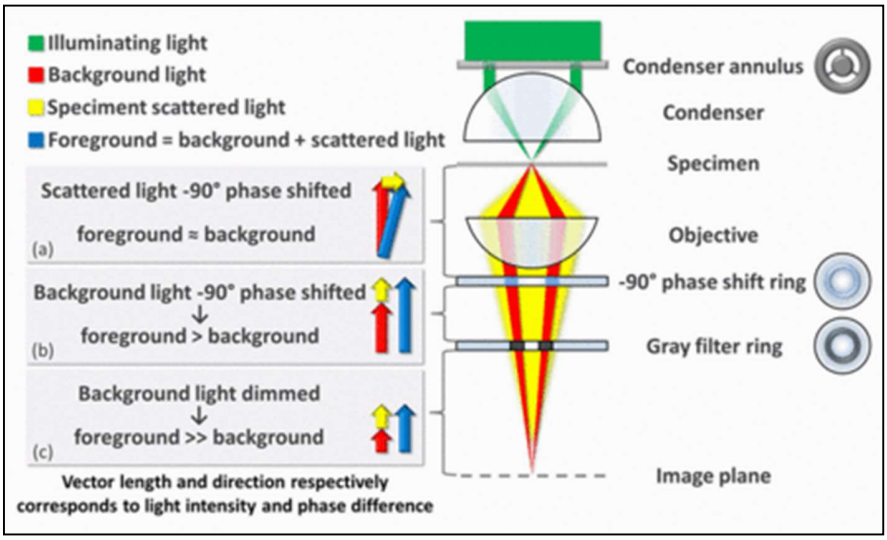
Phase-contrast microscopy is particularly important in biology. It reveals many cellular structures that are not visible with a simpler bright-field microscope, as exemplified in the figure. These structures were made visible to earlier microscopists by staining, but this required additional preparation and thus killing the cells. The phase-contrast microscope made it possible for biologists to study living cells and how they proliferate through cell division. After its invention in the early 1930s, phase-contrast microscopy proved to be such an advancement in microscopy that its inventor Frits Zernike was awarded the Nobel Prize in Physics in 1953.

Working principle

The basic principle to making phase changes visible in phase-contrast microscopy is to separate the illuminating (background) light from the specimen-scattered light (which makes up the foreground details) and to manipulate these differently.

The ring-shaped illuminating light (green) that passes the condenser annulus is focused on the specimen by the condenser. Some of the illuminating light is scattered by the specimen (yellow). The remaining light is unaffected by the specimen and forms the background light (red). When observing an unstained biological specimen, the scattered light is weak and typically phase-shifted by −90° (due to both the typical thickness of specimens and the refractive index difference between biological tissue and the surrounding medium) relative to the background light. This leads to the foreground (blue vector) and background (red vector) having nearly the same intensity, resulting in low image contrast.

In a phase-contrast microscope, image contrast is increased in two ways: by generating constructive interference between scattered and background light rays in regions of the field of view that contain the specimen, and by reducing the amount of background light that reaches the image plane. First, the background light is phase-shifted by −90° by passing it through a phase-shift ring, which eliminates the phase difference between the background and the scattered light rays.



When the light is then focused on the image plane (where a camera or eyepiece is placed), this phase shift causes background and scattered light rays originating from regions of the field of view that contain the sample (i.e., the foreground) to constructively interfere, resulting in an increase in the brightness of these areas compared to regions that do not contain the sample. Finally, the background is dimmed ~70-90% by a gray filter ring—this method maximizes the amount of scattered light generated by the illumination (i.e., background) light, while minimizing the amount of illumination light that reaches the image plane. Some of the scattered light (which illuminates the entire surface of the filter) will be phase-shifted and dimmed by the

rings, but to a much lesser extent than the background light (which only illuminates the phase-shift and gray filter rings).

The above describes negative phase contrast. In its positive form, the background light is instead phase-shifted by +90°. The background light will thus be 180° out of phase relative to the scattered light. The scattered light will then be subtracted from the background light to form an image with a darker foreground and a lighter background, as shown in the first figure.

Interference microscopy

Over the past few years the microscopic techniques had faced with some complications with the observation of living specimens as transparency, light and because of that don't have sufficient contrast.

Thus with the development of these techniques was possible to visualize specimens with adequate contrast.

Interference microscopy or Quantitative interference microscopy

The Interference Microscopy or Quantitative Interference Microscopy is one of these techniques that derive from Phase Contrast Microscopy but is more sensitive than this technique and make possible the easy and clarify viewing of living organisms.

This technique is used by taking light from a condenser and using a prism to separate the light into two beams. Thus, one beam (object beam) goes through the specimen and the objective and the other (reference beam) goes through another objective without touching the specimen. These beams allow a specimen to be seen through the difference in the fields caused by the two beams and the differences of the two images allow details to be seen.

Differential interference contrast microscopy

There is a variation of interference microscopy called Differential Interference Contrast microscopy (DIC), also known as Nomarski Interference Contrast microscopy (NIC) or simply Nomarski microscopy.

This optical microscopy illumination technique used to enhance the contrast in unstained or transparent samples was named after its inventor and also uses two beams produced by a single polarized light.

Initially the polarized light is divided into two rays polarized to each other (sampling and reference rays) when enters in the first Nomarski-modified Wollaston prism. Then this two rays are focused by the condenser for passage through the sample and travel to adjacent areas of the sample, divided by the shear (separation is normally similar to the resolution of the microscope).

After that, they will face different optical way lengths where areas differ in refractive index or thickness which will cause a change in phase of one ray relative to the other according to the delay experienced by the wave in the more optically dense material.

Lastly the rays go through the objective lens and are focused for the second Nomarski-modified Wollaston prism which joins the two rays into one polarized which make an image with a three-dimensional appearance. This final junction of rays leads to interference, brightening or darkening the image at that point according to the optical way difference. These interference techniques have advantages in uses involving living or unstained biological samples, specially their applications in biology, crystallography, mineralogy and chemistry; in standard optical microscopy techniques its resolution and clarity is also visible.

Limitations

On the other hand these techniques also have limitations as its requirement for a transparent sample of similar refractive index. Differential Contrast Microscopy is inadequate for thick samples (tissue slices, pigmented cells) and for most non biological samples because of its polarization dependence.

Polarization microscopy

The polarization **(**polarizing) microscope or "petrographic microscope" is used mainly in geological studies for geological specimens but also in medicine and biology. This type of microscope differs from the normal one by using a polarized light, in which the light waves vibrate in one direction. Unlike the ones from normal light that vibrate in random directions. It's used in anisotropic materials (like minerals) because of their birefringent optical properties – they have several refractive indices. When studying a specimen the light has to pass through a polarizer (polarizing filter) and then in some cases through an analyzer – to increase the quality of image contrast.

What is a polarizing microscope?

A polarizing microscope can be obtain from a compound or a normal one by adding some pieces. The mainly differences between a polarizing microscope and other microscopes:

- A polarizer and analyzer
- A circular rotating stage
- Special plates placed between the object and light path.
- Bertrand lens (if necessary)

A polarizer is a filter that only allows specific light waves or vibrations to pass through it and focus them in a single plane. An analyzer, mainly used as a second polarizer located above the sample, determines the quantity and the direction of the light that illuminates a sample. Due to the use of these filters, the polarized light waves vibrate in one single direction, instead of the normal ones that vibrate in random directions. In this way the polarized light is more concentrated and then more efficient to the study of minerals, for example. By changing the relationship of the polarizer and the analyzer, it's possible to determine the amount of absorbance, reflection and refraction of the light through the microscope.

Important Applications

It is mostly used in the field of geology to study rocks and minerals. Besides that can also be used in medicine, chemistry, biology and some time in metallurgy. it is the best choice to study materials like minerals, polymers, ceramics, wood, urea, substances of natural and synthetic fibers with those birefringent properties, cellophane, and also botanical and insect specimens and fish scales. With polarizing microscopy it is possible to determine the color absorption, structure, composition and refraction of light in isotropic (gases and liquids – one refractive index) and anisotropic substances.

Pathway of Light

The light passes through a polarizing filter called the polarizer (the polarizer is fixed in an east to west vibrational way, but it can be rotated if necessary. There is one more polarizing filter called the analyzer. It is usually situated above the objectives and can be moved in and out of the optical path).

Passes through the birefringent specimen. The polarizer is usually fixed in an east to west vibrational direction, but it can be rotated as required. There is one more polarizing filter called the analyzer. It is usually situated above the objectives and can be moved in and out of the optical path.

Fluorescence microscope

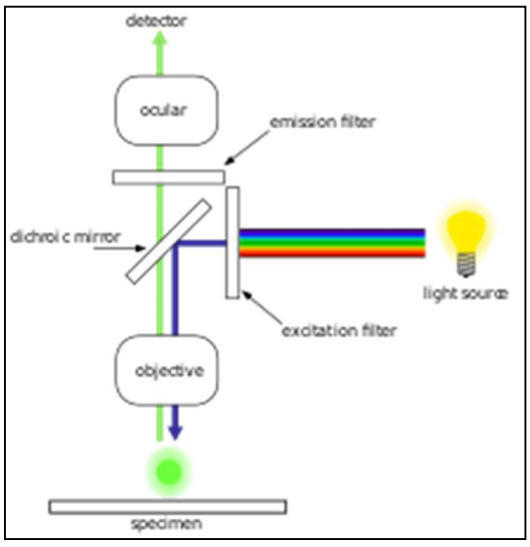
A fluorescence microscope is an optical microscope that uses fluorescence and phosphorescence instead of, or in addition to, reflection and absorption to study properties of organic or inorganic substances. The "fluorescence microscope" refers to any microscope that uses fluorescence to generate an image, whether it is a more simple set up like an epifluorescence microscope, or a more complicated design such as a confocal microscope, which uses optical sectioning to get better resolution of the fluorescent image.

On 8 October 2014, the Nobel Prize in Chemistry was awarded to Eric Betzig, William Moerner and Stefan Hell for "the development of super-resolved fluorescence microscopy," which brings "optical microscopy into the nanodimension

Principle

The specimen is illuminated with light of a specific wavelength (or wavelengths) which is absorbed by the fluorophores, causing them to emit light of longer wavelengths (i.e., of a different color than the absorbed light). The illumination light is separated from the much weaker emitted fluorescence through the use of a spectral emission filter. Typical components of a fluorescence microscope are a light source (xenon arc lampor mercury-vapor lamp are common; more advanced forms are high-power LEDs and lasers), the excitation filter, the dichroic mirror (or dichroic beamsplitter), and the emission filter (see figure below). The filters and the dichroic beamsplitter are chosen to match the spectral excitation and emission characteristics of the fluorophore used to label the specimen.In this manner, the distribution of a single fluorophore (color) is imaged at a time. Multi-color images of several types of fluorophores must be composed by combining several single-color images.

Most fluorescence microscopes in use are epifluorescence microscopes, where excitation of the fluorophore and detection of the fluorescence are done through the same light path (i.e. through the objective). These microscopes are widely used in biology and are the basis for more advanced microscope designs, such as the confocal microscope and the total internal reflection fluorescence microscope (TIRF).



Schematic of a fluorescence microscope.

The majority of fluorescence microscopes, especially those used in the life sciences, are of the epifluorescence design shown in the diagram. Light of the excitation wavelength illuminates the specimen through the objective lens. The fluorescence emitted by the specimen is focused to the detector by the same objective that is used for the excitation which for greater resolution will need objective lens with higher numerical aperture. Since most of the excitation light is transmitted through the specimen, only reflected excitatory light reaches the objective together with the emitted light and the epifluorescence method therefore gives a high signal-to-noise ratio. The dichroic beam splitter acts as a wavelength specific filter, transmitting fluoresced light through to the eyepiece or detector, but reflecting any remaining excitation light back towards the source.

Light source

Fluorescence microscopy requires intense, near-monochromatic, illumination which some widespread light sources, like halogen lamps cannot provide. Four main types of light source are used, including xenon arc lamps or lamps with an excitation filter, lasers, super continuum sources, and high-power LEDs. Lasers are most widely used for more complex fluorescence microscopy techniques like confocal microscopy and total internal reflection fluorescence microscopy while xenon lamps, and mercury lamps, and LEDs with a dichroic excitation filter are commonly used for widefield epifluorescence microscopes. By placing two microlens arrays into the illumination path of a widefield epifluorescence microscope,highly uniform illumination with a coefficient of variation of 1-2% can be achieved.

Sample preparation

In order for a sample to be suitable for fluorescence microscopy it must be fluorescent. There are several methods of creating a fluorescent sample; the main techniques are labelling with fluorescent stains or, in the case of biological samples, expression of a fluorescent protein. Alternatively the intrinsic fluorescence of a sample (i.e., autofluorescence) can be used. In the life sciences fluorescence microscopy is a powerful tool which allows the specific and sensitive staining of a specimen in order to detect the distribution of proteins or other molecules of interest. As a result, there is a diverse range of techniques for fluorescent staining of biological samples.

Limitations

Fluorophores lose their ability to fluoresce as they are illuminated in a process called photobleaching. Photobleaching occurs as the fluorescent molecules accumulate chemical damage from the electrons excited during fluorescence. Photobleaching can severely limit the time over which a sample can be observed by fluorescent microscopy. Several techniques exist to reduce photobleaching such as the use of more robust fluorophores, by minimizing illumination, or by using photoprotective scavenger chemicals.

Fluorescence microscopy with fluorescent reporter proteins has enabled analysis of live cells by fluorescence microscopy, however cells are susceptible to phototoxicity, particularly with short wavelength light. Furthermore, fluorescent molecules have a tendency to generate reactive chemical species when under illumination which enhances the phototoxic effect.

Unlike transmitted and reflected light microscopy techniques fluorescence microscopy only allows observation of the specific structures which have been labeled for fluorescence. For example, observing a tissue sample prepared with a fluorescent DNA stain by fluorescent microscopy only reveals the organization of the DNA within the cells and reveals nothing else about the cell morphologies.

Electron microscopy

An electron microscope is a microscope that uses a beam of accelerated electrons as a source of illumination. As the wavelength of an electron can be up to 100,000 times shorter than that of visible light photons, electron microscopes have a higher resolving power than light microscopes and can reveal the structure of

smaller objects. A scanning transmission electron microscope has achieved better than 50 pm resolution in annular dark-field imaging modeand magnifications of up to about 10,000,000x whereas most light microscopes are limited by diffraction to about 200 nm resolution and useful magnifications below 2000x.

Electron microscopes have electron optical lens systems that are analogous to the glass lenses of an optical light microscope.

Electron microscopes are used to investigate the ultrastructure of a wide range of biological and inorganic specimens including microorganisms, cells, large molecules, biopsy samples, metals, and crystals. Industrially, electron microscopes are often used for quality control and failure analysis. Modern electron microscopes produce electron micrographs using specialized digital cameras and frame grabbers to capture the image.

Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM, also sometimes conventional transmission electron microscopy or CTEM) is a microscopy technique in which a beam of electrons is transmitted through a specimen to form an image. The specimen is most often an ultrathin section less than 100 nm thick or a suspension on a grid. An image is formed from the interaction of the electrons with the sample as the beam is transmitted through the specimen. The image is then magnified and focused onto an imaging device, such as a fluorescent screen, a layer of photographic film, or a sensor such as a charge-coupled device.

Transmission electron microscopes are capable of imaging at a significantly higher resolution than light microscopes, owing to the smaller de Broglie wavelength of electrons. This enables the instrument to capture fine detail—even as small as a single column of atoms, which is thousands of times smaller than a resolvable object seen in a light microscope. Transmission electron microscopy is a major analytical method in the physical, chemical and biological sciences. TEMs find application in cancer research, virology, and materials science as well as pollution, nanotechnology and semiconductor research.

At lower magnifications TEM image contrast is due to differential absorption of electrons by the material due to differences in composition or thickness of the material. At higher magnifications complex wave interactions modulate the intensity of the image, requiring expert analysis of observed images. Alternate modes of use allow for the TEM to observe modulations in chemical identity, crystal orientation, electronic structure and sample induced electron phase shift as well as the regular absorption based imaging.

The first TEM was demonstrated by Max Knoll and Ernst Ruska in 1931, with this group developing the first TEM with resolution greater than that of light in 1933 and the first commercial TEM in 1939. In 1986, Ruska was awarded the Nobel Prize in physics for the development of transmission electron microscopy.

Limitations

There are a number of drawbacks to the TEM technique. Many materials require extensive sample preparation to produce a sample thin enough to be electron transparent, which makes TEM analysis a relatively time consuming process with a low throughput of samples. The structure of the sample may also be changed during the preparation process. Also the field of view is relatively small, raising the possibility that the region analyzed may not be characteristic of the whole sample. There is potential that the sample may be damaged by the electron beam, particularly in the case of biological materials.

Scanning electron microscope (SEM)

A scanning electron microscope (SEM) is a type of electron microscope that produces images of a sample by scanning the surface with a focused beam of electrons. The electrons interact with atoms in the sample, producing various signals that contain information about the surface topography and composition of the

sample. The electron beam is scanned in a raster scan pattern, and the position of the beam is combined with the detected signal to produce an image. SEM can achieve resolution better than 1 nanometer. Specimens are observed in high vacuum in conventional SEM, or in low vacuum or wet conditions in variable pressure or environmental SEM, and at a wide range of cryogenic or elevated temperatures with specialized instruments.

The most common SEM mode is detection of secondary electrons emitted by atoms excited by the electron beam. The number of secondary electrons that can be detected depends, among other things, on specimen topography. By scanning the sample and collecting the secondary electrons that are emitted using a special detector, an image displaying the topography of the surface is created.

Principles and capacities

The signals used by a scanning electron microscope to produce an image result from interactions of the electron beam with atoms at various depths within the sample. Various types of signals are produced including secondary electrons (SE), reflected or back-scattered electrons (BSE), characteristic X-rays and light (cathodoluminescence) (CL), absorbed current (specimen current) and transmitted electrons. Secondary electron detectors are standard equipment in all SEMs, but it is rare for a single machine to have detectors for all other possible signals.

In secondary electron imaging, or SEI, the secondary electrons are emitted from very close to the specimen surface. Consequently, SEI can produce very high-resolution images of a sample surface, revealing details less than 1 nm in size. Back-scattered electrons (BSE) are beam electrons that are reflected from the sample by elastic scattering. They emerge from deeper locations within the specimen and, consequently, the resolution of BSE images is less than SE images. However, BSE are often used in analytical SEM, along with the spectra made from the characteristic X-rays, because the intensity of the BSE signal is strongly related to the atomic number (Z) of the specimen. BSE images can provide information about the distribution, but not the identity, of different elements in the sample. In samples predominantly composed of light elements, such as biological specimens, BSE imaging can image colloidal gold immuno-labels of 5 or 10 nm diameter, which would otherwise be difficult or impossible to detect in secondary electron images. Characteristic X-rays are emitted when the electron beam removes an inner shell electron from the sample, causing a higher-energy electron to fill the shell and release energy. The energy or wavelength of these characteristic X-rays can be measured by Energy-dispersive X-ray spectroscopy or Wavelength-dispersive X-ray spectroscopy and used to identify and measure the abundance of elements in the sample and map their distribution.

Due to the very narrow electron beam, SEM micrographs have a large depth of field yielding a characteristic three-dimensional appearance useful for understanding the surface structure of a sample. This is exemplified by the micrograph of pollen shown above. A wide range of magnifications is possible, from about 10 times (about equivalent to that of a powerful hand-lens) to more than 500,000 times, about 250 times the magnification limit of the best light microscopes.

Fluorescent Microscopes:

Fluorescence microscopy is currently the most widely used contrast technique since it gives superior signal-to-noise ratios (typically white on a black background) for many applications. The most commonly used fluorescence technique is called epifluorescence light microscopy, where ‘epi’ simply means ‘from above’. Here, the light source comes from above the sample, and the objective lens acts as both condenser and objective lens (Fig. 4.10). Fluorescence is popular because of the ability to achieve highly specific labelling of cellular compartments. The images usually consist of distinct regions of fluorescence (white) over large regions of no fluorescence (black), which gives excellent signal-to-noise ratios.

The light source is usually a high-pressure mercury or xenon vapour lamp, and more recently lasers and LED sources, which emit from the UV into the red wavelengths. A specific wavelength of light is used to excite a fluorescent molecule or fluorophore in the specimen. Light of longer wavelength from the excitation of the fluorophore is then imaged. This is achieved in the fluorescence microscope using combinations of filters that are specific for the excitation and emission characteristics of the fluorophore of interest. There are

usually three main filters: an excitation, a dichromatic mirror (often called a dichroic) and a barrier filter, mounted in a single housing above the objective lens. For example, the commonly used fluorophore fluorescein is optimally excited at a wavelength of 488 nm, and emits maximally at 518 nm.

A set of glass filters for viewing fluorescein requires that all wavelengths of light from the lamp be blocked except for the 488 nm light. A filter is available that allows a maximum amount of 488nm light to pass through it (the exciter filter). The 488 nm light is then directed to the specimen via the dichromatic mirror. Any fluorescein label in the specimen is excited by the 488 nm light, and the resulting 518nm light that returns from the specimen passes through both the dichromatic mirror and the barrier filter to the detector. The emission filters only allow light of 518nm to pass through to the detector, and ensure that only the signal emitted from the fluorochrome of interest reaches it. Chromatic mirrors and filters can be designed to filter two or three specific wavelengths for imaging specimens labelled with two or more fluorochromes (multiple labelling). The fluorescence emitted from the specimen is often too low to be detected
by the human eye or it may be out of the wavelength range of detection of the eye, for example, in the far-red wavelengths. A sensitive digital camera easily detects such signals; for example a CCD or a PMT.

More specific dyes are usually used in conjunction with fluorescence microscopy. Immunofluorescence microscopy is used to map the spatial distribution of macromolecules in cells and tissues. The method takes advantage of the highly specific binding of antibodies to proteins. Antibodies are raised to the protein of interest and labelled with a fluorescent probe. This probe is then used to label the protein of interest in the cell and can be imaged using fluorescence microscopy. In practice, cells are usually labelled using indirect immunofluorescence. Here, the antibody to the protein of interest (primary antibody) is further labelled with a second antibody carrying the fluorescent tag (secondary antibody). Such a protocol gives a higher fluorescent signal than using a single fluorescently labelled antibody.

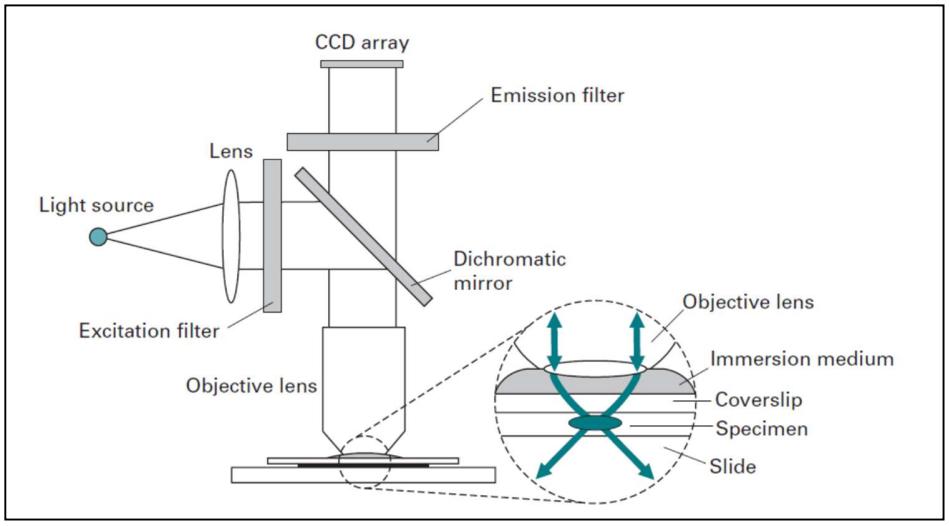


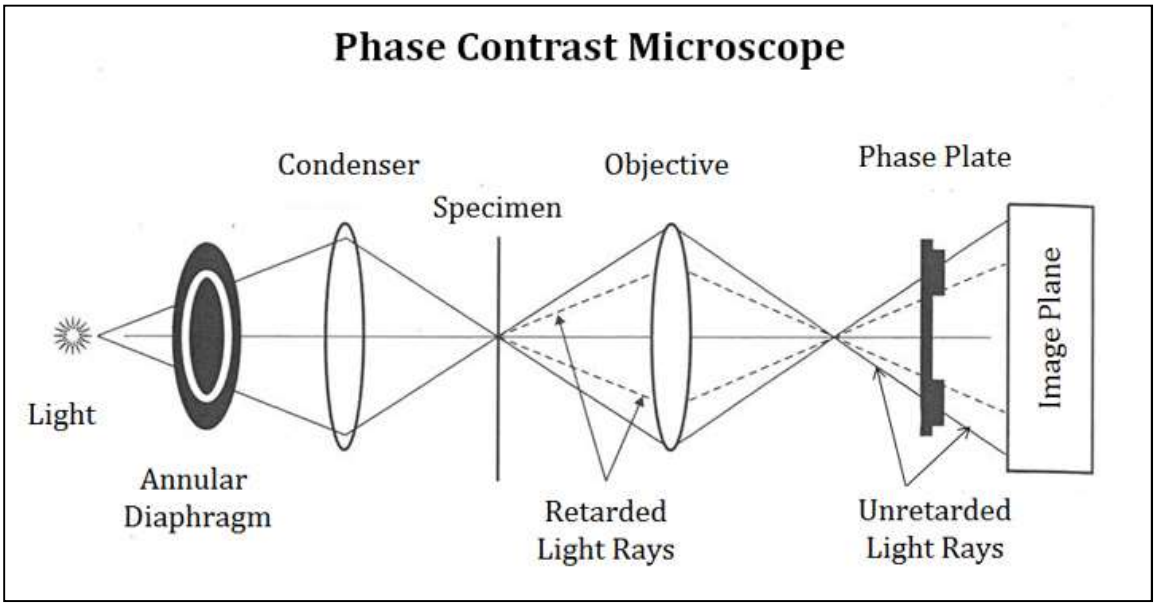
Figure: Epifluorescence microscopy. Light from a xenon or mercury arc lamp (Light source) passes through alens and the excitation filter and reflects off the dichromatic mirror into the objective lens. The objective lensfocusses the light at the specimen via the immersion medium (usually immersion oil) and the glass coverslip(see insert). Any light resulting from the fluorescence excitation in the specimen passes back through theobjective lens, and since it is of longer wavelength than the excitation light, it passes through the dichromaticmirror. The emission filter only allows light of the specific emission wavelength of the fluorochrome of interestto pass through to the CCD array, where an image is formed.

Phase Contrast Microscopy:

Phase contrast is used for viewing unstained cells growing in tissue culture and for testing cell and organelle preparations for lysis. The method images differences in the refractive index of cellular structures. Light that passes through thicker parts of the cell is held up relative to the light that passes through thinner parts of the cytoplasm. It requires a specialized phase condenser and phase objective lenses (both labelled 'ph'). Each phase setting of the condenser lens is matched with the phase setting of the objective lens. These are usually numbered as Phase 1, Phase 2 and Phase 3, and are found on both the condenser and the objective lens.

Applications: Phase contrast is by far the most frequently used method in biological light microscopy. It is an established microscopy technique in cell culture and live cell imaging. When using this inexpensive technique, living cells can be observed in their natural state without previous fixation or labeling.

Principle: Unstained living cells absorb practically no light. Poor light absorption results in extremely small differences in the intensity distribution in the image. This makes the cells barely, or not at all, visible in a brightfield microscope. When light passes through cells, small phase shifts occur, which are invisible to the human eye. In a phase contrast microscope, these phase shifts are converted into changes in amplitude, which can be observed as differences in image contrast. However, this label-free technique is strongly dependent on the correct alignment of components in the optical pathway. This alignment can be disturbed by the naturally occurring meniscus effect, causing weak phase contrast.



Dark Field Microscopy:

Most cells and tissues are colourless and almost transparent, and lack contrast when viewed in a light microscope. Therefore to visualise any details of cellular components it is necessary to introduce contrast into the specimen. This is achieved either by optical means using a specific configuration of microscope components, or by staining the specimen with a dye or, more usually, using a combination of optical and staining methods. Different regions of the cell can be stained selectively with different stains.

Contrast is achieved optically by introducing various elements into the light path of the microscope and using lenses and filters that change the pattern of light passing through the specimen and the optical system. This can be as simple as adding a piece of coloured glass or a neutral density filter into the illuminating light path; by changing the light intensity; or by adjusting the diameter of a condenser aperture.

Usually all of these operations are adjusted until an acceptable level of contrast is achieved for imaging. The most basic mode of the light microscope is called brightfield (bright background),

which can be achieved with the minimum of optical elements. Contrast in brightfield images is usually produced by the colour of the specimen itself. Brightfield is therefore used most often to collect images from pigmented tissues or histological sections or tissue culture cells that have been stained with colourful dyes.

Several configurations of the light microscope have been introduced over the years specifically to add contrast to the final image. Darkfield illumination produces images of brightly illuminated objects on a black background. This technique has traditionally been used for viewing the outlines of objects in liquid media such as living spermatozoa, microorganisms or cells growing in tissue culture, or for a quick check of the status of a biochemical preparation. For lower magnifications, a simple darkfield setting on the condenser will be sufficient. For more critical darkfield imaging at a higher magnification, a darkfield condenser with a darkfield objective lens will be required.

Electron Microscopy:

Principles: Electron microscopy is used when the greatest resolution is required, and when the living state can be ignored. The images produced in an electron microscope reveal the ultrastructure of cells. There are two different types of electron microscope – the transmission electron microscope (TEM) and the scanning electron microscope (SEM). In the TEM, electrons that pass through the specimen are imaged. In the SEM electrons that are reflected back from the specimen (secondary electrons) are collected, and the surfaces of specimens are imaged. The equivalent of the light source in an electron microscope is the electron gun. When a high voltage of between 40,000 and 100, 000 volts (the accelerating voltage) is passed between the cathode and the anode, a tungsten filament emits electrons. The negatively charged electrons pass through a hole in the anode forming an electron beam. The beam of electrons passes through a stack of electromagnetic lenses (the column). Focussing of the electron beam is achieved by changing the voltage across the electromagnetic lenses. When the electron beam passes through the specimen some of the electrons are scattered while others are focussed by the projector lens onto a phosphorescent screen or recorded using photographic film or a digital camera. The electrons have limited penetration power which means that specimens must be thin (50–100 nm) to allow them to pass through.

Preparation of specimens: Contrast in the EM depends on atomic number; the higher the atomic number the greater the scattering and the contrast. Thus heavy metals are used to add contrast in the EM, for example uranium, lead and osmium. Labelled structures appear black or electron dense in the image. All of the water has to be removed from any biological specimen before it can be imaged in the EM. This is because the electron beam can only be produced and focussed in a vacuum. The major drawback of EM observation of biological specimens therefore is the non-physiological conditions necessary for their observation. Nevertheless, the improved resolution afforded by the EM has provided much information about biological structures and biochemical events within cells that could not have been collected using any other microscopical technique.

Extensive specimen preparation is required for EM analysis, and for this reason there can be issues of interpreting the images because of artifacts from specimen preparation. For example, specimens have been traditionally prepared for the TEM by fixation in glutaraldehyde to cross-link proteins followed by osmium tetroxide to fix and stain lipid membranes. This is followed by dehydration in a series of alcohols to remove the water, and then embedding in a plastic such as Epon for thin sectioning.

Small pieces of the embedded tissue are mounted and sectioned on an ultramicrotome using either a glass or a diamond knife. Ultrathin sections are cut to a thickness of approximately 60 nm. The ribbons of sections are floated onto the surface of water and their interference colours are used to assess their thickness. The desired 60 nm section thickness has a silver/gold interference colour on the water surface. The sections are

then mounted onto copper or gold EM grids, and are subsequently stained with heavy metals, for example uranyl acetate and lead citrate.

For the SEM, samples are fixed in glutaraldehyde, dehydrated through a series of solvents and dried completely either in air or by critical point drying. This method removes all of the water from the specimen instantly and avoids surface tension in the drying process thereby avoiding artifacts of drying. The specimens are then mounted onto a special metal holder or stub and coated with a thin layer of gold before viewing in the SEM. Surfaces can also be viewed in the TEM using either negative stains or carbon replicas of air-dried specimens.

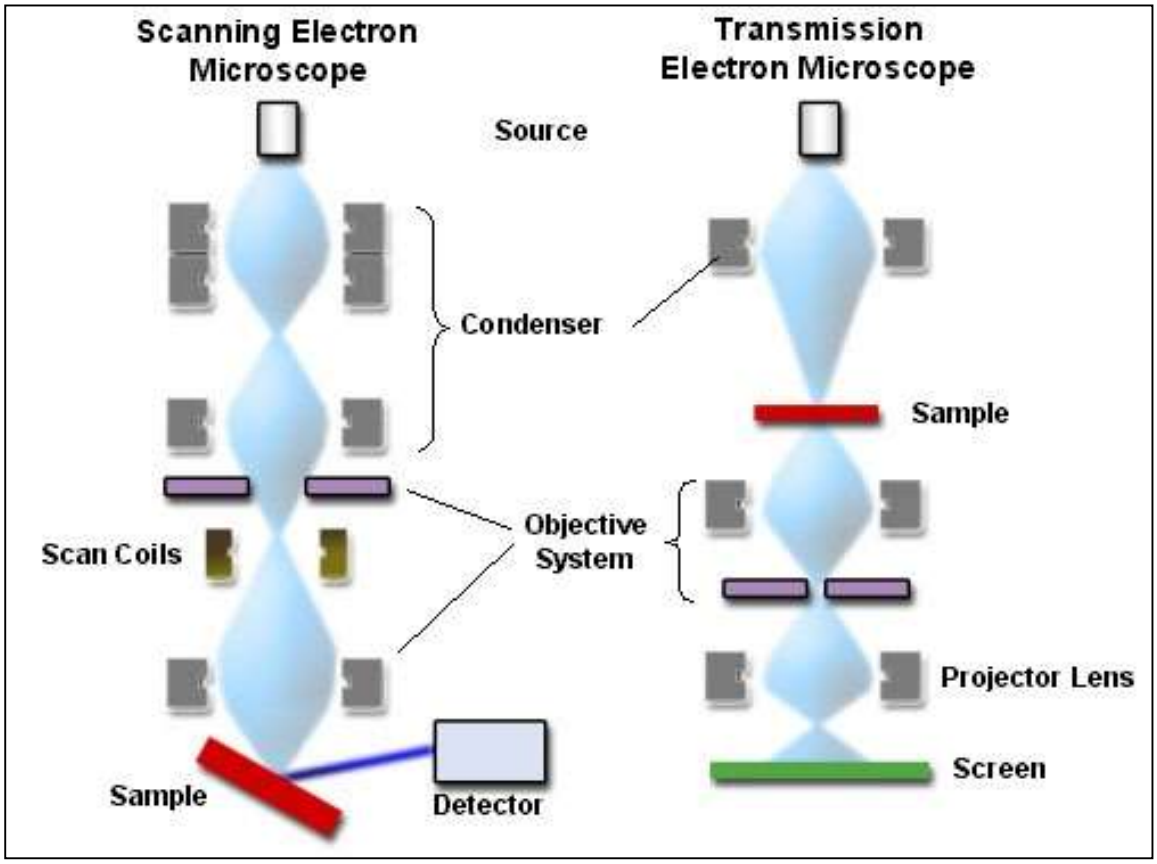


Figure: Diagrammatic representation of scanning and transmission electron microscopes.

Probable questions:

1. What do you mean by the magnification power of a microscope and the limit of resolution?
2. What are the basic difference between LM and EM?
3. Differentiate between dark field and bright field microscopy?
4. State the mechanism of fluorescence microscope.
5. Describe the principle of scanning and transmission electron microscope.

Suggested readings:

Wilson and Walker: Principle and technique of biochemistry and molecular biology, 7th Ed

Unit- VI

Fixation & staining: i) Solutions : Definition, Composition, Expression, Ideal & non-ideal Solution ii) Chemical & physical effects of some primary fixatives: Formalin, alcohol,picric acid, acetic acid

Objective: In this unit, you will learn about Fixation & staining:
i) Solutions : Definition, Composition, Expression, Ideal & non-ideal Solution
ii) Chemical & physical effects of some primary fixatives: Formalin, alcohol, picric acid, acetic acid

Fixation and Staining:

i. Solution: A true solution is made up of at least two components, the dispersed (the solute) and the dispersion (the solvent).

The solute does not settle down and remains evenly dispersed. Its particles are 1 nm or less in size and occur in molecular form. A spoonful of common salt or sucrose when stirred in a beaker full of water gets dissolved to produce a clear solution. Two components are obvious in this experiment; the common salt or sucrose (solute) and the water (solvent).
With constant stirring the solute molecules get evenly dispersed through the solvent in a uniform mixture of the two. In a dilute solution as described above, solute does not settle down but remains evenly dispersed. The solution may be dilute or concentrated depending upon the quantity of the solute added in the solvent.

At a given temperature and pressure, only a specific quantity of solute can be dissolved in a given solvent. The solution in such situations is said to be saturated. In our example, it may be stated that both common salt and sucrose form solutions but these are of different kinds. For instance, sucrose is a non-ionic substance whereas common salt is an ionic substance.

This means that while the former remains intact in water the latter ionizes in water. In other words, common salt (NaCl) breaks into Na^+ and Cl^- ions in water and these are evenly distributed. As a result a true solution is formed.

Depending open the solvent whether it is liquid, solid or gas, following categories of solution may be formed.

(a)	solid—liquid	(b)	liquid—liquid	(c)	gas—liquid
	solid—solid		liquid—solid		gas—solid
	solid—gas		liquid—gas		gas—gas

Water is the most common solvent in the plant and animal cell and its environment. It is universally available and has specific characteristic features. It has highest boiling point and is highly polarized and behaves like dipoles. The heat of vaporization of H_2O is unusually high. Also, water has high surface tension and this accounts for its capillary action.

Three types of solutions occur in the cells:

(i) Solution of a gas in a liquid, carbon dioxide, nitrogen and oxygen are commonly found as gases in solution.

(ii) Solution of liquids in liquids fall into two classes; those in which liquids are freely miscible in all proportions e.g., ethyl alcohol in water and secondly those in which two liquids are sparingly soluble e.g. ether, chloroform in water. Ether and water when shaken thoroughly in a flask and then allowed to stand form distinct layers they are immiscible. The upper layer will be a dilute solution of water in ether and the bottom layer will be a dilute solution of ether in water. The liquid-in-liquid solution is where components are freely miscible; the component in greatest amount is called the solvent.

(iii) Solution of a solid in a liquid is the common type of solution, e.g., solution of sucrose and common salt (NaCl) in water. Sucrose molecule is non-ionic and, therefore, remains as such in water. Sodium chloride (NaCl), on the other hand, is an ionic compound and undergoes ionization in water. Thus, the sodium chloride molecule breaks down to form sodium and chloride ions. These molecules or ions are evenly distributed throughout the water, forming a stable homogenous mixture called a true solution.

Suspension: In a suspension, the particles are greater than 1 µm in size and the particles do not separate into molecules but are aggregates of molecules which can be seen with naked eye. It is an unstable system. When allowed to stand, particles gradually settle down at the bottom of the container separated by a liquid layer. The most common example of suspension is sand in water.

Colloidal System: The sizes of the dispersed particles as well as the properties of the system are midway between the suspension and the true solutions. The size of particles ranges from 0.001 µm to 0.1 µm in diameter and they remain dispersed throughout water in a stable manner, forming a two-phase system.

The liquid phase is called dispersion phase while the solid phase is called the dispersed phase. The colloidal particles are in the form of clusters of molecules. They can be seen under the electron microscope.

Expression of Concentration of a Solution: Concentration of a solution is the relative proportion of the solute in relation to solvent. **It is expressed by any one of the following methods:**

1. Percentage Solution (%) (w/v):
The volume or weight of the solute is calculated as percent of the solution.
This is shown below:
10% sodium chloride = 10 g NaCl dissolved in water till a final volume of 100 ml

2. Molarity (M): Molarity (M) is defined as the number of moles of solute per liter of solution. molarity = moles of solute/liters of solution

3. Molality (m): Molality (m) is defined as the number of moles of solute per kilogram of solvent. molality = moles of solute/kilograms of solvent

Although their spellings are similar, molarity and molality cannot be interchanged. Molarity is a measurement of the moles in the total volume of the solution, whereas molality is a measurement of the moles in relationship to the mass of the solvent.

When water is the solvent and the concentration of the solution is low, these differences can be negligible (d = 1.00 g/mL). However, when the density of the solvent is significantly different than 1 or the concentration of the solution is high, these changes become much more evident.

Example:

Compare the molar and molal volumes of 1 mol of a solute dissolved in CCl_4 (d = 1.59/mL).

For a 1 Molar solution, 1 mol of solute is dissolved in CCl_4 until the final volume of solution is 1 L.

For a 1 molal solution, 1 mol of solute is dissolved in 1 kg of CCl_4.
1 kg of CCl4 × (1,000 g/1 kg) × (mL/1.59 g) = 629 mL CCl_4

4. Normality (N): A normal solution of a substance is obtained by dissolving a gram equivalent weight of that substance in a litre of solution. If we dissolve 2 g equivalent weight in a litre of solution we get a 2N (two normal) solution.
The concentration of acid and alkali solutions is more easily expressed in terms of normality than molarity.

The gram equivalent weight of an acid or base is the quantity that will release or neutralize mole of an hydrogen ions. 1M solution of HCl is also a 1N solution of the acid. However, 1M solution of H_2SO_4 would be 2N since it is capable of releasing 2 moles of hydrogen ions.

5. Parts per Million (ppm):
A gram of solute per million grams of solution or gram of solute per million ml of solution

$$\text{ppm} = \frac{\text{Mass of component}}{\text{Total mass of solution}} \times 10^6$$

or

$$\text{ppm} = \frac{\text{ml of solute}}{\text{ml of solution}} \times 10^6$$

NaCl (1 ppm) in water

1 ppm = 1 mg NaCl/L of solution

= 1 mg NaCl/1000 ml of solution.

- **Ideal and non-ideal Solution:**

The solutions which obey Raoult's law at all compositions of solute in solvent at all temperature are called ideal solution.
Ideal solutions can be obtained by mixing two components with identical molecular size, structure and they should have almost same inter molecular attraction e.g., two liquids A and B form and ideal solution when A – A and B – B molecular attractions will be same and hence A-B molecular attraction will be almost same as A-A and B-B molecular attraction.

Examples of Ideal solutions: Ethyl chloride and ethyl bromide; n-hexane and n-heptane; CCl_4 and $SiCl_4$.The solution which deviate from ideal behaviour are called non ideal solution or real solutions and they do not obey Raoult's law over entire range of composition. It has been found that on increasing dilution, a non ideal solution tend to be ideal. Such a solution show positive deviation from Raoult's Law and the observed boiling point of such solutions is found to be less than the calculated value.e.g. cyclohexane and ethanol. In ethanol the molecules are held together due to hydrogen bonding, when cyclohexane is added to ethanol the molecules of cyclohexane tend to occupy the space between ethanol molecules due to which some hydrogen bonds break due to which inter molecular attraction between cyclohexane and ethanol is reduced and hence show higher vapour pressure. Solutions of above type show negative deviation from Raoult's law and their observed boiling point is found to be higher than the calculated value. For example, when acetone and chloroform are mixed together a hydrogen bond is formed between them which increases inter molecular attraction between them and hence decreases the vapour pressure.

Fixatives:

A large number of chemicals such as ethyl alcohol, formalin, acetic acid, chloroform, mercuric chloride, chromic acid, picric acid, osmic acid, etc. are used singly or in combinations as fixatives for anatomical studies. Amongst these, formalin and formalin-aceto-alcohol (FAA) are most commonly used in Anatomy.
Fixation is the first or the foundation step of the cell/histotechniques done to preserve the tissues in as close a life like state as possible by preventing their autolysis and putrefaction. A number of fixatives exists, either having being in use for decades, or in the case of formaldehyde over a century. Every fixative has different properties, each indicated for a special purpose based on the type of cell component to be studied, the method of sectioning and staining employed and the type of microscopy involved. Thus, a pathologist must have a fair idea of the properties of these commonly available fixatives, so that a correct choice can be made depending upon the desired results. This review aims to give a brief overview of the commonly available fixatives with their merits and their demerits.

The goal of fixation is to preserve structure as faithfully as possible compared to the living state. The three most important parameters to remember about fixation are:

(1) keep the time between killing and fixation to a minimum.
(2) keep the size of the tissue as small as possible without losing information or destroying the tissue. If a large specimen must be fixed, keep one dimension less than 1 mm, or else nick areas of the specimen that can be discarded so that the fixative can penetrate.
(3) keep gross tissue deformation to a minimum by using sharp implements and keeping manipulation of the specimen to the minimum necessary.

- **Formalin Fixative :**

Although various fixatives are available and the information about each fixative is extensive, our focus will be on formalin fixation. The most common and widely accepted fixative is 10% neutral buffered formalin (NBF). This aqueous solution is considered a "universal fixative" since it can be used for a wide variety of stains and techniques.

The properties of formalin are as follows:

- Penetrates tissue quickly, but fixes slowly because cross-linking to tissue proteins takes a long time.
- Causes less tissue shrinkage than other fixatives.
- Hardens tissue, but less than alcohols and acetone.
- Relatively inexpensive and stable.
- Lipids are preserved, but not made insoluble.
- May result in formalin pigment in bloody tissue, but this is prevented by neutralizing the formalin.

Although biopsy specimens may only require several hours to fix properly, larger surgical samples require eight or more hours of fixation prior to the subsequent processing steps. Most small specimens are fixed at room temperature prior to processing, but larger samples will finish fixing in the first step on the tissue processor. Since formalin and paraffin are not miscible (capable of mixing), formalin-fixed tissue must be taken through several intermediate steps before it is placed in paraffin.

- **95% Alcohol**
  PURPOSE: Used as a fixative for frozen sections.

- **Bouin's solution (Yellow)**
  Composition: Picric acid, formaldehyde, and acetic acid.
  Indications: Any tissue (but especially small biopsies).

  Advantages: Fixation in Bouin's will result in sharp Haematoxylin and Eosin staining and is preferred by some pathologists. Bouin's fixation can facilitate finding small lymph nodes. The nodes will remain white and the fat is stained yellow. Prolonged fixation can be used to decalcify tissue.

  Disadvantages: Tissues will become quite brittle and should not be fixed for over 18 hours. Tissues can be transferred to ethanol to avoid this. Large specimens should not be fixed in Bouin's as it will color the entire specimen yellow and it will be difficult to see details grossly. Red cells will be lysed and iron and small calcium deposits dissolved. Immunoperoxidase studies performed on tissues fixed in Bouin's may be less sensitive. Picric acid can cause degradation of DNA and RNA and may interfere with the use of tissues for special studies requiring intact DNA, such as PCR (polymerase chain reaction).

- **Acetic acid**
  Acetic acid (CH_3COOH) is coagulant in action with nucleic acids but generally does not fix proteins. It is incorporated in compound fixatives to help prevent the loss of nucleic acids and, because it swells collagen, to counter the shrinkage caused by other ingredients such as ethanol. Acetic acid penetrates very rapidly but fixatives that contain it will lyse red blood cells.

Probable questions:

1. Define molarity, normality and molality.
2. What do you mean by fixative?
3. Write down the names and mechanism of action of 2 commonly used fixatives.
4. State the advantages and disadvantages of Bouin's fixative.

Suggested readings:

Wilson and Walker: Principle and technique of biochemistry and molecular biology, 7th Ed

Unit- VII

Source and chemical composition of some dyes: Basic fuchsin, carmine, hematin, eosin

Objective: In this unit you will learn about Source and chemical composition of some dyes: Basic fuchsin, carmine, hematin, eosin

Meaning of Stains:
A stain is any colouring organic compound that, when combined with another substance, imparts a colour to that substance. The terms 'dyes' and 'stains' are often used interchangeably by biologists, but they are not the same. The term 'dye' is used to refer to a colouring agent that is used for general purposes, whereas the term 'stain' is used to refer to that dye which is used for biological purposes.

Most of the stains used, particularly for bacteria, are aniline dyes, so called because their derivation from aniline ($C_6H_5NH_2$). The most commonly used aniline dyes are crystal violet, methylene blue, basic fuchsin, safranin, eosin, etc.

Purpose of Staining: Staining is done for any or all of the following three purposes:

(a) To see organism better: Staining enables to see the organism better in contrast with background.

(b) To differentiate one organism from another:
Some microorganisms take colour under the given staining conditions, some do not. Such differences are particularly evident in staining procedures which are therefore called "differential stains", the most common differential stains being the Gram- stain and the acid-fast stain,

(c) To determine particular structures:
There are special stains which react only with certain structures of the organism, e.g., spores, cell wall, nuclei, or others. This is why an organism stained with a cell wall-stain shows only the presence or absence of its cell wall.

Structural Components (Nature) of Stains:
Stains (dyes) usually have complex molecular structure and are chiefly benzene derivatives. A stain consists of three constituents: the organic compound containing a benzene ring, the chromophore, and the auxochrome. Thus a stain may be defined chemically as an organic compound containing both chromophore and auxochrome groups linked to its benzene ring.

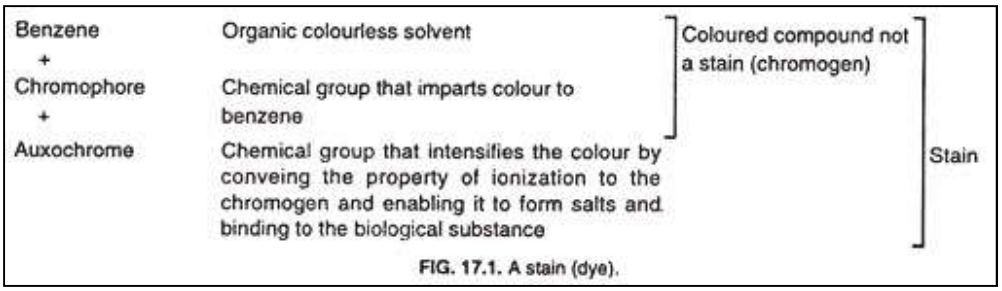


FIG. 17.1. A stain (dye).

Mordant and Its Function:
Mordant is a substance that forms an insoluble compound with a stain and helps to fix the colour to the cell components. Some stains never stain the cells or its components unless treated with a mordant. The mordant

becomes attached to the cell or its components and then combines with the stain to form an insoluble colour complex. This complex is called a lake.

Commonly used mordants are the oxides of aluminium, iron, and chromium. Alizarin is an example of a stain that imparts colour only in collaboration with a mordant. It gives different colours when used with different mordents. It gives red colour with aluminium and tin salts, brownish red colour with a chromium mordant, and black-violets with an iron mordant.

Basic fuchsine:
Basic fuchsine is a mixture of rosaniline, pararosaniline, new fuchsine and Magenta II. Formulations usable for making of Schiff reagent must have high content of pararosanilin. The actual composition of basic fuchsine tends to somewhat vary by vendor and batch, making the batches differently suitable for different purposes.

In solution with phenol (also called carbolic acid) as an accentuator it is called carbol fuchsin and is used for the Ziehl–Neelsen and other similar acid-fast staining of the *Mycobacteria* which cause tuberculosis, leprosy etc. Basic fuchsine is widely used in biology to stain the nucleus, and is also a component of Lactofuchsin, used for Lactofuchsin mounting.

Acetocarmine:
Dry carmine powder … 0-5-1 -0 g
Distilled water … 55 ml
Glacial acetic acid … 45 ml
Preparation: Mix 45 ml glacial acetic acid and 55 ml distilled water and heat to boiling in a conical flask with cotton plugging. Add 0.5 to 1.0 g carmine powder. Shake well, cool and filter and store as stock solution.
Acetocarmine is a non-specific nuclear stain which simply binds the chromosomes and gives colour to them. However, specific nuclear stain (e.g. feulgen) reacts with chromosomes to give them the colour. Acetocarmine is a dye obtained from insects.

Delafield's Haematoxylin:
Reagents:
Saturated aqueous ammonia solution … 400 ml
Haematoxylin … 4 g
90% ethyl alcohol … 25 ml
90% methyl alcohol … 100 ml
Glycerine … 10 ml

Preparation: Dissolve 4 g haematoxylin in 25 ml 90% ethyl alcohol. Mix the solution with 400 ml ammonia solution. Leave the mixture exposed to air and light for 4 days. Add 100 ml 90% methyl alcohol and 10 ml glycerine. Filter and leave to ripen for about 2 months in a bottle plugged with cotton wool.
This haematoxylin is used for staining protozoan parasites. The protozoans are mordented in 3% iron alum first, then stained with haematoxylin.
Hematoxylin and eosin (H&E) stains have been used for at least a century and are still essential for recognizing various tissue types and the morphologic changes that form the basis of contemporary cancer diagnosis. The stain has been unchanged for many years because it works well with a variety of fixatives and displays a broad range of cytoplasmic, nuclear, and extracellular matrix features. Hematoxylin has a deep blue-purple color and stains nucleic acids by a complex, incompletely understood reaction. Eosin is pink and stains proteins nonspecifically. In a typical tissue, nuclei are stained blue, whereas the cytoplasm and extracellular matrix have varying degrees of pink staining. Well-fixed cells show considerable intranuclear detail. Nuclei show varying cell-type- and cancer-type-specific patterns of condensation of heterochromatin (hematoxylin staining) that are diagnostically very important. Nucleoli stain with eosin. If abundant

polyribosomes are present, the cytoplasm will have a distinct blue cast. The Golgi zone can be tentatively identified by the absence of staining in a region next to the nucleus. Thus, the stain discloses abundant structural information, with specific functional implications. A limitation of hematoxylin staining is that it is incompatible with immunofluorescence. It is useful, however, to stain one serial paraffin section from a tissue in which immunofluorescence will be performed. Hematoxylin, generally without eosin, is useful as a counterstain for many immunohistochemical or hybridization procedures that use colorimetric substrates (such as alkaline phosphatase or peroxidase). This protocol describes H&E staining of tissue and cell sections.

Eosin (Aqueous):

Reagents:

Eosin powder ….. 1 g

Distilled water ….. 100 ml

Preparation: Dissolve the eosin powder in 100 ml distilled water.

Probable questions:

1. What are the basic components of a stain?
2. What is mordant?
3. What is H and E staining?
4. Mention the composition of haematoxylin and eosine.

Suggested readings:

Wilson and Walker: Principle and technique of biochemistry and molecular biology, 7th Ed

Unit- VIII

Special application: Finger printing, Southern, Northern & Western transfers

Objective: In this unit you will learn about Special application: Finger printing, Southern, Northern & Western transfers

DNA fingerprinting

This is also known as 'DNA Profiling' o 'DNA Typing'. DNA fingerprinting is a technique to identify a person on the basis of his/her DNA specificity. The practice of using thumbs impression of a person, as an identifying mark is very well known since long. The study of finger, palm and sole prints is called dermatoglyphics and it has been a subject of human interest.

But, the concept of DNA fingerprinting is totally a new approach in the field of molecular biology. Sir Alec Jeffreys (1985-86) invented the DNA fingerprinting technique at Leicester University, United Kingdom.

Meaning:

DNA of an individual carries some specific sequence of bases, which do not carry any information for protein synthesis. Such nucleotide base sequences are repeated many times and are found in many places throughout the length of DNA. The number of repeats is very specific in each individual. The tandem repeats of short sequences are called 'mini satellites' or 'variable number tandem repeats' (VNTRs). Such repeats are used as genetic markers in personal identity.

Technique:

1. The first step is to obtain DNA sample of the individual in question.

2. DNA is also isolated from bloodstains, semen stains or hair root from the body of the victim or from victim's cloth even after many hours of any criminal offence. Even it can be obtained from vaginal swabs of rape victims. The amount of DNA needed for developing fingerprints is very small, only a few nanograms.

3. The DNA is digested with a suitable restriction endonuclease enzyme, which cuts them into fragments.

4. The fragments are subjected to gel electrophoresis by which the fragments are separated according to their size.

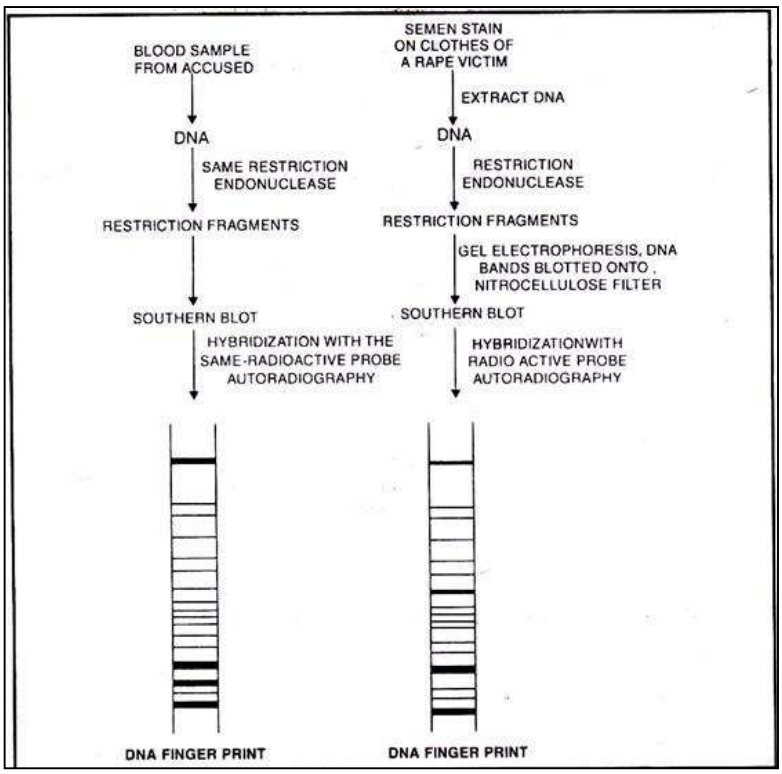


Figure:A Schematic Representation of DNA Fingerprinting of a Person Accused of Rape and of the Semen Stain Recovered from the Clothes of Rape Victim

5. The separated fragments are copied onto a nitrocellulose filter membrane by Southern blotting technique.

6. Special DNA probes are prepared in the laboratory and made radioactive by labeling with radioactive isotopes. These probes contain repeated sequences of bases complimentary to those on mini satellites.

7. The DNA on the nitrocellulose filter membrane is hybridized with the radioactive probes and the free probes are washed off.

8. The bands to which the radioactive probes have been hybridized are detected through autoradiography. This is a technique where an X-ray film is exposed to the nitrocellulose membrane to mark the places where the radioactive DNA probes have bound to the DNA fragments. These places are marked as dark bands when X-ray film is exposed.

9. The dark bands on the X-ray film represent the DNA fingerprints or DNA profiles.

10. Comparison is made between the banding pattern of collected DNA sample and suspected human subject to confirm the criminal with hundred percent accuracy (Fig.5.24).

Significance:

1. The technique is extensively used as confirmatory test in crime detection in cases of rape and murder.

2. Disputed parentage can be solved by the technique.

3. This method can confirm species of more closeness or far apart from evolutionary point of view so that taxonomical problems can be solved.

4. The technique also can be used to study the breeding pattern of endangered animals.

5. Clinically this method can be used in restoring the health of blood cancer patients.

- **Blotting technique:** Blotting technique is an extremely powerful tool for analyzing gene structure and used to study gene expression, once cloned cDNA is isolated. There are three important types of blotting techniques are: 1. Southern Blotting 2. Northern Blotting 3. Western Blotting.

1. Southern Blotting:
Developed by E.M. Southern, the technique of Southern blotting is one of the most important methods used in molecular biology. In Southern blotting, DNA is transferred from a gel to a membrane for hybridization analysis. In this technique, the DNA is cut with suitable restriction enzymes and run on a gel. Treatment with NaOH denatures the DNA to form single strand.

The transfer of DNA from agarose gel to the membrane is performed by capillary action. The gel is placed above the buffer saturated filter paper. The nitrocellulose membrane is placed above the gel and covered by 2-3 layers of dry filter paper towel. A flow of buffer occurs through the gel and membrane to the top papers.

The flow carries the DNA fragment with it. However, DNA cannot pass through the membrane and is fixed firmly to the paper. The membrane, to which the DNA is trapped, is then exposed overnight to a solution containing the radio-labelled cDNA probe. The binding of probe to its complementary sequence is then detected by autoradiography (Fig. 13.3(a)).

Significance:

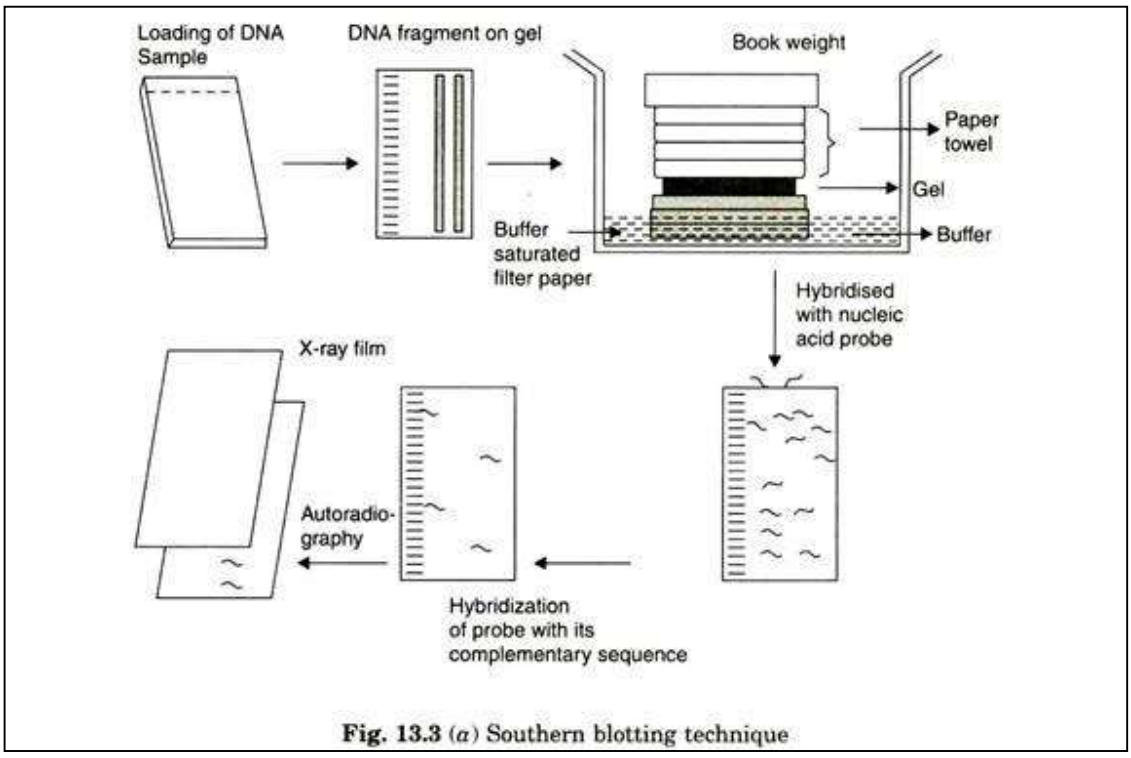


Fig. 13.3 (*a*) Southern blotting technique

Southern blotting is useful for detecting major gene arrangements. This technique plays important role in DNA finger print, identification of novel gene, identification of structurally related genes in the species etc.

2. Northern Blotting:
Northern blotting is a technique used to analyse RNA. In northern blot, RNA is transferred from agarose gel to nitrocellulose paper for hybridization analysis. Total cellular RNA or poly (A) RNA, is separated by size on an agarose gel. The RNA molecules in the gel can be transferred to nitrocellulose or nylon membrane.

The RNA molecules are separated on agarose gel containing formaldehyde or dimethylsulfoxide. The formaldehyde is used to alter secondary structure of RNA molecules. Nitrocellulose filter paper binds strongly to denatured RNA, but not with RNA having secondary structure. The nitrocellulose paper becomes reactive after treating with aminobenzyloxymethyl.

After blotting RNA to chemically reactive paper, they are hybridized to radiolabeled DNA probe. Autoradiography is then carried out to locate RNA bands that are complementary to the probe (Fig. 13.3(b)).

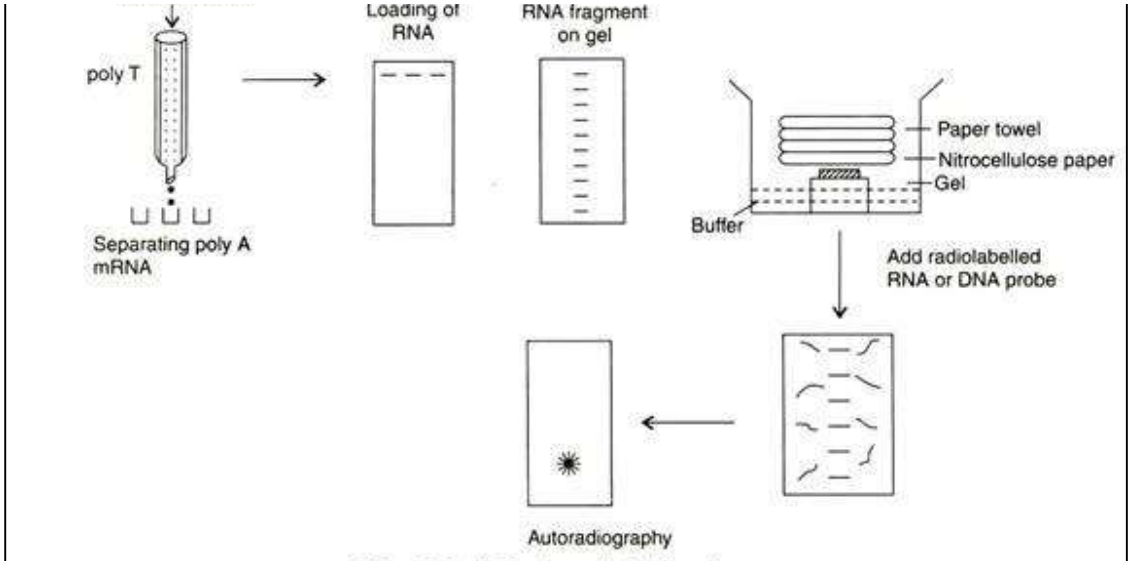


Fig. 13.3 (*b*) Northern hybridization

Significance:
Northern blotting is useful in the identification of a particular gene expression in a tissue or cell type. It is useful in cDNA cloning because the size of a specific mRNA can be compared with the size of cloned cDNA.

3. Western Blotting: Identification of a specific protein in a complex mixture of proteins can be accomplished by a technique known as Western blotting, named for its similarity to Southern blotting, which detects DNA fragments, and Northern blotting, which detects mRNAs.

In Western blotting, a protein is electrophoretically separated on gel (denaturing conditions or native/ non-denaturing conditions).

The proteins are then transferred to a membrane (typically nitrocellulose or PVDF), where they are probed (detected) using antibodies specific to the target protein. The Ag-Ab complexes that form on the band containing the protein recognized by the antibody can be visualized in a variety of ways. If the protein of

interest was bound by a radioactive antibody, its position on the blot can be determined by exposing the membrane to a sheet of X-ray film, a procedure called autoradiography.

However, the most generally used detection procedures employ enzyme-linked antibodies against the protein. After binding of the enzyme-antibody conjugate, addition of a chromogenic substrate that produces a highly coloured and insoluble product causes the appearance of a coloured band at the site of the target antigen. The site of the protein of interest can be determined with much higher sensitivity if a chemiluminescent compound along with suitable enhancing agents is used to produce light at the antigen site.

Western blotting can also identify a specific antibody in a mixture. In this case, known antigens of well-defined molecular weight are separated by SDS-PAGE and blotted onto nitrocellulose. The separated bands of known antigens arc then probed with the sample suspected of containing antibody specific for one or more of these antigens.

Reaction of an antibody with a band is detected by using either radiolabeled or enzyme-linked secondary antibody that is specific for the species of the antibodies in the test sample. The most widely used application of this procedure is in confirmatory testing for HIV, where Western blotting is used to determine whether the patient has antibodies that react with one or more viral proteins.

Steps in a Western Blot:

Tissue Preparation:
Samples may be taken from whole tissue or from cell culture or from cell lysate. In most cases, solid tissues are first broken down mechanically using a blender (for larger sample volumes), using a homogenizer (smaller volumes), or by sonication. Cells may also be broken open by one of the above mechanical methods.

Assorted detergents, salts, and buffers may be employed to encourage lysis of cells and to solubilize proteins. Protease and phosphatase inhibitors are often added to prevent the digestion of the sample by its own enzymes. A combination of biochemical and mechanical techniques — including various types of filtration and centrifugation can be used to separate different cell compartments and organelles.

Gel Electrophoresis:
The proteins of the sample are separated using gel electrophoresis. Separation of proteins may be by isoelectric point (pi), molecular weight, electric charge, or a combination of these factors. The nature of the separation depends on the treatment of the sample and the nature of the gel (for details please refer to "electrophoretic technique").

By far the most common type of gel electrophoresis employs polyacrylamide gels and buffers loaded with sodium dodecyl sulphate (SDS). SDS-PAGE (SDS polyacrylamide gel electrophoresis) maintains polypeptides in a denatured state once they have been treated with strong reducing agents to remove secondary and tertiary structure (e.g., S-S disulphide bonds to SH and SH) and thus allows separation of proteins by their molecular weight.

Sampled proteins become covered in the negatively charged SDS and move to the positively charged electrode through the acrylamide mesh of the gel. Smaller proteins migrate faster through this mesh and the proteins are thus separated according to size (usually measured in kilo daltons, kDa). The concentration of acrylamide determines the resolution of the gel — the greater the acrylamide concentration the better the

resolution of higher molecular weight proteins. Proteins travel only in one dimension along the gel for most blots.

Samples are loaded into wells in the gel. One lane is usually reserved for a marker or ladder, a commercially available mixture of proteins having defined molecular weights, typically stained so as to form visible, coloured bands. When voltage is applied along the gel, proteins migrate into it at different speeds. These different rates of advancement (different electrophoretic mobilities) separate into bands within each lane.

It is also possible to use a two-dimensional (2-D) gel which spreads the proteins from a single sample out in two dimensions. Proteins are separated according to isoelectric point (pH at which they have neutral net charge) in the first dimension, and according to their molecular weight in the second dimension.

Transfer:
In order to make the proteins accessible to antibody detection, they are moved from within the gel onto a membrane made of nitrocellulose or PVDF. The membrane is placed on top of the gel resting on stack of paper towels, and a stack of tissue papers placed on top of that. The entire setup is placed in a buffer solution which moves up the paper by capillary action, bringing the proteins with it.

Another method for transferring the proteins is called electro blotting and uses an electric current to pull proteins from the gel into the PVDF or nitrocellulose membrane. The proteins have now moved from within the gel onto the membrane while maintaining the organization they had within the gel. As a result of this "blotting" process, the proteins are exposed on a thin surface layer for detection.

Both varieties of membrane are chosen for their non-specific protein binding properties (i.e., binds all proteins equally well). Protein binding is based upon hydrophobic interactions as well as charged interactions between the membrane and protein. Nitrocellulose membranes are cheaper than PVDF, but are far more fragile and do not stand up well to repeat probing's.

The uniformity and overall effectiveness of transfer of protein from the gel to the membrane can be checked by staining the membrane with Coomassie or Ponceau S dyes. Coomassie is the more sensitive of the two, although Ponceau S's water solubility makes it easier to subsequently destain and probe the membrane.

Blocking:
Since the membrane has been chosen for its ability to bind protein, and both antibodies and the target are proteins, steps must be taken to prevent interactions between the membrane and the antibody used for detection of the target protein. Blocking of non-specific binding is achieved by placing the membrane in a dilute solution of protein – typically Bovine serum albumin (BSA) or non-fat dry milk (both are inexpensive), with a minute percentage of detergent such as Tween 20.

The protein in the dilute solution attaches to the membrane in all places where the target proteins have not attached. Thus, when the antibody is added, there is no room on the membrane for it to attach other than on the binding sites of the specific target protein. This reduces "noise" in the final product of the Western blot, leading to clearer results, and eliminates false positives.

Detection:
During the detection process the membrane is "probed" for the protein of interest with a modified antibody which is linked to a reported enzyme, which when exposed to an appropriate substrate drives a colorimetric reaction and produces a colour. For a variety of reasons, this traditionally takes place in a two-step process, although there are now one-step detection methods available for certain applications.

(A) Two-step processing:

1. Primary antibody probing:
Antibodies are generated when a host species or immune cell culture is exposed to the protein of interest (or a part thereof). Normally, this is part of the immune response; whereas here they are harvested and used as sensitive and specific detection tools that bind the protein directly.

After blocking, a dilute solution of primary antibody (generally between 0.5 and 5 micrograms/ ml) is incubated with the membrane under gentle agitation. Typically, the solution is comprised of buffered saline solution with a small percentage of detergent, and sometimes with powdered milk or BSA.

The antibody solution and the membrane can be sealed and incubated together for anywhere from 30 minutes to overnight. It can also be incubated at different temperatures, with warmer temperatures being associated with more binding, both specific (to the target protein, the "signal") and non-specific ("noise").

2. Secondary antibody probing:
After rinsing the membrane to remove unbound primary antibody, the membrane is exposed to another antibody, directed at a species-specific portion of the primary antibody. This is known as a secondary antibody, and due to its targeting properties, tends to be referred to as "anti-mouse," "anti-goat," etc. Antibodies come from animal sources (or animal sourced hybridoma cultures); an anti-mouse secondary will bind to just about any mouse-sourced primary antibody.

This allows some cost savings by allowing an entire lab to share a single source of mass-produced antibody, and provides far more consistent results. The secondary antibody is usually linked to biotin or to a reported enzyme such as alkaline phosphatase or horseradish peroxidase. This means that several secondary antibodies will bind to one primary antibody and enhances the signal.

Most commonly, a horseradish peroxidase-linked secondary is used in conjunction with a chemiluminescent agent, and the reaction product produces luminescence in proportion to the amount of protein. A sensitive sheet of photographic film is placed against the membrane, and exposure to the light from the reaction creates an image of the antibodies bound to the blot.

As with the ELISPOT and ELISA procedures, the enzyme can be provided with a substrate molecule that will be converted by the enzyme to a coloured reaction product that will be visible on the membrane. A third alternative is to use a radioactive label rather than an enzyme coupled to the secondary antibody. Since other methods are safer, quicker and cheaper this method is now rarely used.

(B) One step processing:
Historically, the probing process was performed in two steps because of the relative ease of producing primary and secondary antibodies in separate processes. This gives researchers and corporations huge advantages in terms of flexibility, and adds an amplification step to the detection process. Given the advent of high-throughput protein analysis and lower limits of detection, however, there has been interest in developing one-step probing systems that would allow the process to occur faster and with less consumable.

This requires a probe antibody which both recognizes the protein of interest and contains a detectable label probes which are often available for known protein tags. The primary probe is incubated with the membrane in a manner similar to that for the primary antibody in a two-step process, and then is ready for direct detection after a series of wash steps.

Detection:

1. Colorimetric detection:

The colorimetric detection method depends on incubation of the Western blot with a substrate that reacts with the reporter enzyme (such as peroxidase) that is bound to the secondary antibody. This converts the soluble dye into an insoluble form of a different colour that precipitates next to the enzyme and thereby stains the nitrocellulose membrane. Development of the blot is then stopped by washing away the soluble dye. Protein levels are evaluated through densitometry (how intense the stain is) or spectrophotometry.

2. Chemiluminescence:

Chemiluminescent detection methods depend on incubation of the Western blot with a substrate that will luminesce when exposed to the reporter on the secondary antibody. The light is then detected by photographic film, and more recently by CCD cameras which captures a digital image of the Western blot.

The image is analysed by densitometry, which evaluates the relative amount of protein staining and quantifies the results in terms of optical density. Newer software allows further data analysis such as molecular weight analysis if appropriate standards are used. So-called “enhanced chemiluminescent” (ECL) detection is considered to be among the most sensitive detection methods for blotting analysis.

3. Radioactive detection:

Radioactive labels do not require enzyme substrates, but rather allow the placement of medical X-ray film directly against the Western blot which develops as it is exposed to the label and creates dark regions which correspond to the protein bands of interest (see image to the right). The importance of radioactive detection methods is declining, because it is very expensive, health and safety risks are high and ECL provides a useful alternative.

4. Fluorescent detection:

The fluorescently labelled probe is excited by light and the emission of the excitation is then detected by a photo sensor such as CCD camera equipped with appropriate emission filters which captures a digital image of the Western blot and allows further data analysis such as molecular weight analysis and a quantitative Western blot analysis. Fluorescence is considered to be among the most sensitive detection methods for blotting analysis.

Analysis:

After the unbound probes are washed away, the western blot is ready for detection of the probes that are labelled and bound to the protein of interest. In practical terms, not all westerns reveal protein only at one band in a membrane. Size approximations are taken by comparing the stained bands to that of the marker or ladder loaded during electrophoresis.

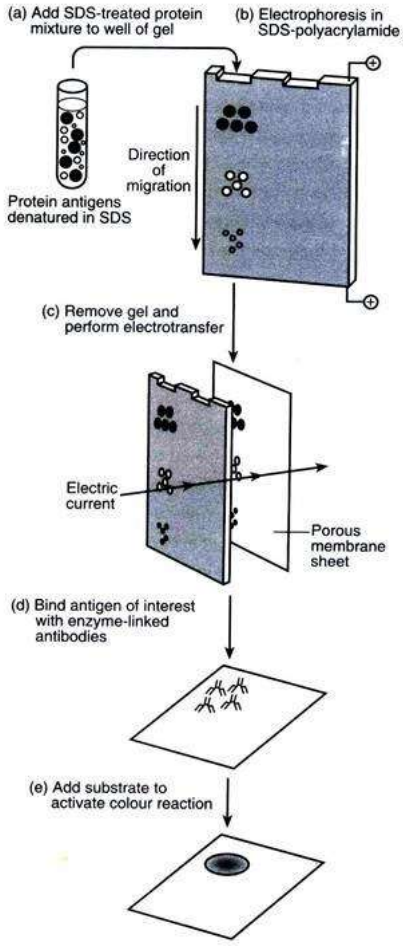


Fig. 6.17: Stern blotting, a protein mixture is (a) treated with SDS, a strong denaturing detergent, (b) then separated by electrophoresis in an SDS polyacrylamide gel (SDS-PAGE) which separates the components according to their molecular weight; lower molecular weight components migrate farther than higher molecular weight ones. (c) The gel is removed from the apparatus and applied to a protein-binding sheet of nitrocellulose or nylon, and the proteins in the gel are transferred to the sheet by the passage of an electric current. (d) Addition of enzyme-linked antibodies detects the antigen of interest, and (e) the position of the antibodies is visualized by means of an ELISA reaction that generates a highly coloured insoluble product that is deposited at the site of the reaction. Alternatively, a chemiluminescent ELISA can be used to generate light that is readily detected by exposure of the blot to a piece of photographic film.

The process is repeated for a structural protein, such as actin or tubulin that should not change between samples. The amount of target protein is indexed to the structural protein to control between groups. This practice ensures correction for the amount of total protein on the membrane in case of errors or incomplete transfers.

Secondary Probing (Stripping):

One major difference between nitrocellulose and PVDF membranes relates to the ability of each to support "stripping" antibodies off and reusing the membrane for subsequent antibody probes. While there are well-established protocols available for stripping nitrocellulose membranes, the sturdier PVDF allows for easier stripping, and for more reuse before background noise limits experiments.

Avoid over stripping the membrane, as target proteins may be lost from the blot during extended incubations. Membrane scanning 9 is done regularly to determine when stripping is complete. If over stripping is a problem, try reducing the amount of SDS in the stripping buffer.

Depending on the strength of the antibody-antigen interactions, one needs to optimize the stringency of stripping protocol. Another difference is that, unlike nitrocellulose, PVDF must be soaked in 95% ethanol, isopropanol or methanol before use. PVDF membranes also tend to be thicker and more resistant to damage during use.

Medical Diagnostic Applications:
1. The confirmatory HIV test employs a Western blot to detect anti-HIV antibody in a human serum sample. Proteins from known HIV-infected cells are separated and blotted on a membrane as above. Then, the serum to be tested is applied in the primary antibody incubation step; free antibody is washed away, and a secondary anti-human antibody linked to an enzyme signal is added. The stained bands then indicate the proteins to which the patient's serum contains antibody.

2. A Western blot is also used as the definitive test for Bovine spongiform encephalopathy (BSE, commonly referred to as 'mad cow disease').

3. Some forms of Lyme disease testing employ Western blotting.

Probable questions:

1. What is northern blotting?
2. Describe the methodology of Southern blotting.
3. What are the advantages of western blotting.
4. State the principle of Western blotting.

Suggested readings:

Wilson and Walker: Principle and technique of biochemistry and molecular biology, 7th Ed

Disclaimer:

The study materials of this book have been collected from various books, e-books, journals and other e sources.

Post-Graduate Degree Programme (CBCS)

in

ZOOLOGY

SEMESTER-III

ELECTIVE THEORY PAPER

CYTOGENETICS AND MOLECULAR BIOLOGY

ZET-301

SELF LEARNING MATERIAL

DIRECTORATE OF OPEN AND DISTANCE LEARNING

UNIVERSITY OF KALYANI

KALYANI, NADIA,

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Director's Message

Satisfying the varied needs of distance learners, overcoming the obstacle of distance and reaching the unreached students are the threefold functions catered by Open and Distance Learning (ODL) systems. The onus lies on writers, editors, production professionals and other personnel involved in the process to overcome the challenges inherent to curriculum design and production of relevant Self Learning Materials (SLMs). At the University of Kalyani a dedicated team under the able guidance of the Hon'ble Vice-Chancellor has invested its best efforts, professionally and in keeping with the demands of Post Graduate CBCS Programmes in Distance Mode to devise a self-sufficient curriculum for each course offered by the Directorate of Open and Distance Learning (DODL), University of Kalyani.

Development of printed SLMs for students admitted to the DODL within a limited time to cater to the academic requirements of the Course as per standards set by Distance Education Bureau of the University Grants Commission, New Delhi, India under Open and Distance Mode UGC Regulations, 2017 had been our endeavour. We are happy to have achieved our goal.

Utmost care and precision have been ensured in the development of the SLMs, making them useful to the learners, besides avoiding errors as far as practicable. Further suggestions from the stakeholders in this would be welcome.

During the production-process of the SLMs, the team continuously received positive stimulations and feedback from Professor (Dr.) Sankar Kumar Ghosh, Hon'ble Vice- Chancellor, University of Kalyani, who kindly accorded directions, encouragements and suggestions, offered constructive criticism to develop it within proper requirements. We gracefully, acknowledge his inspiration and guidance.

Sincere gratitude is due to the respective chairpersons as well as each and every member of PGBOS (DODL), University of Kalyani. Heartfelt thanks is also due to the Course Writers-faculty members at the DODL, subject-experts serving at University Post Graduate departments and also to the authors and academicians whose academic contributions have enriched the SLMs. We humbly acknowledge their valuable academic contributions. I would especially like to convey gratitude to all other University dignitaries and personnel involved either at the conceptual or operational level of the DODL of University of Kalyani.

Their persistent and co-ordinated efforts have resulted in the compilation of comprehensive, learner-friendly, flexible texts that meet the curriculum requirements of the Post Graduate Programme through Distance Mode.

All the Self Learning Materials are self writing and collected from e-book, journals and websites.

Prof Manas Mohan Adhikary
Director
Directorate of Open and Distance Learning
University of Kalyani

ELECTIVE PAPER (ZET-301)

Cytogenetics and Molecular Biology

Module	Unit	Content	Credit	Class	Time (h)	Page No.
ZET - 301 (Cytogenetics and Molecular Biology)	I	Eukaryotic chromosome organization: Packaging of DNA in eukaryotic cell; chromatin structure; histones and non histones; nucleosome; higher order structure of chromatin; domains and scaffold; organization of active chromatin and assembly of chromatin during replication.	2.0	1	1	
	II	Mapping genomes - physical maps, EST, SNPs as physical markers, radiation hybrids, FISH, optical mapping, gene maps, integration of physical and genetic maps; sequencing genomes: high-throughput sequencing, strategies of sequencing, recognition of coding and non-coding regions and annotation of genes, quality of genome-sequence data, base calling and sequence accuracy.		1	1	
	III	Large scale mutagenesis and interference - genome wide gene targeting; systematic approach, random mutagenesis, insertional mutagenesis, libraries of knock-down phenocopies created by RNA interference; transcriptome analysis, DNA micro-array profiling, data processing and presentation, expression profiling, proteomics - expression analysis, protein structure analysis, protein-protein interaction.		1	1	

	IV	Protein folding and processing: Chaperones and folding; enzymes and protein folding, protein cleavage, glycosylation, attachment of lipids.		**1**	**1**	
	V	Current development of chromosome banding techniques and SCE		**1**	**1**	
	VI	Microbial genetics: organization of prokaryotic genome; single stranded DNA phages; RNA phages; cycle and gene expression in SV40 virus; Lytic and lysogenic phage morphogenesis; bacterial conjugation, transduction and transformation.		**1**	**1**	
	VII	Cell signalling: Modes of cell-cell signalling; steroid hormones and steroid hormone superfamily, neurotransmitters; peptide hormones and growth factors; eicosanoids, functions of cell surface receptors.		**1**	**1**	
	VIII	G-protein coupled receptors, tyrosine kinases, cytokine receptors; pathways of intercellular signal transduction, camp, C GMP pathways; Phospholipids and Ca ion, Ras, Raf and MAP kinase pathway, JAK/STAT pathway.		**1**	**1**	

Unit-I

Eukaryotic chromosome organization: Packaging of DNA in eukaryotic cell; chromatin structure; histones and non-histones; nucleosome; higher order structure of chromatin; domains and scaffold; organization of active chromatin and assembly of chromatin during replication

Objective: In this unit you will learn about eukaryotic chromosome organization. You will learn about Packaging of DNA in eukaryotic cell; chromatin structure; histones and nonhistones; nucleosome; higher order structure of chromatin; domains and scaffold; organization of active chromatin and assembly of chromatin during replication.

Introduction:

Eukaryotic genomes contain levels of complexity that are not encountered in prokaryotes. In contrast to prokaryotes, most eukaryotes are diploid, having two complete sets of genes, one from each parent. Although eukaryotes have only about 2 to 15 times as many genes as *E. coli,* they have orders of magnitude more DNA. Moreover, much of this DNA does not contain genes, at least not genes encoding proteins or RNA molecules. Not only do most eukaryotes contain many times the amount of DNA in prokaryotes, but also this DNA is packaged into several chromosomes, and each chromosome is present in two (diploids) or more (polyploids) copies. Recall that the chromosome of *E. coli* has a contour length of 1500μm, or about 1.5 mm. Now consider that the haploid chromosome complement, or genome, of a human contains about 1000 mm of DNA (or about 2000 mm per diploid cell). Moreover, this meter of DNA is subdivided among 23 chromosomes of variable size and shape, with each chromosome containing 15 to 85 mm of DNA. In the past, geneticists had little information as to how this DNA was arranged in the chromosomes. Is there one molecule of DNA per chromosome as in prokaryotes, or are there many? If many, how are the molecules arranged relative to each other? How does the 85 mm (85,000 μm) of DNA in the largest human chromosome get condensed into a mitotic metaphase structure that is about 0.5 μm in diameter and 10 μm long?

CHEMICAL COMPOSITIONOF EUKARYOTIC CHROMOSOMES:

Interphase chromosomes are usually not visible with the light microscope. However, chemical analysis, electron microscopy, and X-ray diffraction studies of isolated chromatin (the complex of the DNA, chromosomal proteins, and other chromosome constituents isolated from nuclei) have provided valuable information about the structure of eukaryotic chromosomes. When chromatin is isolated from interphase nuclei, the individual chromosomes are not recognizable. Instead, one observes an irregular aggregate of nucleoprotein. Chemical analysis of isolated chromatin shows that it consists primarily of DNA and proteins with lesser amounts of RNA (Figure 9.16). The proteins are of two major classes: (1) basic (positively charged at neutral pH) proteins called histones and (2) a heterogeneous, largely acidic (negatively charged at neutral pH) group of proteins collectively referred to as nonhistone chromosomal proteins.

Histones play a major structural role in chromatin. They are present in the chromatin of all eukaryotes in amounts equivalent to the amounts of DNA. This relationship suggests that an interaction occurs between histones and DNA that is conserved in eukaryotes. The histones of all plants and animals consist of five classes of proteins.

These five major histone types, called *H1, H2a, H2b, H3*, and *H4*, are present in almost all cell types. A few exceptions exist, most notably some sperm, where the histones are replaced by another class of small basic proteins called protamines.

The five histone types are present in molar ratios of approximately 1 H1:2 H2a:2H2b:2 H3:2 H4. Four of the five types of histones are specifically complexed with DNA to produce the basic structural subunits of chromatin, small (approximately 11 nm in diameter by 6.5 nm high) ellipsoidal beads called nucleosomes. The histones have been highly conserved during evolution—four of the five types of histone are similar in all eukaryotes.

Most of the 20 amino acids in proteins are neutral in charge; that is, they have no charge at pH 7. However, a few are basic and a few are acidic. The histones are basic because they contain 20 to 30 percent arginine and lysine, two positively charged amino acids (see Figure 12.1). The exposed -$NH3^{+}$groups of arginine and lysine allow histones to act as polycations. The positively charged side groups on histones are important in their interaction with DNA, which is polyanionic because of the negatively charged phosphate groups.

The remarkable constancy of histones H2a, H2b, H3, and H4 in all cell types of an organism and even among widely divergent species is consistent with the idea that they are important in chromatin structure (DNA packaging) and are only non-specifically involved in the regulation of gene expression. However, as will be discussed later, chemical modifications of histones can alter chromosome structure, which, in turn, can enhance or decrease the level of expression of genes located in the modified chromatin.
In contrast, the nonhistone protein fraction of chromatin consists of a large number of heterogeneous proteins. Moreover, the composition of the nonhistone chromosomal protein fraction varies widely among different cell types of the same organism. Thus, the nonhistone chromosomal proteins probably do not play central roles in the packaging of DNA into chromosomes. Instead, they are likely candidates for roles in regulating the expression of specific genes or sets of genes.

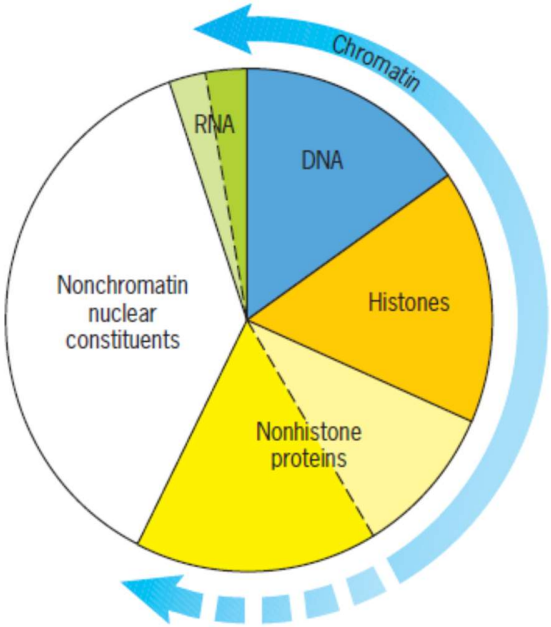


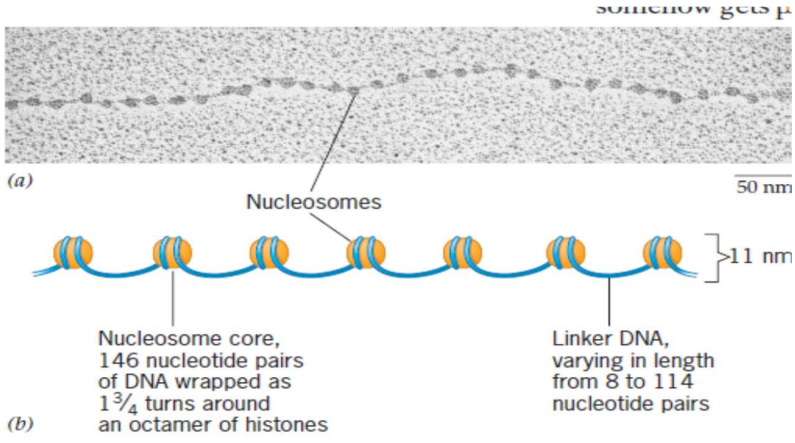
Figure 9.16 The chemical composition of chromatin as a function of the total nuclear content. The DNA and histone contents of chromatin are relatively constant, but the amount of nonhistone proteins present depends on the procedure used to isolate the chromatin (dashed arrow).

ONE LARGE DNA MOLECULE PER CHROMOSOME:

A typical eukaryotic chromosome contains 1 to 20 cm of DNA. During metaphase of meiosis and mitosis, this DNA is packaged in a chromosome with a length of only 1 to 10 um. How is all of this DNA condensed into the compact chromosomes that are present during mitosis and meiosis?
Considerable evidence now indicates that each chromosome contains a single, giant molecule of DNA that extends from one end through the centromere all the way to the other end of the chromosome. However, as we will discuss in the following section, this giant DNA molecule is highly condensed (coiled and folded) within the chromosome.

THREE LEVELS OF DNA PACKAGINGIN EUKARYOTIC CHROMOSOMES:

The largest chromosome in the human genome contains about 85 mm (85,000 um, or8.5 _ 107 nm) of DNA that is believed to exist as one giant molecule. This DNA molecule somehow gets packaged into a metaphase structure that is about 0.5 um in diameter and about 10 um in length—a condensation of almost 104-fold in length from the naked DNA molecule to the metaphase chromosome. How does this condensation occur? What components of the chromosomes are involved in the packaging processes? Is there a universal packaging scheme? Are there different levels of packaging? Clearly, meiotic and mitotic chromosomes are more extensively condensed than interphase chromosomes. What additional levels of condensation occur in these special structures that are designed to assure the proper segregation of the genetic material during cell divisions?
Are DNA sequences of genes that are being expressed packaged differently from those of genes that are not being expressed? Let us investigate some of the evidence that establishes the existence of three different levels of packaging of DNA into chromosomes. When isolated chromatin from interphase cells is examined by electron microscopy, it is found to consist of a series of ellipsoidal beads (about 11 nm in diameter and 6.5 nm high) joined by thin threads (Figure 9.17*a*). Further evidence for a regular, periodic packaging of DNA has come from studies on the digestion of chromatin with various nucleases. Partial digestion of chromatin with these nucleases yielded fragments of DNA in a set of discrete sizes that were integral multiples of the smallest size fragment. These results are nicely explained if chromatin has a repeating structure, supposedly the bead seen by electron microscopy(Figure 9.17*a*), within which the DNA is packaged in a nuclease-resistant form (Figure 9.17*b*). This "bead" or chromatin subunit is called the nucleosome. According to the present concept of chromatin structure, the linkers, or interbead threads of DNA, are susceptible to nuclease attack.



■ **FIGURE 9.17** Electron micrograph (*a*) and low-resolution diagram (*b*) of the beads-on-a-string nucleosome substructure of chromatin isolated from interphase nuclei. *In vivo*, the DNA linkers are probably wound between the nucleosomes forming a condensed 11-nm fiber.

Figure 9.17 Electron micrograph (a) and low-resolution diagram(b) of the beads-on-a-string nucleosome substructure of chromatin isolated from interphase nuclei. In vivo, the DNA linkers are probably wound between the nucleosomes forming a condensed 11-nm fibre.

After partial digestion of the DNA in chromatin with an endonuclease (an enzyme that cleaves DNA internally), DNA approximately 200 nucleotide pairs in length is associated with each nucleosome (produced by a cleavage in each linker region). After extensive nuclease digestion, a 146-nucleotide-pair-longsegment of DNA remains present in each nucleosome. This nuclease-resistant structure is called the nucleosome core. Its structure—essentially invariant in eukaryotes—consists of a 146-nucleotide-pair length of DNA and two molecules each of histones H2a, H2b, H3, and H4. The histones protect the segment of DNA in the nucleosome core from cleavage by endonucleases. Physical studies (X-ray diffraction and similar analyses) of nucleosome-core crystals have shown that the DNA is wound as 1.65 turns of a superhelix around the outside of the histone octamer (Figure 9.18*a*).

The complete chromatin subunit consists of the nucleosome core, the linker DNA, and the associated nonhistone chromosomal proteins, all stabilized by the binding of one molecule of histone H1 to the outside of the structure(Figure 9.18*b*).

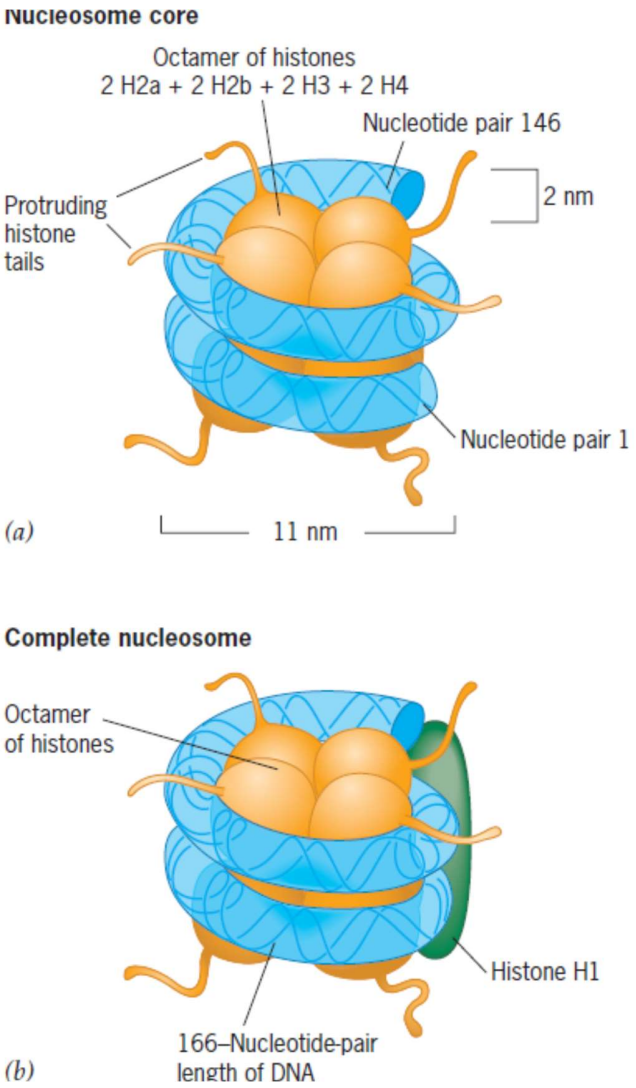


Figure 9.18 Diagrams of the gross structure of (a)the nucleosome core and (b) the complete nucleosome. The nucleosome core contains 146 nucleotide pairs wound as 1.65 turns of negatively supercoiled DNA around an octamer of histones—two molecules each of histones H2a, H2b, H3, and H4. The complete nucleosome contains 166 nucleotide pairs that form almost two superhelical turns of DNA around the histone octamer. One molecule of histone H1 is thought to stabilize the complete nucleosome.

The size of the linker DNA varies from species to species and from one cell type to another. Linkers as short as eight nucleotide pairs and as long as 114 nucleotide pairs have been reported. Evidence suggests that the complete nucleosome (as opposed to the nucleosome core) contains two full turns of DNA superhelix (a 166-nucleotide-pair length of DNA) on the surface of the histone octamer and the stabilization of this structure by the binding of one molecule of histone H1 (Figure 9.18*b*).
The structure of the nucleosome core has been determined with resolution to 0.28 nm by X-ray diffraction studies. The resulting high-resolution map of the nucleosome core shows the precise location of all eight histone molecules

and the 146 nucleotide pairs of negatively supercoiled DNA (Figure 9.19*a* and *b*). Some of the terminal segments of the histones pass over and between the turns of the DNA superhelix to add stability to the nucleosome. The interactions between the various histone molecules and between the histones and DNA are seen most clearly in the structure of one-half of the nucleosome core (Figure 9.19*c*),which contains only 73 nucleotide pairs of supercoiled DNA.

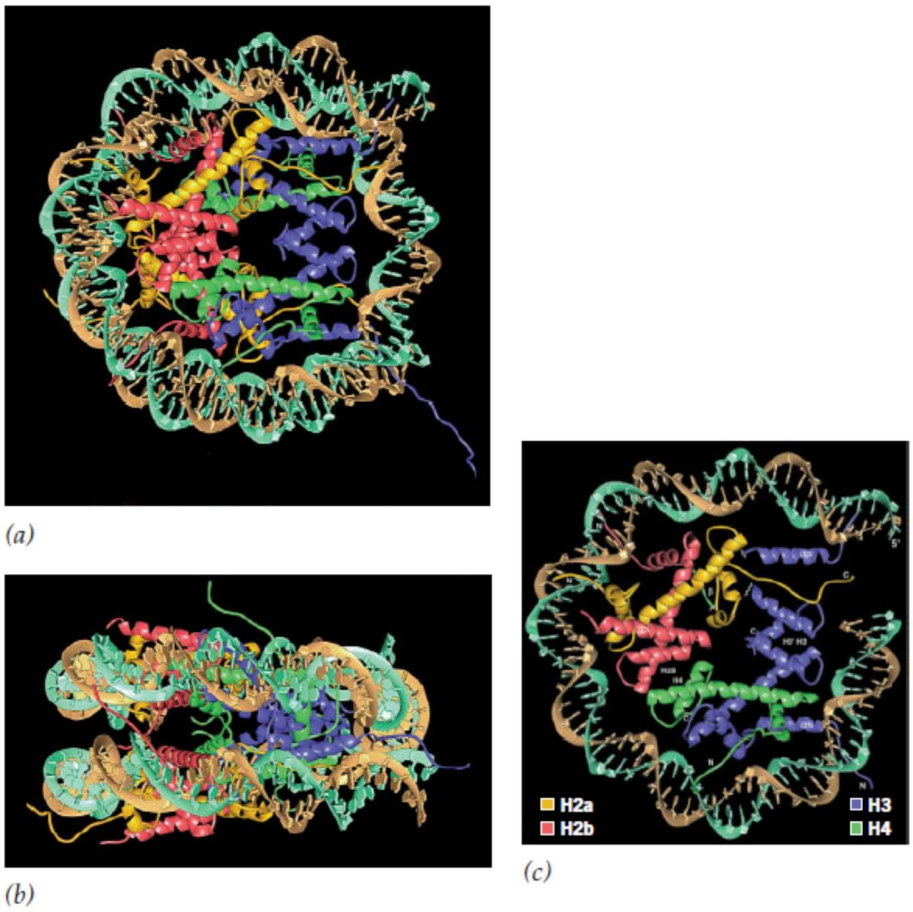


Figure 9.19 Structure of the nucleosome core based on X-ray diffraction studies with 0.28-nm resolution. The macromolecular composition of the nucleosome core is shown looking along (a) or perpendicular to (b) the axis of the superhelix. (c) Diagram of the structure of a half nucleosome, which shows the relative positions of the DNA superhelix and the histones more clearly. The complementary strands of DNA are shown in brown and green, and histones H2a, H2b, H3, and H4 are shown in yellow, red, blue, and green, respectively.

The basic structural component of eukaryotic chromatin is the nucleosome. But are the structures of all nucleosomes the same? What role(s), if any, does nucleosome structure play in gene expression and regulation of gene expression? The structure of nucleosomes in transcriptionally active regions of chromatin is known to differ from that of nucleosomes in transcriptionally inactive regions. But what are the details of this structure–function relationship? The tails of some of the histone molecules protrude from the nucleosome and are accessible to enzymes that add and remove chemical groups such as methyl (—CH3) and acetyl groups. The addition of these groups can change the level of expression of genes packaged in nucleosomes containing the modified histones.
Electron micrographs of isolated metaphase chromosomes show masses of tightly coiled or folded lumpy fibres (Figure 9.20). These chromatin fibres have an average diameter of 30 nm. When the structures seen by light and electron microscopy during earlier stages of meiosis are compared, it becomes clear that the light microscope simply permits one to see those regions where these 30-nmfibres are tightly packed or condensed. Indeed, when interphase chromatin is isolated using very gentle procedures, it also consists of 30-nm fibres (Figure 9.21*a*).

However, the structure of these fibres seems to be quite variable and depends on the procedures used. When observed by cryo-electron microscopy(microscopy using quickly frozen chromatin rather than fixed chromatin),the 30-nm fibres show less tightly packed "zigzag" structures (Figure 9.21*b*).

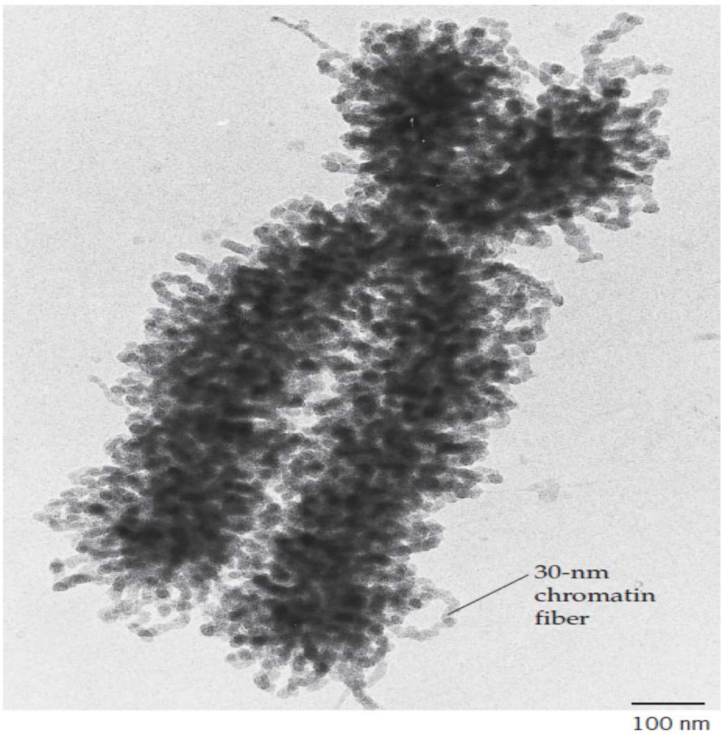


Figure 9.20 Electron micrograph of a human metaphase chromosome showing the presence of 30-nm chromatin fibres. The available evidence indicates that each chromatid contains one large, highly coiled or folded 30-nm fibre.

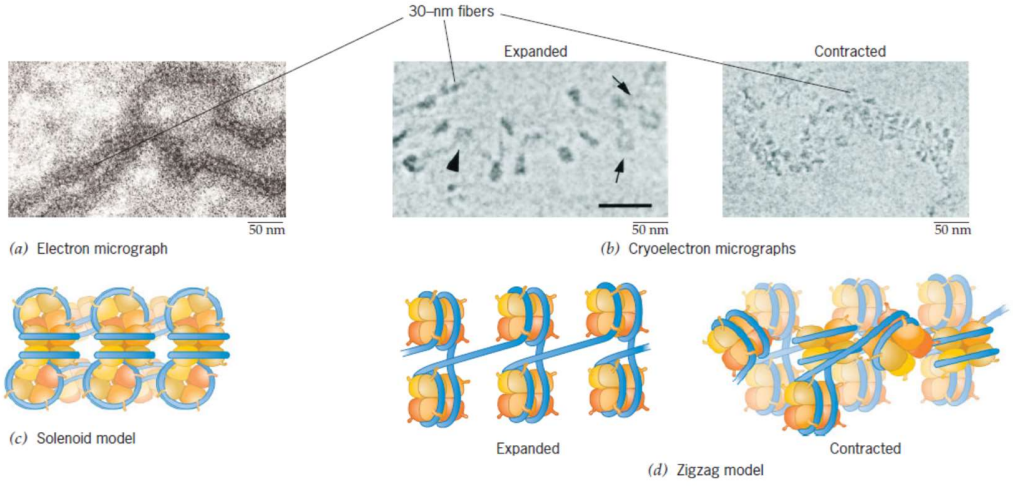


Figure 9.21 Electron micrograph (a) and cryo-electron micrographs (b) of the 30-nmchromatin fibres in eukaryotic chromosomes. The structure of 30-nm chromatin fibres seems to vary based on the procedures used to isolate and photograph them. (c) According to one popular model, the 30-nm fibre is produced by coiling the 11-nm nucleosome fibre into a solenoid structure with six nucleosomes per turn. (d) However, when chromatin is visualized after cryopreservation (quick freezing) without fixation, it exhibits a zigzag structure whose density—expanded versus contracted—varies with ionic strength and with chemical modifications of the histone molecules.

The two most popular models are the solenoid model (Figure 9.21*c*) and the zigzag model (Figure 9.21*d*). *In vivo,* the nucleosomes clearly interact with one another to condense the 11-nmnucleosomes into 30-nm chromatin fibres. Whether these have solenoid structures or zigzag structures, or both, depending on the conditions, is still uncertain. What is certain is that chromatin structure is not static; chromatin can expand and contract in response to chemical modifications of histone H1 and the histone tails that protrude from the nucleosomes.
Metaphase chromosomes are the most condensed of normal eukaryotic chromosomes. Clearly, the role of these highly condensed chromosomes is to organize and package the giant DNA molecules of eukaryotic chromosomes into structures that will facilitate their segregation to daughter nuclei without the DNA molecules of different chromosomes becoming entangled and, as a result, being broken during the anaphase separation of the daughter chromosomes. As we noted in the preceding section, the basic structural unit of the metaphase chromosome is the 30-nm chromatin fibre. However, how are these30-nm fibres further condensed into the observed metaphase structure?
Unfortunately, there is still no clear answer to this question. There is evidence that the gross structure of metaphase chromosomes is not dependent on histones. Electron micrographs of isolated metaphase chromosomes from which the histones have been removed reveal a scaffold**,** or central core, which is surrounded by a huge pool or halo of DNA.

This chromosome scaffold must be composed of nonhistone chromosomal proteins. Note the absence of any apparent ends of DNA molecules in the micrograph shown in Figure 9.22; this finding again supports the concept of one giant DNA molecule per chromosome. In summary, at least three levels of condensation are required to package the103 to 105 um of DNA in a eukaryotic chromosome into a metaphase structure a few microns long (Figure 9.23).

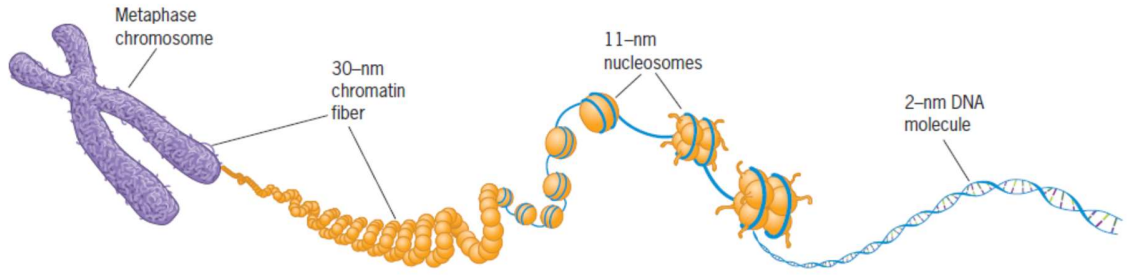


Figure 9.23. Diagram showing the different levels of DNA packaging in chromosomes. The 2-nm DNA molecule is first condensed into 11-nm nucleosomes, which are further condensed into 30-nm chromatin fibres. The 30-nm fibres are then segregated into supercoiled domains or loops via their attachment to chromosome scaffolds composed of nonhistone chromosomal proteins.

1. The first level of condensation involves packaging DNA as a negative supercoil into nucleosomes, to produce the 11-nm-diameter interphase chromatin fibre. This clearly involves an octamer of histone molecules, two each of histones H2a, H2b,H3, and H4.

2. The second level of condensation involves an additional folding or supercoiling of the 11-nm nucleosome fibre, to produce the 30-nm chromatin fibre. Histone H1 is involved in this supercoiling of the 11-nm nucleosome fibre to produce the 30-nmchromatin fibre.

3. Finally, nonhistone chromosomal proteins form a scaffold that is involved in condensing the 30-nm chromatin fibre into the tightly packed metaphase chromosomes. This third level of condensation appears to involve the separation of segments of the giant DNA molecules present in eukaryotic chromosomes into independently supercoiled domains or loops. The mechanism by which this third level of condensation occurs is not known.

REPEATED DNA SEQUENCES:

The centromeres and telomeres discussed in this chapter contain DNA sequences that are repeated many times. Indeed, the chromosomes of eukaryotes contain many DNA sequences that are repeated in the haploid chromosome complement, sometimes as many as a million times. DNA containing such repeated sequences, called **repetitive DNA,** is a major component (15 to 80 percent) of eukaryotic genomes.

The first evidence for repetitive DNA came from centrifugation studies of eukaryotic DNA. When the DNA of a prokaryote, such as *E. coli*, is isolated, fragmented, and centrifuged at high speeds for long periods of time in a 6*M* cesium chloride(CsCl) solution, the DNA will form a single band in the centrifuge tube at the position where its density is equal to the density of the CsCl solution. For *E. coli*, this band will form at a position where the CsCl density is equal to the density of DNA containing about 50 percent A:T and 50 percent G:C base pairs.

DNA density increases with increasing G:C content. The extra hydrogen bond in a G:C base pair results in a tighter association between the bases and thus a higher density than for A:T base pairs. The centrifugation of DNAs from eukaryotes to equilibrium conditions in such CsCl solutions usually reveals the presence of one large main band of DNA and one to several small bands. These small bands of DNA are called satellite bands (from the Latin word *satelles,* meaning "an attendant" or "subordinate")and the DNAs in these bands are often referred to as satellite DNAs**.** For example, the genome of *Drosophila virilis*, a distant relative of *Drosophila melanogaster,* contains three distinct satellite DNAs, each composed of a repeating sequence of seven base pairs.

Other satellite DNAs in eukaryotes have long repetitive sequences. Much of what we know about the types of repeated DNA sequences in the chromosomes of various eukaryotic species resulted from DNA renaturation experiments. The two strands of a DNA double helix are held together by a large number of relatively weak hydrogen bonds between complementary bases. When DNA molecules in aqueous solution are heated to near 100_C, these bonds are broken and the complementary strands of DNA separate. This process is called denaturation. If the complementary single strands of DNA are cooled slowly under the right conditions, the complementary base sequences will find each other and will re-form base-paired double helices. This reformation of double helices from the complementary single strands of DNA is called renaturation.

If a DNA sequence is repeated many times, denaturation will yield a large number of complementary single strands that will renature rapidly, faster than the rate of renaturation of sequences that are present only once in the genome. Indeed, the rate of DNA renaturation is directly proportional to copy number (the number of copies of the sequence in the genome)—the higher the copy number, the faster the rate and the less time required for renaturation. Mathematical analyses of the rates of renaturation of DNA sequences in eukaryotic genomes provided strong evidence for the presence of different classes of repeated DNA sequences, or repetitive DNA, in eukaryotic chromosomes. The recent genome sequencing projects have provided additional information about the different types of repetitive DNA sequences in eukaryotic genomes, and ongoing sequencing projects are providing information about the sequence variability that occurs in human populations. The locations of different DNA sequences in chromosomes can be determined directly by procedures similar to the renaturation experiments described here. With this procedure, called *in situ* hybridization, labelled strands of DNA form double helices with denatured DNA still present in chromosomes.

The most highly repeated sequences in eukaryotic genomes do not encode proteins. Indeed, they are not even transcribed. Other less repetitive sequences encode proteins, such as ribosomal proteins and the muscle proteins actin and myosin that are needed in large amounts and are each encoded by several genes. The genes that specify ribosomal RNAs are also multicopy genes because cells need large amounts of ribosomal RNA to produce the ribosomes required for protein synthesis.

The most prevalent of the repeated DNA sequences are transposable genetic elements**,** DNA sequences that can move from one location in a chromosome to another or even to a different chromosome, or inactive sequences derived from transposable elements. In *D. melanogaster,* about 90 different families of transposable elements have been characterized and been given interesting names such as *hobo, pogo,* and *gypsy* that suggest their mobility. A much larger proportion—between 40 and 50 percent—of the human genome contains transposable elements or sequences derived from them. As much as 80 percent of the corn genome may consist of transposable genetic elements or their derivatives.

DUPLICATION OF NUCLEOSOMES AT REPLICATION FORKS:

The DNA in eukaryotic interphase chromosomes is packaged in approximately 11-nm beads called nucleosomes. Each nucleosome contains 166 nucleotide pairs of DNA wound in two turns around an octamer of histone molecules. Given the size of nucleosomes and the large size of DNA replisomes, it seems unlikely that a replication fork can move past an intact nucleosome. Yet, electron micrographs of replicating chromatin in *Drosophila* clearly show nucleosomes with approximately normal structure and spacing on both sides of replication forks (Figure 10.33*a*); that is, nucleosomes appear to have the same structure and spacing immediately behind a replication fork (post replicative DNA) as they do in front of a replication fork (prereplicative DNA). This observation suggests that nucleosomes must be disassembled to let the replisome duplicate the DNA packaged in them and then be quickly reassembled; that
is, DNA replication and nucleosome assembly must be tightly coupled. Since the mass of the histones in nucleosomes is equivalent to that of the DNA, large quantities of histones must be synthesized during each cell generation in order for the nucleosomes to duplicate. Although histone synthesis occurs throughout the cell cycle, there is a burst of histone biosynthesis during S phase that generates enough histones for chromatin duplication. When density-transfer experiments were performed to examine the mode of nucleosome duplication, the nucleosomes on both progeny DNA molecules were found to contain both old (prereplicative) histone complexes and new (postreplicative) complexes. Thus, at the protein level, nucleosome duplication appears to occur by a dispersive mechanism.

A number of proteins are involved in the disassembly and assembly of nucleosomes during chromosome replication in eukaryotes. Two of the most important are *n*ucleosome *a*ssembly *p*rotein-*1* (Nap-1) and *c*hromatin *a*ssembly *f*actor-*1* (CAF-1). Nap-1 transports histones from their site of synthesis in the cytoplasm to the nucleus, and CAF-1 carries them to the chromosomal sites of nucleosome assembly (Figure 10.33*b*). CAF-1 delivers histones to the sites of DNA replication by binding to PNCA (*p*roliferating *c*ell *n*uclear *a*ntigen)—the clamp that tethers DNA polymerase to the DNA template (Figure 10.32). CAF-1 is an essential protein in *Drosophila,* but not in yeast where other proteins can perform some of its functions.

Many other proteins affect nucleosome structure. Some are involved in chromatin remodeling—changing nucleosome structure in ways that activate or silence the expression of the genes packaged therein. Others modify nucleosome structure by adding methyl or acetyl groups to specific histones. In addition, eukaryotes contain several minor histones with structures slightly different from the major histones, and the incorporation of these minor histones into nucleosomes can change their structure. In *Drosophila,* for example, the incorporation of histone H3.3 into nucleosomes results in high levels of transcription of the genes therein. Thus, nucleosome structure is not invariant; to the contrary, it plays an important role in modulating gene expression.

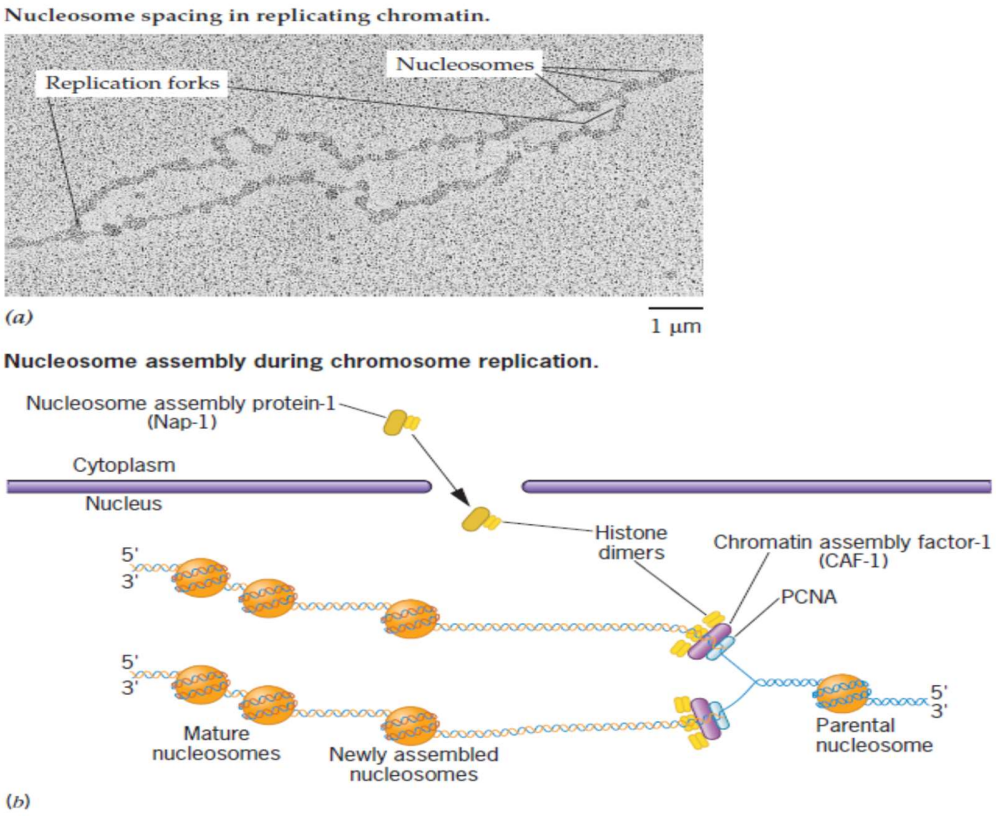


Figure 10.33 The disassembly and assembly of nucleosomes during the replication of chromosomes in eukaryotes. (a) An electron micrograph showing nucleosomes on both sides of two replication forks in Drosophila. Recall that DNA replication is bidirectional; thus, each branch point is a replication fork. (b) The assembly of new nucleosomes during chromosome replication requires proteins that transport histones from the cytoplasm to the nucleus and that concentrate them at the site of nucleosome assembly. PCNA - proliferating cell nuclear antigen.

Assembly of Chromatin in replication:

The duplication of a chromosome requires replication of the DNA and the reassembly of the associated proteins on each daughter DNA molecule. The latter process is tightly linked to DNA replication to ensure that the newly replicated DNA is rapidly packaged into nucleosomes. Although the replication of DNA requires the nucleosome disassembly, the DNA is rapidly repackaged into nucleosomes in an ordered series of events. The first step in the assembly of a nucleosome is the binding of an H3.H4 tetramer to the DNA. Once the tetramer is bound, two H2A.H2B dimers associate to form the final nucleosome. H1 joins this complex last, presumably during the formation of higher-order chromatin assemblies.

To duplicate a chromosome, at least half of the nucleosomes on the daughter chromosomes must be newly synthesized. The fate of the old histones is a particularly important issue given the effects that histone modification can have on the accessibility of the resulting chromatin. If the old histones were lost completely, then chromosome duplication would erase any “memory” of the previously modified nucleosomes. In contrast, if the old histones were retained on a single chromosome, that chromosome would have a distinct set of modifications relative to the other copy of the chromosome. Mixing is not entirely random, however. H3.H4 tetramers and H2A.H2B dimers are composed of either all new or all old histones. Thus, as the replication fork passes, nucleosomes are broken down into their

component subassemblies. H3.H4 tetramers appear to remain bound to one of the two daughter duplexes at random and are never released from DNA into the free pool of histones. In contrast, the H2A.H2B dimers are released and enter the local pool, available for new nucleosome assembly.
The distributive inheritance of old histones during chromosome duplication provides a mechanism for the propagation of the parental pattern of histone modification. By this mechanism, old modified histones will tend to rebind one of the daughter chromosomes at a position near their previous position on the parental chromosome. The old histones have an equal probability of binding either daughter chromosome. This localized inheritance of modified histones ensures that a subset of the modified histones is located in similar positions on each daughter chromosome

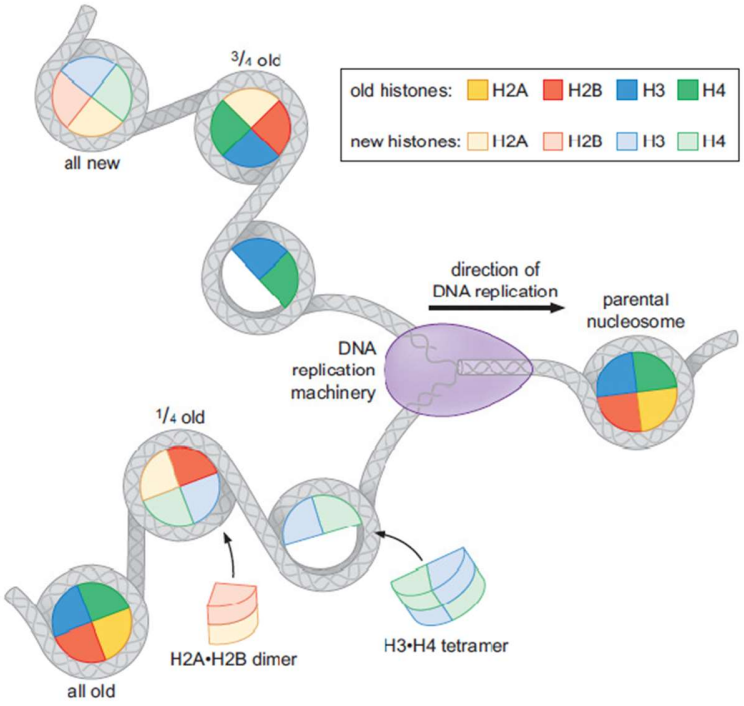


Figure: Inheritance of histone and assembly of chromatin during replication.

Probable questions:

1. Write down the chemical composition of Eukaryotic chromosome.
2. How DNA are packed into chromosomes.
3. Name histone proteins which took part in nucleosome formation.
4. What is scaffold?
5. What are the role of histone and non histone proteins in DNA packaging?
6. How duplication of nucleosomes occur at replication forks?
7. What do you now about repeated DNA sequences?

Suggested readings:

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7th Edition.
6. Gene cloning and DNA Analysis by T. A. Brown, Sixth Edition.
7. Genetics . Verma and Agarwal.

Unit-II

Mapping genomes - physical maps, EST, SNPs as physical markers, radiation hybrids, FISH, optical mapping, gene maps, integration of physical and genetic maps; sequencing genomes: high-throughput sequencing, strategies of sequencing, recognition of coding and non-coding regions and annotation of genes, quality of genome-sequence data, base calling and sequence accuracy

Objective: In this unit you will learn physical maps, EST, SNPs as physical markers, radiation hybrids, FISH, optical mapping, gene maps, integration of physical and genetic maps; sequencing genomes: high-throughput sequencing, strategies of sequencing, recognition of coding and non-coding regions and annotation of genes, quality of genome-sequence data, base calling and sequence accuracy

Gene mapping describes the methods used to identify the locus of a gene and the distances between genes.The essence of all genome mapping is to place a collection of molecular markers onto their respective positions on the genome. Molecular markers come in all forms. Genes can be viewed as one special type of genetic markers in the construction of genome maps, and mapped the same way as any other markers.

Genetic and Physical Maps

The convention is to divide genome mapping methods into two categories.

- Genetic mapping is based on the use of genetic techniques to construct maps showing the positions of genes and other sequence features on a genome. Genetic techniques include cross-breeding experiments or, in the case of humans, the examination of family histories (pedigrees).
- Physical mapping uses molecular biology techniques to examine DNA molecules directly in order to construct maps showing the positions of sequence features, including genes.

Genetic Mapping

As with any type of map, a genetic map must show the positions of distinctive features. In a geographic map these markers are recognizable components of the landscape, such as rivers, roads and buildings. What markers can we use in a genetic landscape?

Genes were the first markers to be used

The first genetic maps, constructed in the early decades of the 20th century for organisms such as the fruit fly, used genes as markers. This was many years before it was understood that genes are segments of DNA molecules. Instead, genes were looked upon as abstract entities responsible for the transmission of heritable characteristics from parent to offspring. To be useful in genetic analysis, a heritable characteristic has to exist in at least two alternative forms or phenotypes, an example being tall or short stems in the pea plants originally studied by Mendel. Each phenotype is specified by a different allele of the corresponding gene. To begin with, the only genes that could be studied were those specifying phenotypes that were distinguishable by visual examination. So, for example, the first fruit-fly maps showed the positions of genes for body color, eye color, wing shape and suchlike, all of these phenotypes being visible simply by looking at the flies with a

low-power microscope or the naked eye. This approach was fine in the early days but geneticists soon realized that there were only a limited number of visual phenotypes whose inheritance could be studied, and in many cases their analysis was complicated because a single phenotype could be affected by more than one gene. For example, by 1922 over 50 genes had been mapped onto the four fruit-fly chromosomes, but nine of these were for eye color; in later research, geneticists studying fruit flies had to learn to distinguish between fly eyes that were colored red, light red, vermilion, garnet, carnation, cinnabar, ruby, sepia, scarlet, pink, cardinal, claret, purple or brown. To make gene maps more comprehensive it would be necessary to find characteristics that were more distinctive and less complex than visual ones.

The answer was to use biochemistry to distinguish phenotypes. This has been particularly important with two types of organisms - microbes and humans. Microbes, such as bacteria and yeast, have very few visual characteristics so gene mapping with these organisms has to rely on biochemical phenotypes such as those listed in With humans it is possible to use visual characteristics, but since the 1920s studies of human genetic variation have been based largely on biochemical phenotypes that can be scored by blood typing. These phenotypes include not only the standard blood groups such as the ABO series (Yamamoto et al., 1990), but also variants of blood serum proteins and of immunological proteins such as the human leukocyte antigens (the HLA system). A big advantage of these markers is that many of the relevant genes have multiple alleles. For example, the gene called *HLA-DRB1* has at least 290 alleles and *HLA- B* has over 400. This is relevant because of the way in which gene mapping is carried out with humans. Rather than setting up many breeding experiments, which is the procedure with experimental organisms such as fruit flies or mice, data on inheritance of human genes have to be gleaned by examining the phenotypes displayed by members of a single family. If all the family members have the same allele for the gene being studied then no useful information can be obtained. It is therefore necessary for the relevant marriages to have occurred, by chance, between individuals with different alleles. This is much more likely if the gene being studied has 290 rather than two alleles.

DNA markers for genetic mapping

Genes are very useful markers but they are by no means ideal. One problem, especially with larger genomes such as those of vertebrates and flowering plants, is that a map based entirely on genes is not very detailed. This would be true even if every gene could be mapped because, as we saw in Chapter 2, in most eukaryotic genomes the genes are widely spaced out with large gaps between them (see Figure). The problem is made worse by the fact that only a fraction of the total number of genes exist in allelic forms that can be distinguished conveniently. Gene maps are therefore not very comprehensive. We need other types of marker.

Mapped features that are not genes are called DNA markers. As with gene markers, a DNA marker must have at least two alleles to be useful. There are three Types of DNA sequence feature that satisfy this requirement: restriction fragment length polymorphisms (RFLPs), simple sequence length polymorphisms (SSLPs), and single nucleotide polymorphisms (SNPs). Discussed underneath is the SNPs which is a comparatively modern technique used in current times:

Single nucleotide polymorphisms (SNPs)

These are positions in a genome where some individuals have one nucleotide (e.g. a G) and others have a different nucleotide (e.g. a C) (Figure). There are vast numbers of SNPs in every genome, some of which also give rise to RFLPs, but many of which do not because the sequence in which they lie is not recognized by any restriction enzyme. In the human genome there are at least 1.42 million SNPs, only 100 000 of which result in an RFLP (SNP Group,2001).

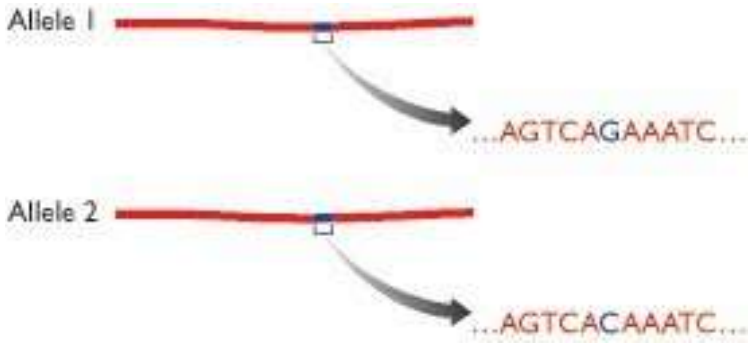
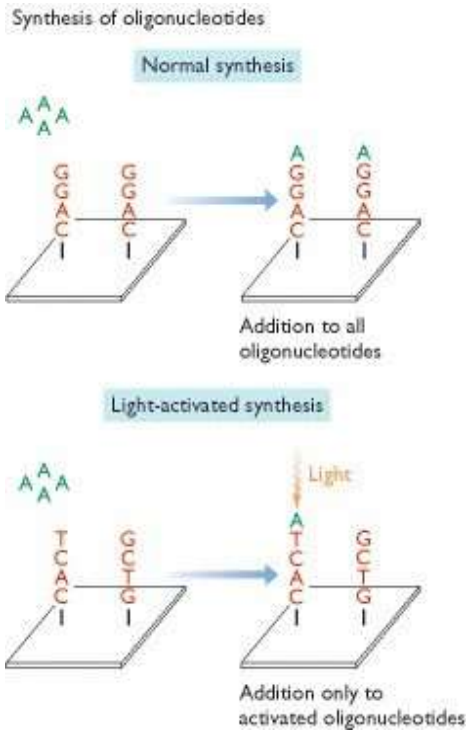


Figure : A single nucleotide polymorphism (SNP)

Although each SNP could, potentially, have four alleles (because there are four nucleotides), most exist in just two forms, so these markers suffer from the same drawback as RFLPs with regard to human genetic mapping: there is a high possibility that a SNP does not display any variability in the family that is being studied. The advantages of SNPs are their abundant numbers and the fact that they can be typed by methods that do not involve gel electrophoresis. This is important because gel electrophoresis has proved difficult to automate so any detection method that uses it will be relatively slow and labor-intensive. SNP detection is more rapid because it is based on oligonucleotide hybridization analysis. An oligonucleotide is a short single-stranded DNA molecule, usually less than 50 nucleotides in length, that is synthesized in the test tube. If the conditions are just right, then an oligonucleotide will hybridize with another DNA molecule only if the oligonucleotide forms a completely base-paired structure with the second molecule. If there is a single mismatch - a single position within the oligonucleotide that does not form a base pair - then hybridization does not occur (Figure). Oligonucleotide hybridization can therefore discriminate between the two alleles of an SNP. Various screening strategies have been devised (Mir and Southern, 2000), including DNA chip technology and solution hybridization technique



A DNA chip is a wafer of glass or silicon, 2.0 cm^2 or less in area, carrying many different oligonucleotides in a high-density array. The DNA to be tested is labeled with a fluorescent marker and pipetted onto the surface of the chip. Hybridization is detected by examining the chip with a fluorescence microscope, the positions at which the fluorescent signal is emitted indicating which oligonucleotides have hybridized with the test DNA. Many SNPs can therefore be scored in a single experiment (Wang et al., 1998; Gerhold et al.,1999).

Solution hybridization techniques are carried out in the wells of a microtiter tray, each well containing a different oligonucleotide, and use a detection system that can discriminate between unhybridized single-stranded DNA and the double-stranded product that results when an oligonucleotide hybridizes to the test DNA. Several systems have been developed, one of which makes use of a pair of labels comprising a fluorescent dye and a compound that quenches the fluorescent signal when brought into close proximity with the dye. The dye is attached to one end of an oligonucleotide and the quenching compound to the other end. Normally there is no fluorescence because the oligonucleotide is designed in such a way that the two ends base-pair to one another, placing the quencher next to the dye (Figure 5.9). Hybridization between oligonucleotide and test DNA disrupts this base pairing, moving the quencher away from the dye and enabling the fluorescent signal to be generated (Tyagi et al.,1998).

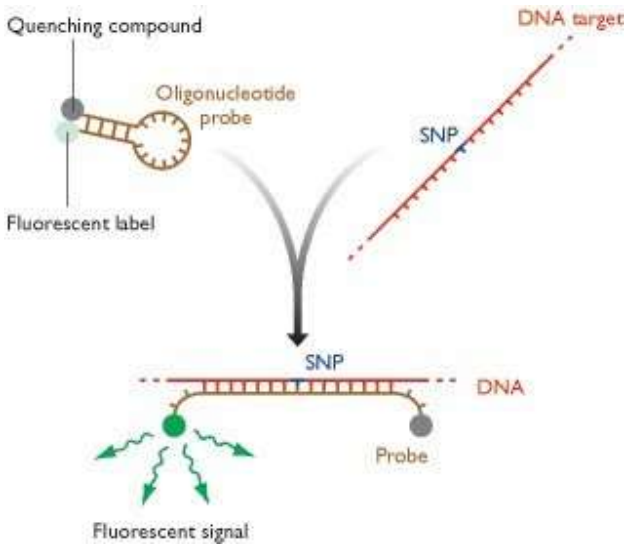


Figure: One way of detecting an SNP by solution hybridization.

Linkage analysis is the basis of genetic mapping

Now that we have assembled a set of markers with which to construct a genetic map we can move on to look at the mapping techniques themselves. These techniques are all based on genetic linkage, which in turn derives from the seminal discoveries in genetics made in the mid 19th century by Gregor Mendel.

Working out a genetic map from recombination frequencies

Once Morgan had understood how partial linkage could be explained by crossing-over during meiosis he was able to devise a way of mapping the relative positions of genes on a chromosome. In fact the most

important work was done not by Morgan himself, but by an undergraduate in his laboratory, Arthur Sturtevant (Sturtevant, 1913). Sturtevant assumed that crossing-overwasarandomevent,therebeinganequalchanceofitoccurringatanypositionalong a pair of lined-up chromatids. If this assumption is correct then two genes that are close together will be separated by crossovers less frequently than two genes that are more distant from one another. Furthermore, the frequency with which the genes are unlinked by crossovers will be directly proportional to how far apart they are on their chromosome. The recombination frequency is therefore a measure of the distance between two genes. If you work out the recombination frequencies for different pairs of genes, you can construct a map of their relative positions on the chromosome.

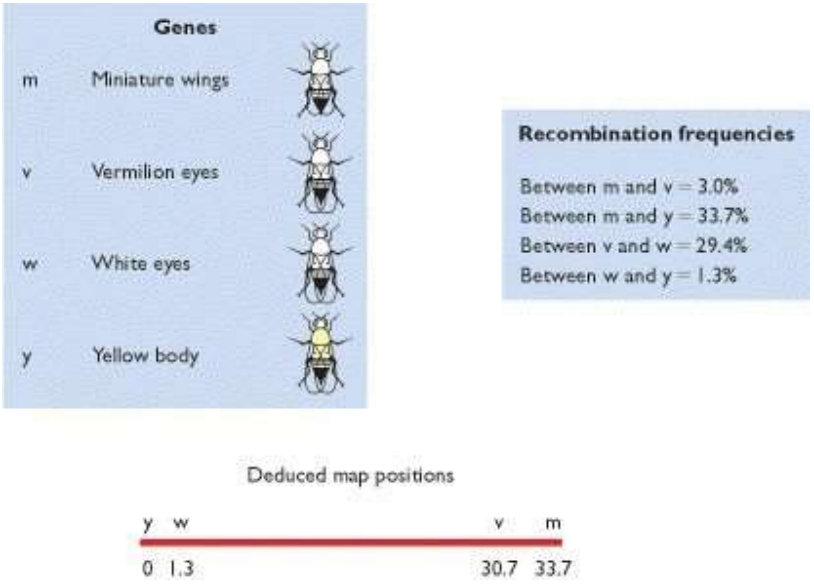


Figure: Working out a genetic map from recombination frequencies. The example is taken from the original experiments carried out with fruit flies by Arthur Sturtevant. All four genes are on the X chromosome of the fruit fly.

It turns out that Sturtevant's assumption about the randomness of crossovers was not entirely justified. Comparisons between genetic maps and the actual positions of genes on DNA molecules, as revealed by physical mapping and DNA sequencing, have shown that some regions of chromosomes, called recombination hotspots, are more likely to be involved in crossovers than others. This means that a genetic map distance does not necessarily indicate the physical distance between two markers (see Figure). Also, we now realize that a single chromatid can participate in more than one crossover at the same time, but that there are limitations on how close together these crossovers can be, leading to more inaccuracies in the mapping procedure. Despite these qualifications, linkage analysis usually makes correct deductions about gene order, and distance estimates are sufficiently accurate to generate genetic maps that are of value as frameworks for genome sequencing projects.

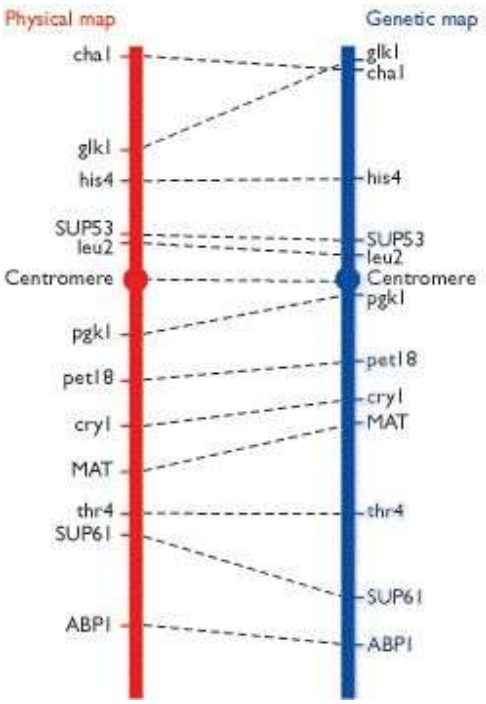


Figure: Comparison between the genetic and physical maps of *Saccharomyces cerevisiae* chromosome III. The comparison shows the discrepancies between the genetic and physical maps, the latter determined by DNA sequencing.

Physical Mapping

A map generated by genetic techniques is rarely sufficient for directing the sequencing phase of a genome project. This is for two reasons:

- **The resolution of a genetic map depends on the number of crossovers that have been scored .**This is not a major problem for microorganisms because these can be obtained in huge numbers, enabling many crossovers to be studied, resulting in a highly detailed genetic map in which the markers are just a few kb apart. For example, when the *Escherichia coli* genome sequencing project began in 1990, the latest genetic map for this organism comprised over 1400 markers, an average of one per 3.3 kb. This was sufficiently detailed to direct the sequencing program without the need for extensive physical mapping. Similarly, the *Saccharomyces cerevisiae* project was supported by a fine-scale genetic map (approximately 1150 genetic markers, on average one per 10 kb). The problem with humans and most other eukaryotes is that it is simply not possible to obtain large numbers of progeny, so relatively few meiosis can be studied and the resolving power of linkage analysis is restricted. This means that genes that are several tens of kb apart may appear at the same position on the genetic map.

- **Genetic maps have limited accuracy .** Sturtevant's assumption that crossovers occur at random along chromosomes is only partly correct because the presence of recombination hotspots means that crossovers are more likely to occur at some points rather than at others. The effect that this can have on the accuracy of a genetic map was illustrated in 1992 when the complete sequence for S. *cerevisiae* chromosome III was published (Oliver et al., 1992), enabling the first direct comparison to be made between a genetic map and the actual positions of markers as shown by

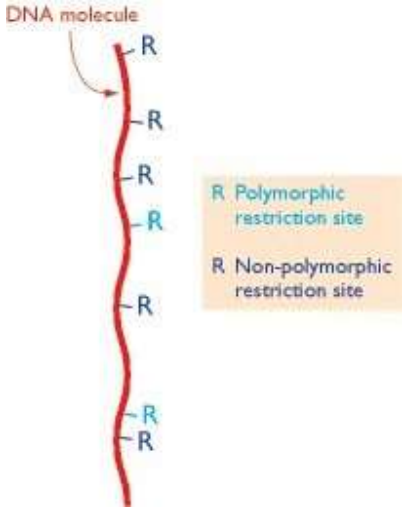
DNA sequencing (Figure). There were considerable discrepancies, even to the extent that one pair of genes had been ordered incorrectly by genetic analysis. Bear in mind that *S. cerevisiae* is one of the two eukaryotes (fruit fly is the second) whose genomes have been subjected to intensive genetic mapping. If the yeast genetic map is inaccurate then how precise are the genetic maps of organisms subjected to less detailed analysis?

These two limitations of genetic mapping mean that for most eukaryotes a genetic map must be checked and supplemented by alternative mapping procedures before large-scale DNA sequencing begins. A plethora of physical mapping techniques has been developed to address this problem, the most important being:

- Restriction mapping, which locates the relative positions on a DNA molecule of the recognition sequences for restriction endonucleases;
- **Fluorescent *in situ* hybridization (**FISH**)**, in which marker locations are mapped by hybridizing a probe containing the marker to intact chromosomes;
- Sequence tagged site **(**STS**) mapping**, in which the positions of short sequences are mapped by PCR and/or hybridization analysis of genome fragments.

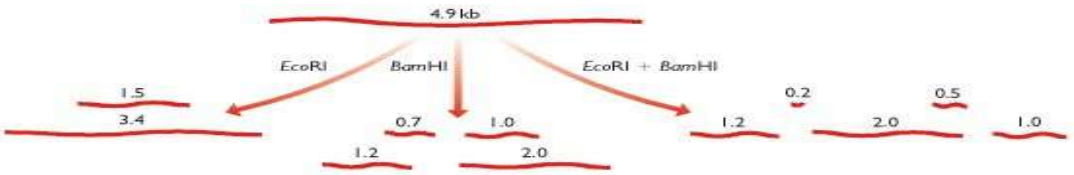
Restriction mapping

Genetic mapping using RFLPs as DNA markers can locate the positions of polymorphic restriction sites within a genome , but very few of the restriction sites in a genome are polymorphic, so many sites are not mapped by this technique (Figure). Could we increase the marker density on a genome map by using an alternative method to locate the positions of some of the non-polymorphic restriction sites? This is what restriction mapping achieves, although in practice the technique has limitations which mean that it is applicable only to relatively small DNA molecules. We will look first at the technique and then consider its relevance to genome mapping.



The basic methodology for restriction mapping:

The simplest way to construct a restriction map is to compare the fragment sizes produced when a DNA molecule is digested with two different restriction enzymes that recognize different target sequences. An example using the restriction enzymes *Eco*RI and *Bam*HI is shown in Figure. First, the DNA molecule is digested with just one of the enzymes and the sizes of the resulting fragments are measured by agarose gel electrophoresis. Next, the molecule is digested with the second enzyme and the resulting fragments again sized in an agarose gel. The results so far enable the number of restriction sites for each enzyme to be worked out, but do not allow their relative positions to be determined. Additional information is therefore obtained by cutting the DNAmoleculewithbothenzymestogether.IntheexampleshowninFigure,thisdoublerestriction enables three of the sites to be mapped. However, a problem arises with the larger *Eco*RI fragment because this contains two *Bam*HI sites and there are two alternative possibilities for the map location of the outer one of these. The problem is solved by going back to the original DNA molecule and treating it again with *Bam*HI on its own, but this time preventing the digestion from going to completion by, for example, incubating the reaction for only a short time or using a suboptimal incubation temperature. This is called a partial restriction and leads to a more complex set of products, the complete restriction products now being supplemented with partially restricted fragments that still contain one or more uncut *Bam*HI sites. In the example shown in Figure, the size of one of the partial restriction fragments is diagnostic and the correct map can be identified.



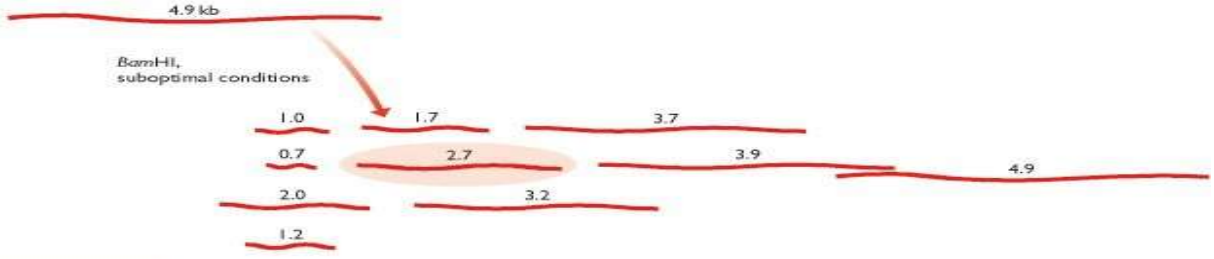
INTERPRETATION OF THE DOUBLE RESTRICTION

Fragments	Conclusions
0.2 kb, 0.5 kb	These must derive from the 0.7 kb *Bam*HI fragment, which therefore has an internal *Eco*RI site:
1.0 kb	This must be a *Bam*HI fragment with no internal *Eco*RI site. We can account for the 1.5 kb *Eco*RI fragment if we place the 1.0 kb fragment thus:
1.2 kb, 2.0 kb	These must also be *Bam*HI fragments with no internal *Eco*RI sites. They must lie within the 3.4 kb *Eco*RI fragment. There are two possibilities:

MAP I MAP II

PREDICTED RESULTS OF A PARTIAL *Bam*HI RESTRICTION

If Map I is correct, then the partial restriction products will include a fragment of 1.2 + 0.7 = 1.9 kb
If Map II is correct, then the partial restriction products will include a fragment of 2.0 + 0.7 = 2.7 kb



CONCLUSION
Map II is correct

Figure : Restriction mapping. The objective is to map the EcoRI (E) and BamHI (B) sites in a linear DNA molecule of 4.9 kb. The results of single and double restrictions are shown at the top. The sizes of the fragments given after double restriction enable two alternative maps to be constructed, as explained in the central panel, the unresolved issue being the position of one of the three BamHI sites. The two maps are tested by a partial BamHI restriction (bottom), which shows that Map II is the correct one.

A partial restriction usually gives the information needed to complete a map, but if there are many restriction sites then this type of analysis becomes unwieldy, simply because there are so many different fragments to consider. An alternative strategy is simpler because it enables the majority of the fragments to be ignored. This is achieved by attaching a radioactive or other type of marker to each end of the starting DNA molecule before carrying out the partial digestion. The result is that many of the partial restriction products become 'invisible' because they do not contain an end-fragment and so do not show up when the agarose gel is screened for labeled products. The sizes of the partial restriction products that are visible enable unmapped sites to be positioned relative to the ends of the startingmolecule.

The scale of restriction mapping is limited by the sizes of the restriction fragments

Restriction maps are easy to generate if there are relatively few cut sites for the enzymes being used. However, as the number of cut sites increases, so also do the numbers of single, double and partial restriction products whose sizes must be determined and compared in order for the map to be constructed. Computer analysis can be brought into play but problems still eventually arise. A stage will be reached when a digest contains so many fragments that individual bands merge on the agarose gel, increasing the chances of one or more fragments being measured incorrectly or missed out entirely. If several fragments have similar sizes then even if they can all be identified, it may not be possible to assemble them into an unambiguous map.

Restriction mapping is therefore more applicable to small rather than large molecules, with the upper limit for the technique depending on the frequency of the restriction sites in the molecule being mapped. In practice, if a DNA molecule is less than 50 kb in length it is usually possible to construct an unambiguous restriction map for a selection of enzymes with six-nucleotide recognition sequences. Fifty kb is of course way below the minimum size for bacterial or eukaryotic chromosomes, although it does cover a few viral and organelle genomes, and whole- genome restriction maps have indeed been important in directing sequencing projects with these small molecules. Restriction maps are equally useful after bacterial or eukaryotic genomic DNA has been cloned, if the cloned fragments are less than 50 kb, because a detailed restriction map can then be built up as a preliminary to sequencing the cloned region. This is an important application of restriction mapping in sequencing projects with large genomes, but is there any possibility of using restriction analysis for the more general mapping of entire genomes larger than 50 kb?

The answer is a qualified 'yes', because the limitations of restriction mapping can be eased slightly by choosing enzymes expected to have infrequent cut sites in the target DNA molecule. These 'rare cutters' fall into two categories:

- **Enzymes with seven- or eight-nucleotide recognition sequences .** A few restriction enzymes cut at seven- or eight-nucleotide recognition sequences. Examples are SapI (5′- GCTCTTC-3′) and SgfI (5′-GCGATCGC-3′). The seven-nucleotide enzymes would be expected, on average, to cut a DNA molecule with a GC content of 50% once every 4^7 = 16 384 bp. The eight-nucleotide enzymes should cut once every 4^8 = 65 536 bp. These figures compare with 4^6 = 4096 bp for six-nucleotide enzymes such as BamHI and EcoRI. Seven- and eight-nucleotide cutters are often used in restriction mapping of large molecules but the approach is not as useful as it might be simply because not many of these enzymes are known.
-

- **Enzymes whose recognition sequences contain motifs that are rare in the target** DNA **.** Genomic DNA molecules do not have random sequences and some are significantly deficient in certain motifs. For example, the sequence 5′-CG-3′ is rare in human DNA because human cells possess an enzyme that adds a methyl group to carbon 5 of the C nucleotide in this sequence. The resulting 5-methylcytosine is unstable and tends to undergo deamination to give thymine (Figure). The consequence is that during human evolution many of the 5′-CG-3′ sequences that were originally in our genome have become converted to 5′-TG-3′. Restriction enzymes that recognize a site containing 5′-CG-3′ therefore cut human DNA relatively infrequently. Examples are SmaI (5′- CCCGGG-3′), which cuts human DNA on average once every 78 kb, and BssHII (5′-GCGCGC-3′) which cuts once every 390 kb. Note that NotI, an eight-nucleotide cutter, also targets 5′-CG-3′ sequences (recognition sequence 5′-GCGGCCGC-3′) and cuts human DNA very rarely - approximately once every 10Mb.

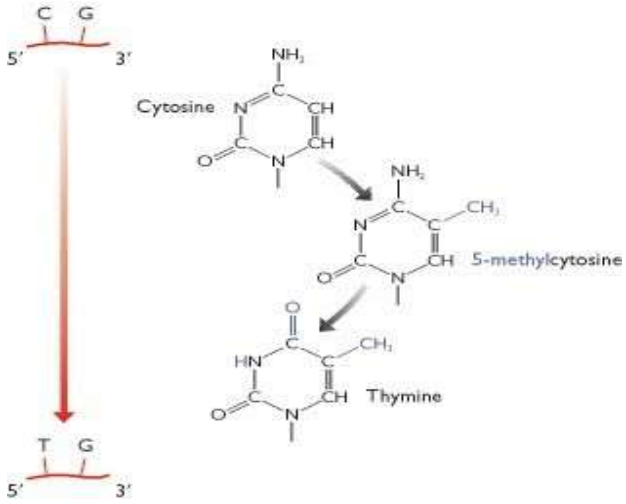


Figure: The sequence 5′-CG-3′ is rare in human DNA because of methylation of the C, followed by deamination to give T.

The potential of restriction mapping is therefore increased by using rare cutters. It is still not possible to construct restriction maps of the genomes of animals and plants, but it is feasible to use the technique with large cloned fragments, and the smaller DNA molecules of prokaryotes and lower eukaryotes such as yeast and fungi.

If a rare cutter is used then it may be necessary to employ a special type of agarose gel electrophoresis to study the resulting restriction fragments. This is because the relationship between the length of a DNA molecule and its migration rate in an electrophoresis gel is not linear, the resolution decreasing as the molecules get longer (Figure A). This means that it is not possible to separate molecules more than about 50 kb in length because all of these longer molecules run as a single slowly migrating band in a standard agarose gel. To separate them it is necessary to replace the linear electric field used in conventional gel electrophoresis with a more complex field. An example is provided by orthogonal field alternation gel electrophoresis (OFAGE), in which the electric field alternates between two pairs of electrodes, each positioned at an angle of 45° to the length of the gel (Figure B). The DNA molecules still move down through the gel, but each change in the field forces the molecules to realign. Shorter molecules realign more quickly than longer ones and so migrate more rapidly through the gel. The overall result is that molecules much longer than those separated by conventional gel electrophoresis can be resolved. Related techniques include CHEF (contour clamped homogeneous electric fields) and FIGE (field inversion gel electrophoresis).

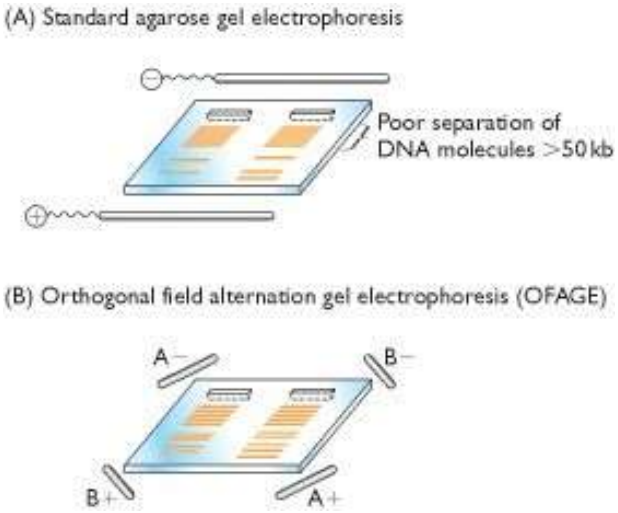


Figure :Conventional and non-conventional agarose gel electrophoresis. (A) In standard agarose gel electrophoresis the electrodes are placed at either end of the gel and the DNA molecules migrate directly towards the positive electrode. Molecules longer than about 50 kb cannot be separated from one another in this way. (B) In OFAGE, the electrodes are placed at the corners of the gel, with the field pulsing between the A pair and the B pair.

Optical mapping:

It is also possible to use methods other than electrophoresis to map restriction sites in DNA molecules. With the technique called optical mapping (Schwartz et al., 1993), restriction sites are directly located by looking at the cut DNA molecules with a microscope. The DNA must first be attached to a glass slide in such a way that the individual molecules become stretched out, rather than clumped together in a mass. There are two ways of doing this: gel stretching and molecular combing. To prepare gel-stretched DNA fibres (Schwartz et al., 1993), chromosomal DNA is suspended in molten agarose and placed on a microscope slide. As the gel cools and solidifies, the DNA molecules become extended (Figure A). To utilize gel stretching in optical mapping, the microscope slide onto which the molten agarose is placed is first coated with a restriction enzyme. The enzyme is inactive at this stage because there are no magnesium ions, which the enzyme needs in order to function. Once the gel has solidified it is washed with a solution containing magnesium chloride, which activates the restriction enzyme. A fluorescent dye is added, such as DAPI (4,6-diamino-2-phenylindole dihydrochloride), which stains the DNA so that the fibres can be seen when the slide is examined with a high-power fluorescence microscope. The restriction sites in the extended molecules gradually become gaps as the degree of fibre extension is reduced by the natural springiness of the DNA, enabling the relative positions of the cuts to be recorded.

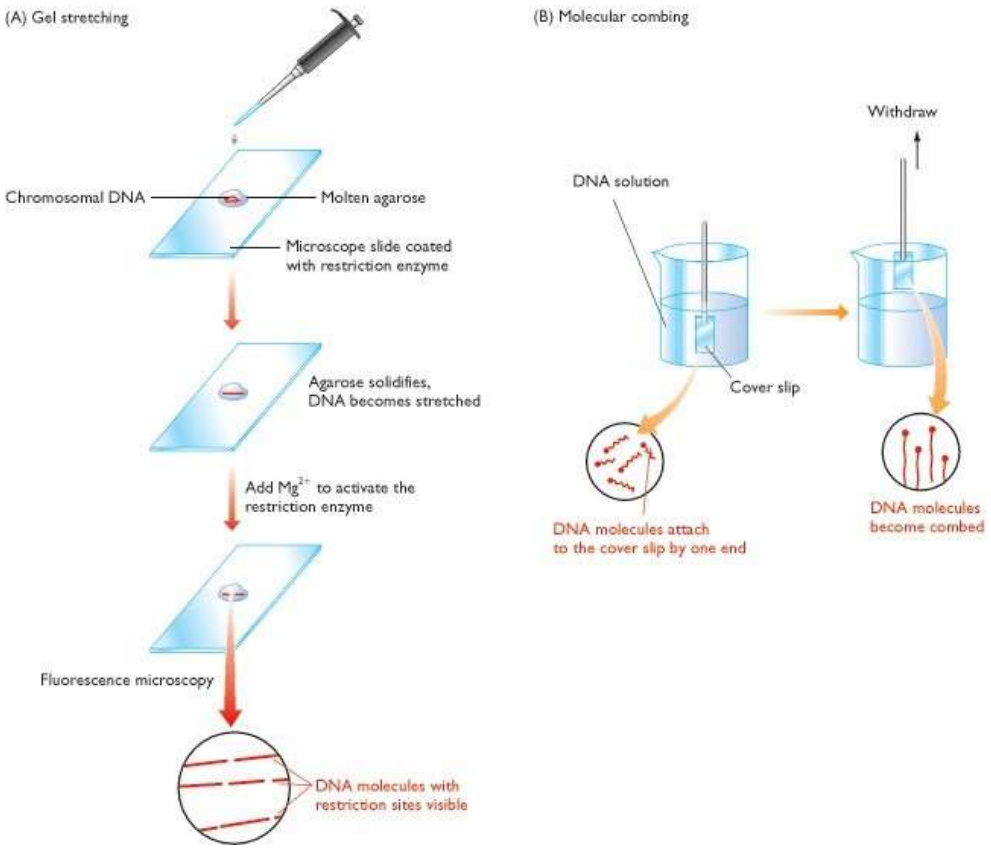


Figure: Gel stretching and molecular combing. (A) To carry out gel stretching, molten agarose containing chromosomal DNA molecules is pipetted onto a microscope slide coated with a restriction enzyme. As the gel solidifies, the DNA molecules become stretched.

In molecular combing (Michalet et al., 1997), the DNA fibres are prepared by dipping a silicone- coated cover slip into a solution of DNA, leaving it for 5 minutes (during which time the DNA molecules attach to the cover slip by their ends), and then removing the slip at a constant speed of 0.3 mm s^{-1} (Figure B). The force required to pull the DNA molecules through the meniscus causes them to line up. Once in the air, the surface of the cover slip dries, retaining the DNA molecules as an array of parallel fibres.

Optical mapping was first applied to large DNA fragments cloned in YAC and BAC vectors . More recently, the feasibility of using this technique with genomic DNA has been proven with studies of a 1-Mbchromosome of the malaria parasite Plasmodium falciparum (Jing et al., 1999), and the two chromosomes and single mega plasmid of the bacterium *Deinococcus radiodurans* (Lin et al.,1999;

Fluorescent in situ hybridization (FISH)

The optical mapping method described above provides a link to the second type of physical mapping procedure that we will consider - FISH (Heiskanen et al., 1996). As in optical mapping, FISH enables the position of a marker on a chromosome or extended DNA molecule to be directly visualized. In optical mapping the marker is a restriction site and it is visualized as a gap in an extended DNA fibre. In FISH, the marker is a DNA sequence that is visualized by hybridization with a fluorescent probe.

In situ hybridization with radioactive or fluorescent probes

In situ hybridization is a version of hybridization analysis in which an intact chromosome is examined by probing it with a labeled DNA molecule. The position on the chromosome at which hybridization occurs provides information about the map location of the DNA sequence used as the probe (Figure). For the method to work, the DNA in the chromosome must be made single stranded ('denatured') by breaking the base pairs that hold the double helix together. Only then will the chromosomal DNA be able to hybridize with the probe. The standard method for denaturing chromosomal DNA without destroying the morphology of the chromosome is to dry the preparation onto a glass microscope slide and then treat with formamide.

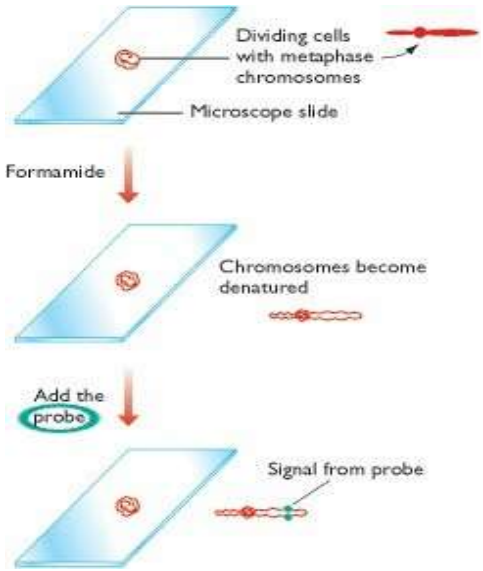


Figure :Fluorescent in situ hybridization. A sample of dividing cells is dried onto a microscope slide and treated with formamide so that the chromosomes become denatured but do not lose their characteristic metaphase morphologies (see Section 2.2.1). The position at which the probe hybridizes to the chromosomal DNA is visualized by detecting the fluorescent signal emitted by the labeled DNA.

In the early versions of in situ hybridization the probe was radioactively labeled but this procedure was unsatisfactory because it is difficult to achieve both sensitivity and resolution with a radioactive label, two critical requirements for successful in situ hybridization. Sensitivity requires that the radioactive label has a high emission energy (an example of such a radiolabel is ^{32}P), but if the radiolabel has a high emission energy then it scatters its signal and so gives poor resolution. High resolution is possible if a radiolabel with low emission energy, such as ^{3}H, is used, but these have such low sensitivity that lengthy exposures are needed, leading to a high background and difficulties in discerning the genuine signal.

These problems were solved in the late 1980s by the development of non-radioactive fluorescent DNA labels. These labels combine high sensitivity with high resolution and are ideal for in situ hybridization. Fluorolabels with different colored emissions have been designed, making it possible to hybridize a number of different probes to a single chromosome and distinguish their individual hybridization signals, thus enabling the relative positions of the probe sequences to be mapped. To maximize sensitivity, the probes must be labeled as heavily as possible, which in the past has meant that they must be quite lengthy DNA molecules - usually cloned DNA fragments of at least 40 kb. This requirement is less important now

that techniques for achieving heavy labeling with shorter molecules have been developed. As far as the construction of a physical map is concerned, a cloned DNA fragment can be looked upon as simply another type of marker, although in practice the use of clones as markers adds a second dimension because the cloned DNA is the material from which the DNA sequence is determined. Mapping the positions of clones therefore provides a direct link between a genome map and its DNA sequence.

If the probe is a long fragment of DNA then one potential problem, at least with higher eukaryotes, is that it is likely to contain examples of repetitive DNA sequences and so may hybridize to many chromosomal positions, not just the specific point to which it is perfectly matched. To reduce this non-specific hybridization, the probe, before use, is mixed with unlabeled DNA from the organism being studied. This DNA can simply be total nuclear DNA (i.e. representing the entire genome) but it is better if a fraction enriched for repeat sequences is used. The idea is that the unlabeled DNA hybridizes to the repetitive DNA sequences in the probe, blocking these so that the subsequent *in situ* hybridization is driven wholly by the unique sequences (Lichter et al., 1990). Non-specific hybridization is therefore reduced or eliminated entirely (Figure).

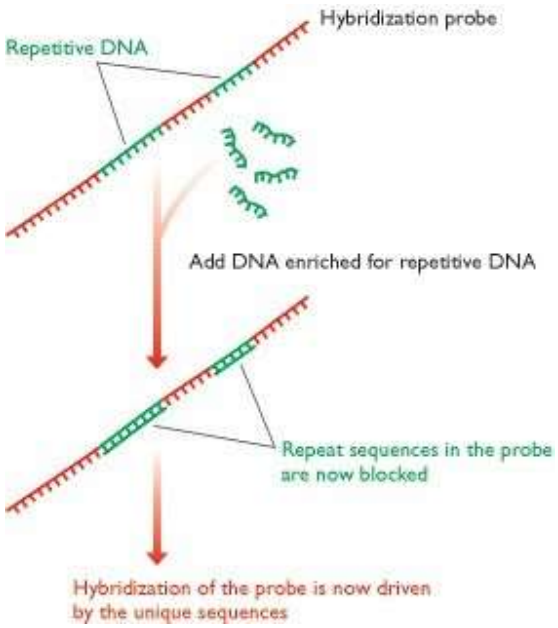


Figure :A method for blocking repetitive DNA sequences in a hybridization probe. In this example the probe molecule contains two genome-wide repeat sequences (shown in green). If these sequences are not blocked then the probe will hybridize to any copies of these genome-wide repeats in the target DNA. To block the repeat sequences, the probe is prehybridized with a DNA fraction enriched for repetitive DN

FISH in action

FISH was originally used with metaphase chromosomes . These chromosomes, prepared from nuclei that are undergoing division, are highly condensed and each chromosome in a set takes up a recognizable appearance, characterized by the position of its centromere and the banding pattern that emerges after the chromosome preparation is stained (see Figure). With metaphase chromosomes, a fluorescent signal obtained by FISH is mapped by measuring its position relative to the end of the short arm of the chromosome (the FLpter value). A disadvantage is that the highly condensed nature of metaphase chromosomes means that only low-resolution mapping is possible, two markers having to be at least 1 Mb apart to be resolved as separate hybridization signals (Trask et al., 1991). This degree of resolution is insufficient for the construction of useful chromosome maps, and the main application of metaphase FISH has been in determining the chromosome on which a new marker is located, and providing a rough idea of its map position, as a preliminary to finer scale mapping by other methods.

For several years these 'other methods' did not involve any form of FISH, but since 1995 a range of higher resolution FISH techniques has been developed. With these techniques, higher resolution is achieved by changing the nature of the chromosomal preparation being studied. If metaphase chromosomes are too condensed for fine-scale mapping then we must use chromosomes that are more extended. There are two ways of doing this (Heiskanen et al., 1996):

- **Mechanically stretched chromosomes** can be obtained by modifying the preparative method used to isolate chromosomes from metaphase nuclei. The inclusion of a centrifugation step generates shear forces which can result in the chromosomes becoming stretched to up to 20 times their normal length. Individual chromosomes are still recognizable and FISH signals can be mapped in the same way as with normal metaphase chromosomes. The resolution is significantly improved and markers that are 200– 300 kb apart can be distinguished.
- **Non-metaphase chromosomes** can be used because it is only during metaphase that chromosomes are highly condensed: at other stages of the cell cycle the chromosomes are naturally unpacked. Attempts have been made to use prophase nuclei because in these the chromosomes are still sufficiently condensed for individual ones to be identified. In practice, however, these preparations provide no advantage over mechanically stretched chromosomes. Interphase chromosomes are more useful because this stage of the cell cycle (between nuclear divisions) is when the chromosomes are most unpacked. Resolution down to 25 kb is possible, but chromosome morphology is lost so there are no external reference points against which to map the position of the probe. This technique is therefore used after preliminary map information has been obtained, usually as a means of determining the order of a series of markers in a small region of a chromosome.

Interphase chromosomes contain the most unpacked of all cellular DNA molecules. To improve the resolution of FISH to better than 25 kb it is therefore necessary to abandon intact chromosomes and instead use purified DNA. This approach, called fibre-FISH, makes use of DNA prepared by gel stretching or molecular combing and can distinguish markers that are less than 10 kb apart.

Sequence tagged site (STS)mapping

To generate a detailed physical map of a large genome we need, ideally, a high-resolution mapping procedure that is rapid and not technically demanding. Neither of the two techniques that we have considered so far - restriction mapping and FISH - meets these requirements. Restriction mapping is

rapid, easy, and provides detailed information, but it cannot be applied to large genomes. FISH can be applied to large genomes, and modified versions such as fibre-FISH can give high-resolution data, but FISH is difficult to carry out and data accumulation is slow, map positions for no more than three or four markers being obtained in a single experiment. If detailed physical maps are to become a reality then we need a more powerful technique.

At present the most powerful physical mapping technique, and the one that has been responsible for generation of the most detailed maps of large genomes, is STS mapping. A sequence tagged site or **STS** is simply a short DNA sequence, generally between 100 and 500 bp in length, that is easily recognizable and occurs only once in the chromosome or genome being studied. To map a set of STSs, a collection of overlapping DNA fragments from a single chromosome or from the entire genome is needed. In the example shown in Figure, a fragment collection has been prepared from a single chromosome, with each point along the chromosome represented on average five times in the collection. The data from which the map will be derived are obtained by determining which fragments contain which STSs. This can be done by hybridization analysis but PCR is generally used because it is quicker and has proven to be more amenable to automation. The chances of two STSs being present on the same fragment will, of course, depend on how close together they are in the genome. If they are very close then there is a good chance that they will always be on the same fragment; if they are further apart then sometimes they will be on the same fragment and sometimes they will not (Figure). The data can therefore be used to calculate the distance between two markers, in a manner analogous to the way in which map distances are determined by linkage analysis (Section). Remember that in linkage analysis a map distance is calculated from the frequency at which crossovers occur between two markers. STS mapping is essentially the same, except that each map distance is based on the frequency at which *breaks* occur between two markers.

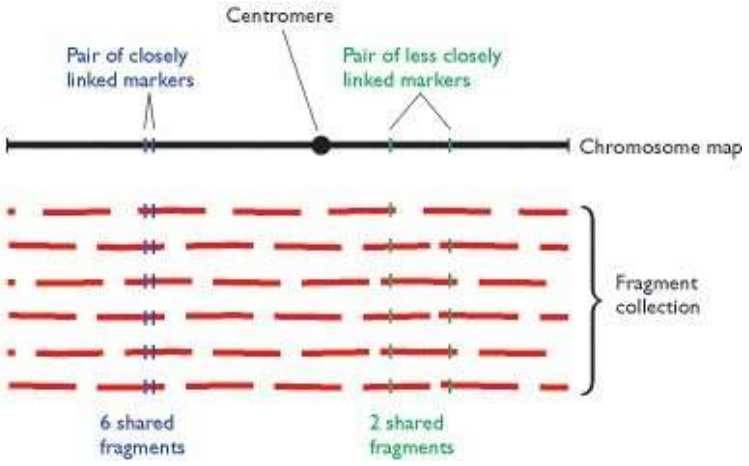


Figure :A fragment collection suitable for STS mapping. The fragments span the entire length of a chromosome, with each point on the chromosome present in an average of five fragments. The two blue markers are close together on the chromosome map and there is a high probability that they will be found on the same fragment. The two green markers are more distant from one another and so are less likely to be found on the same fragment

Any unique DNA sequence can be used as an STS

To qualify as an STS, a DNA sequence must satisfy two criteria. The first is that its sequence must be known, so that a PCR assay can be set up to test for the presence or absence of the STS on different DNA fragments. The second requirement is that the STS must have a unique location in the chromosome being studied, or in the genome as a whole if the DNA fragment set covers the entire genome. If the STS sequence occurs at more than one position then the mapping data will be ambiguous. Care must therefore be taken to ensure that STSs do not include sequences found in repetitive DNA.

These are easy criteria to satisfy and STSs can be obtained in many ways, the most common sources being **expressed sequence tags (ESTs)**, SSLPs, and **random genomic sequences**.

- Expressed sequence tags ***(ESTs)***. These are short sequences obtained by analysis of cDNA clones (Marra et al., 1998). Complementary DNA is prepared by converting an mRNA preparation into double-stranded DNA (Figure). Because the mRNA in a cell is derived from protein-coding genes, cDNAs and the ESTs obtained from them represent the genes that were being expressed in the cell from which the mRNA was prepared. ESTs are looked upon as a rapid means of gaining access to the sequences of important genes, and they are valuable even if their sequences are incomplete. An EST can also be used as an STS, assuming that it comes from a unique gene and not from a member of a gene family in which all the genes have the same or very similar sequences.
- ***SSLPs .*** In EARLIER sections, we examined the use of microsatellites and other SSLPs in genetic mapping. SSLPs can also be used as STSs in physical mapping. SSLPs that are polymorphic and have already been mapped by linkage analysis are particularly valuable as they provide a direct connection between the genetic and physical maps.
- **Random genomic sequences.** These are obtained by sequencing random pieces of cloned genomic DNA, or simply by downloading sequences that have been deposited in the databases.

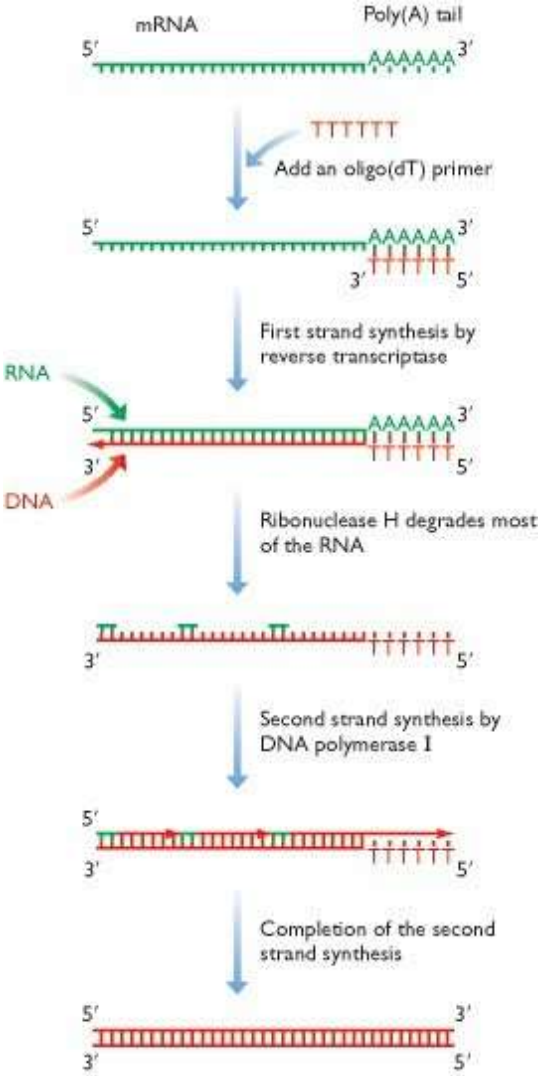

mRNA
Poly(A) tail
5′
AAAAAA
3′
TTTTTT
Add an oligo(dT) primer
3′
5′
First strand synthesis by reverse transcriptase
RNA
DNA
Ribonuclease H degrades most of the RNA
Second strand synthesis by DNA polymerase I
Completion of the second strand synthesis

Figure : Most eukaryotic mRNAs have a poly(A) tail at their 3′ end . This series of A nucleotides is used as the priming site for the first stage of cDNA synthesis, carried out by reverse transcriptase - a DNA polymerase that copies an RNA template . The primer is a short synthetic DNA oligonucleotide, typically 20 nucleotides in length, made up entirely of Ts (an 'oligo(dT)' primer). When the first strand synthesis has been completed, the preparation is treated with ribonuclease H, which specifically degrades the RNA component of an RNA-DNA hybrid. Under the conditions used, the enzyme does not degrade all of the RNA, instead leaving short segments that prime the second DNA strand synthesis reaction, this one catalyzed by DNA polymerase-I.

Fragments of DNA for STS mapping

The second component of an STS mapping procedure is the collection of DNA fragments spanning the chromosome or genome being studied. This collection is sometimes called the mapping reagent and at present there are two ways in which it can be assembled: as a clone library and as a panel of radiation hybrids. We will consider radiation hybrids first.

A radiation hybrid is a rodent cell that contains fragments of chromosomes from a second organism (McCarthy, 1996). The technology was first developed in the 1970s when it was discovered that exposure of human cells to X-ray doses of 3000–8000 rads causes the chromosomes to break up randomly into fragments, larger X-ray doses producing smaller fragments (Figure). This treatment is of course lethal for the human cells, but the chromosome fragments can be propagated if the irradiated cells are subsequently fused with non-irradiated hamster or other rodent cells. Fusion is stimulated either chemically with polyethylene glycol or by exposure to Sendai virus. Not all of the hamster cells take up chromosome fragments so a means of identifying the hybrids is needed. The routine selection process is to use a hamster cell line that is unable to make either thymidine kinase (TK) or hypoxanthine phosphoribosyl transferase (HPRT), deficiencies in either of these two enzymes being lethal when the cells are grown in a medium containing a mixture of hypoxanthine, aminopterin and thymidine (HAT medium). After fusion, the cells are placed in HAT medium. Those that grow are hybrid hamster cells that have acquired human DNA fragments that include genes for the human TK and HPRT enzymes, which are synthesized inside the hybrids, enabling these cells to grow in the selective medium. The treatment results in hybrid cells that contain arandomselection of human DNA fragments inserted into the hamster chromosomes. Typically the fragments are 5–10 Mb in size, with each cell containing fragments equivalent to 15–35% of the human genome. The collection of cells is called a radiation hybrid panel and can be used as a mapping reagent in STS mapping, provided that the PCR assay used to identify the STS does not amplify the equivalent region of DNA from the hamster genome.

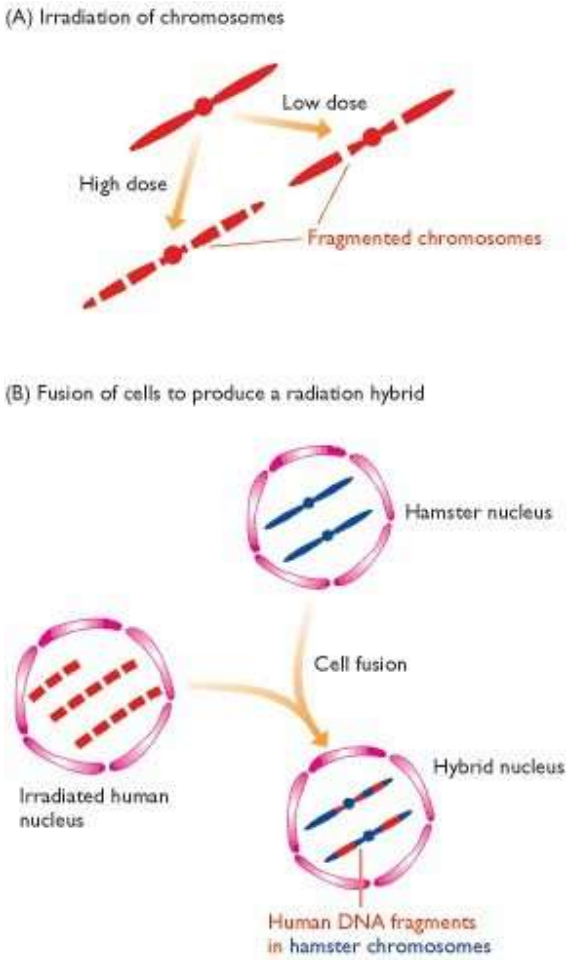


Figure : Radiation hybrids. (A) The result of irradiation of human cells: the chromosomes break into fragments, smaller fragments generated by higher X-ray doses. In (B), a radiation hybrid is produced by fusing an irradiated human cell with an untreated hamster cell. For clarity, only the nuclei are shown.

A second type of radiation hybrid panel, containing DNA from just one human chromosome, can be constructed if the cell line that is irradiated is not a human one but a second type of rodent hybrid. Cytogeneticists have developed a number of rodent cell lines in which a single human chromosome is stably propagated in the rodent nucleus. If a cell line of this type is irradiated and fused with hamster cells, then the hybrid hamster cells obtained after selection will contain either human or mouse chromosome fragments, or a mixture of both. The ones containing human DNA can be identified by probing with a human-specific genome-wide repeat sequence, such as the short interspersed nuclear element (SINE) called Alu, which has a copy number of just over 1 million and so occurs on average once every 4 kb in the human genome. Only cells containing human DNA will hybridize to Alu probes, enabling the uninteresting mouse hybrids to be discarded and STS mapping to be directed at the cells containing human chromosome fragments.

Radiation hybrid mapping of the human genome was initially carried out with chromosome- specific rather than whole-genome panels because it was thought that fewer hybrids would be needed to map a single chromosome than would be needed to map the entire genome. It turns out that a high-resolution map of a single human chromosome requires a panel of 100–200 hybrids, which is about the most that can be handled conveniently in a PCR screening program. But whole-genome and single-chromosome panels are constructed differently, the former involving irradiation of just human DNA, and the latter requiring

irradiation of a mouse cell containing much mouse DNA and relatively little human DNA. This means that the human DNA content per hybrid is much lower in a single-chromosome panel than in a whole-genome panel. It transpires that detailed mapping of the entire human genome is possible with fewer than 100 whole-genome radiation hybrids, so whole-genome mapping is no more difficult than single- chromosome mapping. Once this was realized, whole-genome radiation hybrids became a central component of the mapping phase of the Human Genome Project . Whole-genome libraries are also being used for STS mapping of other mammalian genomes and for those of the zebra fish and the chicken (McCarthy,1996).

A clone library can also be used as the mapping reagent for STS analysis

A preliminary to the sequencing phase of a genome project is to break the genome or isolated chromosomes into fragments and to clone each one in a high-capacity vector, one able to handle large fragments of DNA . This results in a clone library, a collection of DNA fragments, which, in this case, have an average size of several hundred kb. As well as supporting the sequencing work, this type of clone library can also be used as a mapping reagent in STS analysis.

As with radiation hybrid panels, a clone library can be prepared from genomic DNA, and so represents the entire genome, or a chromosome-specific library can be made if the starting DNA comes from just one type of chromosome. The latter is possible because individual chromosomes can be separated by flow cytometry. To carry out this technique, dividing cells (ones with condensed chromosomes) are carefully broken open so that a mixture of intact chromosomes is obtained. The chromosomes are then stained with a fluorescent dye. The amount of dye that a chromosome binds depends on its size, so larger chromosomes bind more dye and fluoresce more brightly than smaller ones. The chromosome preparation is diluted and passed through a fine aperture, producing a stream of droplets, each one containing a single chromosome. The droplets pass through a detector that measures the amount of fluorescence, and hence identifies which droplets contain the particular chromosome being sought. An electric charge is applied to these drops, and no others (Figure), enabling the droplets containing the desired chromosome to be deflected and separated from the rest. What if two different chromosomes have similar sizes, as is the case with human chromosomes 21 and 22? These can usually be separated if the dye that is used is not one that binds non-specifically to DNA, but instead has a preference for AT- or GC-rich regions. Examples of such dyes are Hoechst 33258 and chromomycin A_3, respectively. Two chromosomes that are the same size rarely have identical GC contents, and so can be distinguished by the amounts of AT- or GC-specific dye that they bind.

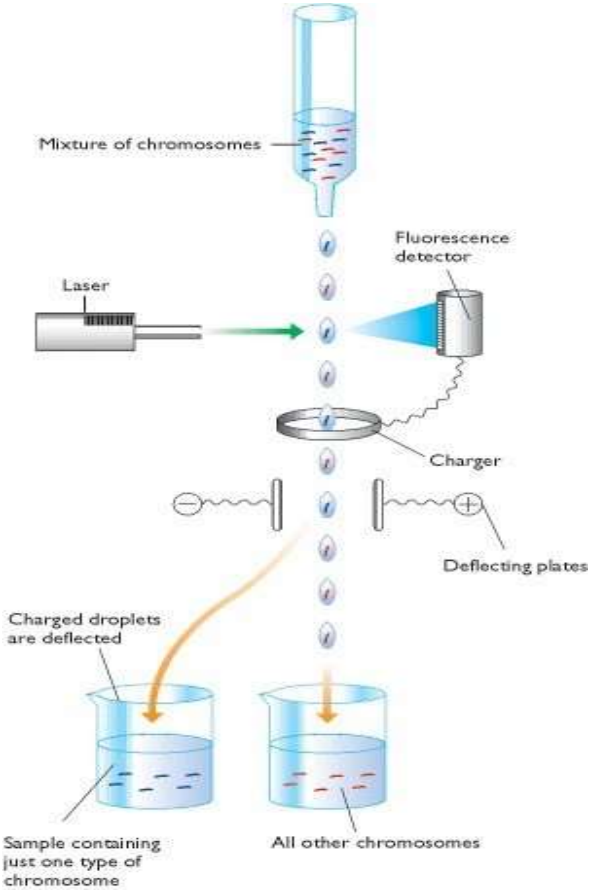


Figure :Separating chromosomes by flow cytometry A mixture of fluorescently stained chromosomes is passed through a small aperture so that each drop that emerges contains just one chromosome. The fluorescence detector identifies the signal from drops containing the correct chromosome and applies an electric charge to these drops. When the drops reach the electric plates, the charged ones are deflected into a separate beaker. All other drops fall straight through the deflecting plates and are collected in the waste beaker.

Compared with radiation hybrid panels, clone libraries have one important advantage for STS mapping. This is the fact that the individual clones can subsequently provide the DNA that is actually sequenced. The data resulting from STS analysis, from which the physical map is generated, can equally well be used to determine which clones contain overlapping DNA fragments, enabling a clone contig to be built up (Figure); for other methods for assembling clone contigs see). This assembly of overlapping clones can be used as the base material for a lengthy, continuous DNA sequence, and the STS data can later be used to anchor this sequence precisely onto the physical map. If the STSs also include SSLPs that have been mapped by genetic linkage analysis then the DNA sequence, physical map and genetic map can all be integrated.

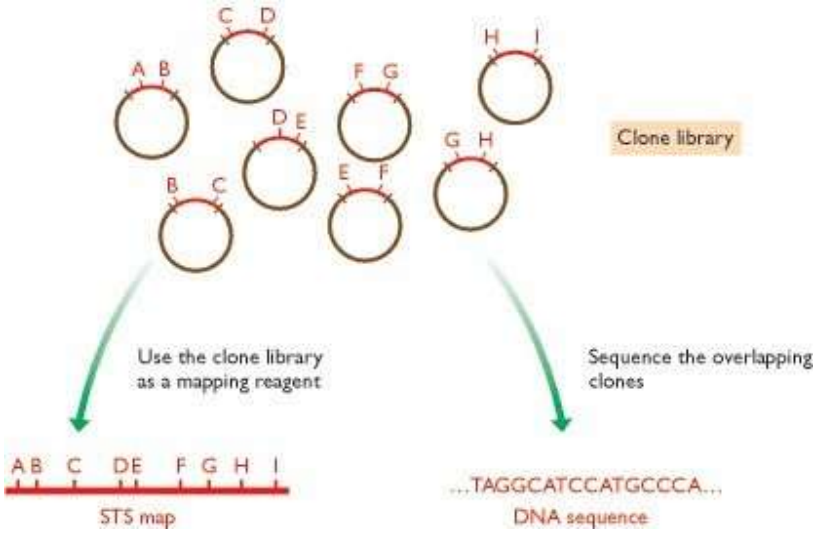


Figure The value of clone libraries in genome projects. The small clone library shown in this example contains sufficient information for an STS map to be constructed, and can also be used as the source of the DNA that will besequenced.

Genome Sequencing:

The objective of a genome project is the complete DNA sequence for the organism being studied, ideally integrated with the genetic and/or physical maps of the genome so that genes and other interesting features can be located within the DNA sequence. This chapter describes the techniques and research strategies that are used during the sequencing phase of a genome project, when this ultimate objective is being directly addressed. Techniques for sequencing DNA are clearly of central importance in this context and we will begin the chapter with a detailed examination of sequencing methodology. This methodology is of little value however, unless the short sequences that result from individual sequencing experiments can be linked together in the correct order to give the master sequences of the chromosomes that make up the genome. The middle part of this chapter describes the strategies used to ensure that the master sequences are assembled correctly. Finally, we will review the way in which mapping and sequencing were used to produce the two draft human genome sequences that were published in February 2001.

The Methodology for DNA Sequencing

Rapid and efficient methods for DNA sequencing were first devised in the mid-1970s. Two different procedures were published at almost the same time:

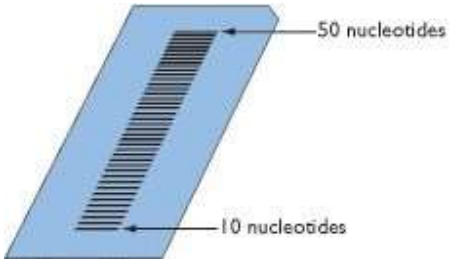
- The chain termination method (Sanger et al., 1977), in which the sequence of a single-stranded DNA molecule is determined by enzymatic synthesis of complementary polynucleotide chains, these chains terminating at specific nucleotide positions;
- The **chemical degradation method** (Maxam and Gilbert, 1977), in which the sequence of a double-stranded DNA molecule is determined by treatment with chemicals that cut the molecule at specific nucleotide positions.

Both methods were equally popular to begin with but the chain termination procedure has gained

ascendancy in recent years, particularly for genome sequencing. This is partly because the chemicals used in the chemical degradation method are toxic and therefore hazardous to the health of the researchers doing the sequencing experiments, but mainly because it has been easier to automate chain termination sequencing. As we will see later in this chapter, a genome project involves a huge number of individual sequencing experiments and it would take many years to perform all these by hand. Automated sequencing techniques are therefore essential if the project is to be completed in a reasonable time-span.

Chain termination DNA sequencing

Chain termination DNA sequencing is based on the principle that single-stranded DNA molecules that differ in length by just a single nucleotide can be separated from one another by polyacrylamide gel electrophoresis. This means that it is possible to resolve a family of molecules, representing all lengths from 10 to 1500 nucleotides, into a series of bands(Figure).



Figure; The banding pattern is produced after separation of single- stranded DNA molecules by denaturing polyacrylamide gel electrophoresis. The molecules are labeled with a radioactive marker and the bands visualized by autoradiography. The bands gradually get closer together towards the top of the ladder. In practice, molecules up to about 1500 nucleotides in length can be separated if the electrophoresis is continued for long enough. Chain termination sequencing in outline

The starting material for a chain termination sequencing experiment is a preparation of identical single-stranded DNA molecules. The first step is to anneal a short oligonucleotide to the same position on each molecule, this oligonucleotide subsequently acting as the primer for synthesis of a new DNA strand that is complementary to the template (Figure). The strand synthesis reaction, which is catalyzed by a DNA polymerase enzyme and requires the four deoxyribonucleotide triphosphates (dNTPs - dATP, dCTP, dGTP and dTTP) as substrates, would normally continue until several thousand nucleotides had been polymerized. This does not occur in a chain termination sequencing experiment because, as well as the four dNTPs, a small amount of a dideoxynucleotide (e.g. ddATP) is added to the reaction. The polymerase enzyme does not discriminate between dNTPs and ddNTPs, so the dideoxynucleotide can be incorporated into the growing chain, but it then blocks further elongation because it lacks the 3′-hydroxyl group needed to form a connection with the next nucleotide (Figure B).

If ddATP is present, chain termination occurs at positions opposite thymidines in the template DNA. Because dATP is also present the strand synthesis does not always terminate at the first T in the template; in fact it may continue until several hundred nucleotides have been polymerized before a ddATP is eventually incorporated. The result is therefore a set of new chains, all of different

lengths, but each ending in ddATP. Now the polyacrylamide gel comes into play. The family of molecules generated in the presence of ddATP is loaded into one lane of the gel, and the families generated with ddCTP, ddGTP and ddTTP loaded into the three adjacent lanes. After electrophoresis, the DNA sequence can be read directly from the positions of the bands in the gel (Figure D). The band that has moved the furthest represents the smallest piece of DNA, this being the strand that terminated by incorporation of a ddNTP at the first position in the template. In the example shown in Figure this band lies in the 'G' lane (i.e. the lane containing the molecules terminated with ddGTP), so the first nucleotide in the sequence is 'G'. The next band, corresponding to the molecule that is one nucleotide longer than the first, is in the 'A' lane, so the second nucleotide is 'A' and the sequence so far is 'GA'. Continuing up through the gel we see that the next band also lies in the 'A' lane (sequence GAA), then we move to the 'T' lane (GAAT), and so on. The sequence reading can be continued up to the region of the gel where individual bands are not separated.

Chain termination sequencing requires a single-stranded DNA template

The template for a chain termination experiment is a single-stranded version of the DNA molecule to be sequenced. There are several ways in which this can be obtained:

- **The** DNA **can be cloned in a plasmid vector** . The resulting DNA will be double stranded so cannot be used directly in sequencing. Instead, it must be converted into single-stranded DNA by denaturation with alkali or by boiling. This is a common method for obtaining template DNA for DNA sequencing, largely because cloning in a plasmid vector is such a routine technique. A shortcoming is that it can be difficult to prepare plasmid DNA that is not contaminated with small quantities of bacterial DNA and RNA, which can act as spurious templates or primers in the DNA sequencing experiment.

- **The** DNA **can be cloned in a bacteriophage M13 vector.** Vectors based on M13 bacteriophagearedesignedspecificallyfortheproductionofsingle-strandedtemplatesfor DNA sequencing. M13 bacteriophage has a single-stranded DNA genome which, after infection of Escherichia coli bacteria, is converted into a double-stranded replicative form. The replicative form is copied until over 100 molecules are present in the cell, and when the cell divides the copy number in the new cells is maintained by further replication. At the same time, the infected cells continually secrete new M13 phage particles, approximately 1000 per generation, these phages containing the single-stranded version of the genome. Cloning vectors based on M13 vectors are double-stranded DNA molecules equivalent to the replicative form of the M13 genome. They can be manipulated in exactly the same way as a plasmid cloning vector. The difference is that cells that have been transfected with a recombinant M13 vector secrete phage particles containing single-stranded DNA, this DNA comprising the vector molecule plus any additional DNA that has been ligated into it. The phages therefore provide the template DNA for chain termination sequencing. The one disadvantage is that DNA fragments longer than about 3 kb suffer deletions and rearrangements when cloned in an M13 vector, so the system can only be used with short pieces of DNA.

- **The** DNA **can be cloned in a phagemid.** This is a plasmid cloning vector that contains, in addition to its plasmid origin of replication, the origin from M13 or another phage with a single-stranded DNA genome. If an E. coli cell contains both a phagemid and the replicative form of a helper phage, the latter carrying genes for the phage replication enzymes and coat proteins, then the phage origin of the phagemid becomes activated, resulting in synthesis of phage particles containing the single-stranded version of the phagemid. The double-stranded

plasmid DNA is therefore converted into single-stranded template DNA for DNA sequencing. This system avoids the instabilities of M13 cloning and can be used with fragments up to 10 kb ormore.

- PCR **can be used to generate single-stranded** DNA**.** There are various ways of generating single-stranded DNA by PCR, the most effective being to modify one of the two primers so that DNA strands synthesized from this primer are easily purified. One possibility is to attach small metallic beads to the primer and then use a magnetic device to purify the resulting strands.

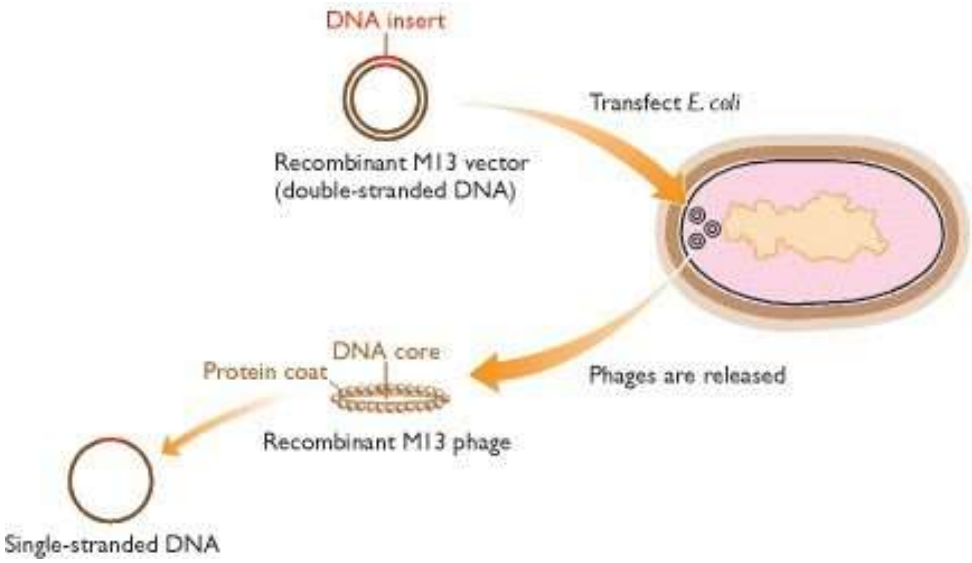


Figure 6.3. M13 vectors can be obtained in two forms: the double-stranded replicative molecule and the single-stranded version found in bacteriophage particles. The replicative form can be manipulated in the same way as a plasmid cloning vector (Section 4.2.1) with new DNA inserted by restriction followed by ligation. The recombinant vector is introduced into Escherichia coli cells by transfection. Once inside an E. coli cell, the double-stranded vector replicates and directs synthesis of single-stranded copies, which are packaged into phage particles and secreted from the cell. The phage particles can be collected from the culture medium after centrifuging to pellet the bacteria. The protein coats of the phages are removed by treating with phenol, and the single-stranded version of the recombinant vector is purified for use in DNA sequencing.

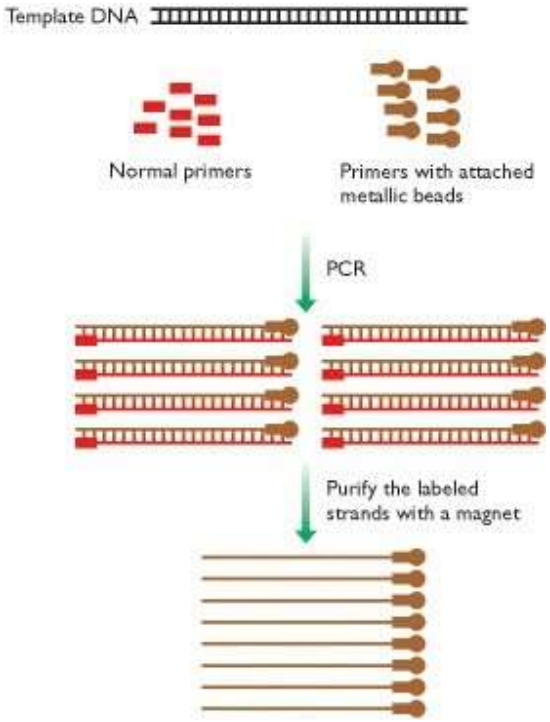


Figure :The PCR is carried out with one normal primer (shown in red), and one primer that is labelled with a metallic bead (shown in brown). After PCR, the labelled strands are purified with a magnetic device. For more details about PCR The primer determines the region of the template DNA that will be sequenced

To begin a chain termination sequencing experiment, an oligonucleotide primer is annealed onto the template DNA. The primer is needed because template-dependent DNA polymerases cannot initiate DNA synthesis on a molecule that is entirely single-stranded: there must be a short double-stranded region to provide a 3′ end onto which the enzyme can add new nucleotides .

The primer also plays the critical role of determining the region of the template molecule that will be sequenced. For most sequencing experiments a ‘universal’ primer is used, this being one that is complementary to the part of the vector DNA immediately adjacent to the point into which new DNA is ligated (Figure 6.5 A). The same universal primer can therefore give the sequence of any piece of DNA that has been ligated into the vector. Of course if this inserted DNA is longer than 750 bp or so then only a part of its sequence will be obtained, but usually this is not a problem because the project as a whole simply requires that a large number of short sequences are generated and subsequently assembled into the contiguous master sequence. It is immaterial whether or not the short sequences are the complete or only partial sequences of the DNA fragments used as templates. If double-stranded plasmid DNA is being used to provide the template then, if desired, more sequence can be obtained from the other end of the insert. Alternatively, it is possible to extend the sequence in one direction by synthesizing a non- universal primer, designed to anneal at a position within the insert DNA (Figure B). An experiment with this primer will provide a second short sequence that overlaps the previous one.

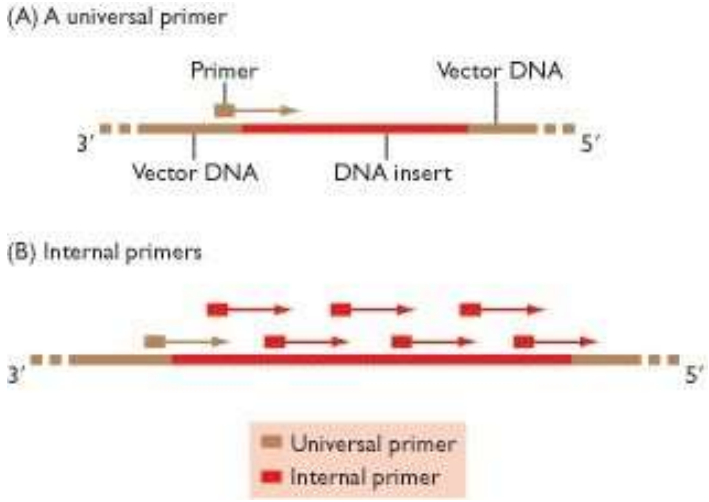


Figure 6.5(A) A universal primer anneals to the vector DNA, adjacent to the position at which new DNA is inserted. A single universal primer can therefore be used to sequence any DNA insert, but only provides the sequence of one end of the insert. (B) One way of obtaining a longer sequence is to carry out a series of chain termination experiments, each with a different internal primer that anneals within the DNAinsert.

Thermal cycle sequencing offers an alternative to the traditional methodology

The discovery of thermostable DNA polymerases, which led to the development of PCR , has also resulted in new methodologies for chain termination sequencing. In particular, the innovation called thermal cycle sequencing (Sears et al., 1992) has two advantages over traditionalchainterminationsequencing.First,itusesdouble-strandedratherthansingle-stranded DNA as the starting material. Second, very little template DNA is needed, so the DNA does not have to be cloned before being sequenced.

Thermal cycle sequencing is carried out in a similar way to PCR but just one primer is used and each reaction mixture includes one of the ddNTPs (Figure below). Because there is only one primer, only one of the strands of the starting molecule is copied, and the product accumulates in a linear fashion, not exponentially as is the case in a real PCR. The presence of the ddNTP in the reaction mixture causes chain termination, as in the standard methodology, and the family of resulting strands can be analyzed and the sequence read in the normal manner by polyacrylamide gel electrophoresis.

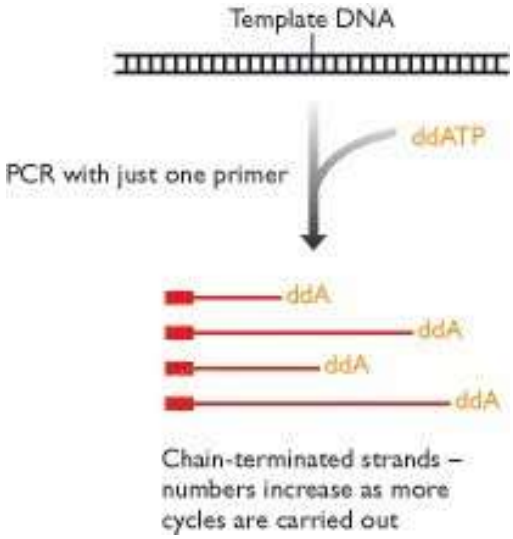


Figure PCR is carried out with just one primer and with a dideoxynucleotide present in the reaction mixture. The result is a family of chain-terminated strands - the 'A' family in the reaction shown. These strands, along with the products of the C, G and T reactions, are electrophoresed as in the standard methodology

The standard chain termination sequencing methodology employs radioactive labels, and the banding pattern in the polyacrylamide gel is visualized by autoradiography. Usually one of the nucleotides in the sequencing reaction is labeled so that the newly synthesized strands contain radiolabels along their lengths, giving high detection sensitivity. To ensure good band resolution, ^{33}P or ^{35}S is generally used, as the emission energies of these isotopes are relatively low, in contrast to ^{32}P, which has a higher emission energy and gives poorer resolution because of signal scattering.

Previously we saw how the replacement of radioactive labels by fluorescent ones has given a new dimension to in situ hybridization techniques. Fluorolabeling has been equally important in the development of sequencing methodology, in particular because the detection system for fluorolabels has opened the way to automated sequence reading (Prober et al., 1987). The label is attached to the ddNTPs, with a different fluorolabel used for each one (Figure below). Chains terminated with A are therefore labeled with one fluorophore, chains terminated with C are labeled with a second fluorophore, and so on. Now it is possible to carry out the four sequencing reactions - for A, C, G and T - in a single tube and to load all four families of molecules into just one lane of the polyacrylamide gel, because the fluorescent detector can discriminate between the different labels and hence determine if each band represents an A, C, G or T. The sequence can be read directly as the bands pass in front of the detector and either printed out in a form readable by eye (Figure B) or sent straight to a computer for storage. When combined with robotic devices that prepare the sequencing reactions and load the gel, the fluorescent detection system provides a major increase in throughput and avoids errors that might arise when a sequence is read by eye and then entered manually into a computer. It is only by use of these automated techniques that we can hope to generate sequence data rapidly enough to complete a genome project in a reasonable length of time.

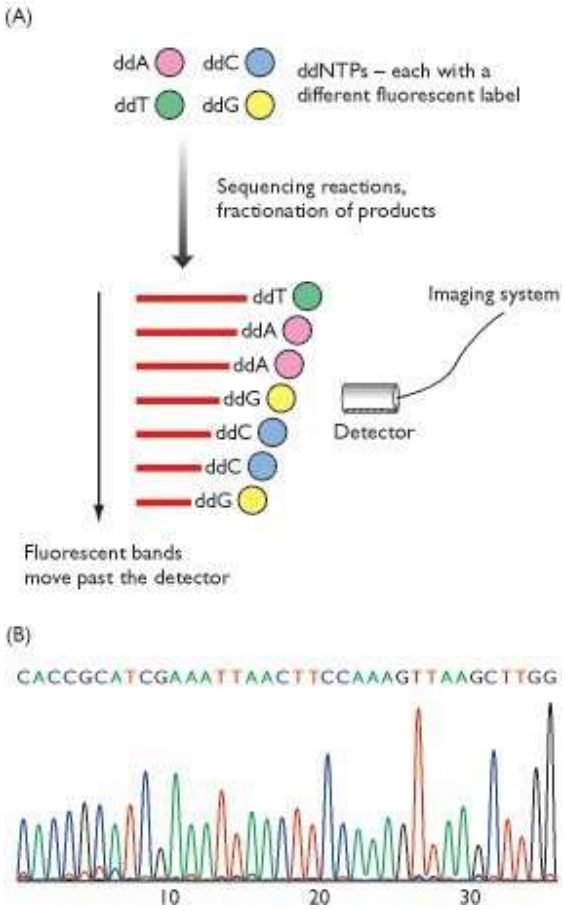


Figure (A) The chain termination reactions are carried out in a single tube, with each dideoxynucleotide labeled with a different fluorophore. In the automated sequencer, the bands in the electrophoresis gel move past a fluorescence detector, which identifies which dideoxynucleotide is present in each band. The information is passed to the imaging system. (B) The printout from an automated sequencer. The sequence is represented by a series of peaks, one for each nucleotide position. In this example, a green peak is an 'A', blue is 'C', black is 'G', and red is 'T'.

High throughput sequencing methods

In spite of the development of automated techniques, conventional DNA sequencing suffers from the limitation that only a few hundred bp of sequence can be determined in a single experiment. In the context of the Human Genome Project, this means that each experiment provides only one five-millionth of the total genome sequence. Attempts are continually being made to modify the technology so that sequence acquisition is more rapid, a recent example being the introduction of new automated sequencers that use capillary separation rather than a polyacrylamide gel. These have 96 channels so 96 sequences can be determined in parallel, and each run takes less than 2 hours to complete, enabling up to 1000 sequences to be obtained in a single day (Mullikan and McMurray, 1999). Other systems that are being developed will increase data generation even further by enabling 384 or 1024 sequences to be run at the same time (Rogers, 1999).

There have also been attempts to make sequence acquisition more rapid by devising new sequencing methodologies. One possibility is pyrosequencing, which does not require electrophoresis or any

other fragment separation procedure and so is more rapid than chain termination sequencing (Ronaghi et al., 1998). In pyrosequencing, the template is copied in a straightforward manner without added ddNTPs. As the new strand is being made, the order in which the dNTPs are incorporated is detected, so the sequence can be 'read' as the reaction proceeds. The addition of a nucleotide to the end of the growing strand is detectable because it is accompanied by release of a molecule of pyrophosphate, which can be converted by the enzyme sulfurylase into a flash of chemiluminescence. Of course, if all four dNTPs were added at once then flashes of light would be seen all the time and no useful sequence information would be obtained. Each dNTP is therefore added separately, one after the other, with a nucleotidase enzyme also present in the reaction mixture so that if a dNTP is not incorporated into the polynucleotide then it is rapidly degraded before the next dNTP is added (Figure). This procedure makes it possible to follow the order in which the dNTPs are incorporated into the growing strand. The technique sounds complicated, but it simply requires that a repetitive series of additions be made to the reaction mixture, precisely the type of procedure that is easily automated, with the possibility of many experiments being carried out in parallel.

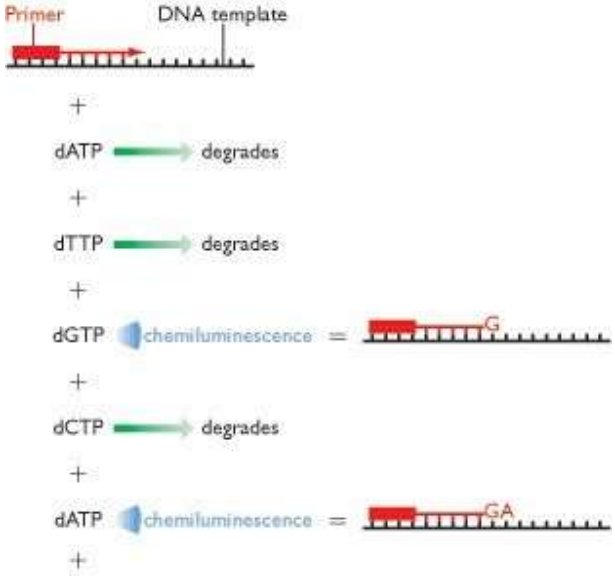


Figure Pyrosequencing. The strand synthesis reaction is carried out in the absence of dideoxynucleotides. Each dNTP is added individually, along with a nucleotidase enzyme that degrades the dNTP if it is not incorporated into the strand being synthesized. Incorporation of a nucleotide is detected by a flash of chemiluminescence induced by the pyrophosphate released from the dNTP. The order in which nucleotides are added to the growing strand can therefore be followed.

A very different approach to DNA sequencing through the use of DNA chips might one day be possible. A chip carrying an array of different oligonucleotides could be used in DNA sequencing by applying the test molecule - the one whose sequence is to be determined - to the array and detecting the positions at which it hybridizes. Hybridization to an individual oligonucleotide would indicate the presence of that particular oligonucleotide sequence in the test molecule, and comparison of all the oligonucleotides to which hybridization occurs would enable the sequence of the test molecule to be deduced (Figure). The problem with this approach is that the maximum length of the molecule that can be sequenced is given by the square root of the number of oligonucleotides in the array, so if every possible 8-mer oligonucleotide (ones containing eight nucleotides) were attached to the chip - all 65 536 of them - then the maximum length of readable sequence would be only 256 bp(Southern, 1996). Even if the chip carried all the 1 048 576 different 10-mer sequences, it could still only be used to sequence a 1 kb molecule. To sequence a 1 Mb molecule (this being the sort of advance in sequence capability that is really needed) the chip would have to carry all of the 1×10^{12} possible 20-

mers. This may sound an outlandish proposition but advances in miniaturization, together with the possibility of electronic rather than visual detection of hybridization, could bring such an array within reach in the future.

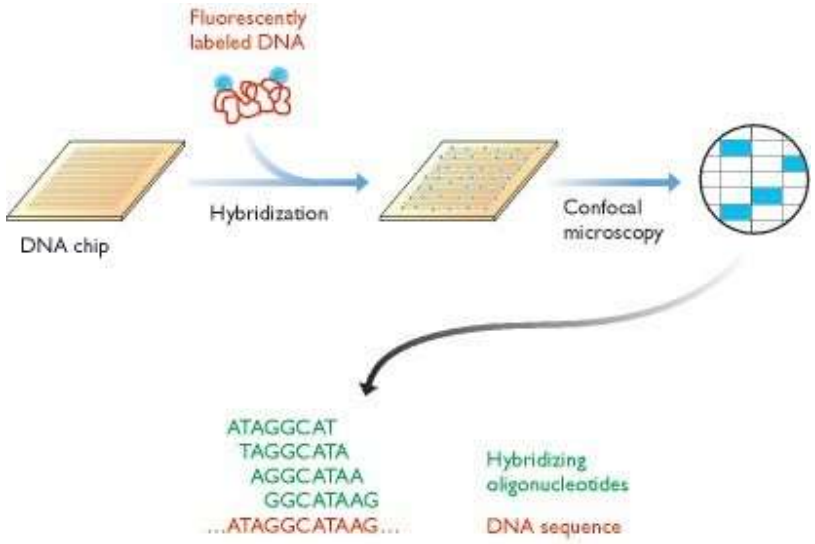


Figure The chip carries an array of every possible 8-mer oligonucleotide. The DNA to be sequenced is labeled with a fluorescent marker and applied to the chip, and the positions of hybridizing oligonucleotides determined by confocal microscopy. Each hybridizing oligonucleotide represents an 8-nucleotide sequence motif that is present in the probe DNA. The sequence of the probe DNA can therefore be deduced from the overlaps between the sequences of these hybridizing oligonucleotides.

DNA Annotation

DNA annotation or genome annotation is the process of identifying the locations of genes and all of the coding regions in a genome and determining what those genes do. An annotation (irrespective of the context) is a note added by way of explanation or commentary. Once a genome is sequenced, it needs to be annotated to make sense of it.

For DNA annotation, a previously unknown sequence representation of genetic material is enriched with information relating genomic position to intron-exon boundaries, regulatory sequences, repeats, gene names and protein products. This annotation is stored in genomic databases such as Mouse Genome Informatics, FlyBase, and Worm Base. Educational materials on some aspects of biological annotation from the 2006 Gene Ontology annotation camp and similar events are available at the Gene Ontology website.[2]

The National Center for Biomedical Ontology (www.bioontology.org) develops tools for automated annotation[3] of database records based on the textual descriptions of those records.

As a general method, dcGO has an automated procedure for statistically inferring associations between ontology terms and protein domains or combinations of domains from the existing gene/protein-level annotations.

Process

Genome annotation consists of three main steps:.

1. identifying portions of the genome that do not code for proteins
2. identifying elements on the genome, a process called gene prediction, and
3. attaching biological information to these elements.

Automatic annotation tools try to perform all this by computer analysis, as opposed to manual annotation (a.k.a. curation) which involves human expertise. Ideally, these approaches co-exist and complement each other in the same annotation pipeline.

The simplest way to perform gene annotation relies on homology based search tools, like BLAST, to search for homologous genes in specific databases, the resulting information is then used to annotate genes and genomes. However, nowadays more and more additional information is added to the annotation platform. The additional information allows manual annotators to deconvolute discrepancies between genes that are given the same annotation. Some databases use genome context information, similarity scores, experimental data, and integrations of other resources to provide genome annotations through their Subsystems approach. Other databases (e.g. Ensembl) rely on both curated data sources as well as a range of different software tools in their automated genome annotation pipeline.

Structural annotation consists of the identification of genomic elements.

- ORFs and their localization
- Gene structure
- Coding regions
- location of regulatory motifs

Functional annotation consists of attaching biological information to genomic elements.

- Biochemical function
- Biological function
- involved regulation and interactions
- expression

These steps may involve both biological experiments and in silico analysis. Proteo-genomics based approaches utilize information from expressed proteins, often derived from mass spectrometry, to improve genomics annotations.

A variety of software tools have been developed to permit scientists to view and share genome annotations. Genome annotation remains a major challenge for scientists investigating the human genome, now that the genome sequences of more than a thousand human individuals and several model organisms are largely complete. Identifying the locations of genes and other genetic control elements is often described as defining the biological "parts list" for the assembly and normal operation of an organism. Scientists are still at an early stage in the process of delineating this parts list and in understanding how all the parts "fit together".[

Genome annotation is an active area of investigation and involves a number of different organizations in the life science community which publish the results of their efforts in publicly available biological databases accessible via the web and other electronic means. Here is an alphabetical listing of on-going projects relevant to genome annotation:

- Encyclopedia of DNA elements(ENCODE)
- Entrez Gene
- Ensembl
- GENCODE
- Gene Ontology Consortium
- GeneRIF
- RefSeq
- Uniprot
- Vertebrate and Genome Annotation Project (Vega)

Base calling and sequence accuracy

Base calling is the process by which an order of nucleotides in a template is inferred during a sequencing reaction. Next generation sequencing platforms that use fluorescently labeled reversible terminators have a unique color for each base. These are incorporated into the complementary strand of the DNA template and captured with a sensitive CCD camera. These images are processed into signals which are used to infer the order of nucleotides, also known as base calling. While sequencing platforms typically have integrated base calling software, the development of high performing base calling algorithms is an area of ongoing research.

Base calling accuracy is typically measured by a Q score (Phred quality score), a common metric to assess the accuracy of a sequencing run. Q scores are defined as logarithmically related to base calling error probability.

$Q = - 10 \log P / \log 10$

If a sequencing run is assigned a Q score of 40, this is equal to the probability of an incorrect base call of 1 in 10,000 times, or 99.99% base calling accuracy.

Q Score 10 - Base calling accuracy 1 in 10 - Probability of incorrect base 90% Q Score 20 - Base calling accuracy 1 in 100 - Probability of incorrect base 99%
Q Score 30 - Base calling accuracy 1 in 1,000 - Probability of incorrect base 99.9%

Q Score 40 - Base calling accuracy 1 in 10,000 - Probability of incorrect base 99.99%

Q Score 50 - Base calling accuracy 1 in 100,000 - Probability of incorrect base 99.999%

A lower Q score of 10 means, there is the probability of an incorrect call in 1 of 10 bases. Lower Q scores can lead to increases in false positive variant calls and reduces the overall confidence an investigator has in their sequencing data

Probable Questions:

1. How DNA markers can be used for genetic mapping?
2. Write down the importance of SNP markers in genetic mapping.
3. Describe basic methodology of restriction mapping.
4. What is optical mapping. Describe the technique.
5. Describe FISH method. What are its significance.
6. Describe STTS mapping.
7. Describe the methodology of DNA sequencing?
8. What is high throughput DNA sequencing?
9. What do you mean by base calling?
10. What is DNA annotation?

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Unit-III

Large scale mutagenesis and interference - genome wide gene targeting; systematic approach, random mutagenesis, insertional mutagenesis, libraries of knock-down phenocopies created by RNA interference; transcriptome analysis, DNA micro-array profiling, data processing and presentation, expression profiling, proteomics - expression analysis, protein structure analysis, protein-protein interaction

Objective: In this unit you will learn about Large scale mutagenesis and interference - genome wide gene targeting; systematic approach, random mutagenesis, insertional mutagenesis, libraries of knock-down phenocopies created by RNA interference; transcriptome analysis, DNA micro-array profiling, data processing and presentation, expression profiling, proteomics - expression analysis, protein structure analysis, protein-protein interaction.

Gene Targeting:

Gene targeting (also, replacement strategy based on homologous recombination) is a genetic technique that uses homologous recombination to modify an endogenous gene. The method can be used to delete a gene, remove exons, add a gene and modify individual base pairs (introduce point mutations). Gene targeting can be permanent or conditional. Conditions can be a specific time during development / life of the organism or limitation to a specific tissue, for example. Gene targeting requires the creation of a specific vector for each gene of interest. However, it can be used for any gene, regardless of transcriptional activity or gene size. In general, DNA containing part of the gene to be targeted, a reporter gene, and a (dominant) selectable marker is assembled in bacteria. Gene targeting methods are established for several model organisms and may vary depending on the species used. To target genes in mice, the DNA is inserted into mouse embryonic stem cells in culture. Cells with the insertion can contribute to a mouse's tissue via embryo injection. Finally, chimeric mice where the modified cells make up the reproductive organs are bred. After this step the entire body of the mouse is based on the selected embryonic stem cell.

To target genes in moss, the DNA is incubated together with freshly isolated protoplasts and with polyethylene glycol. As mosses are haploid organisms, moss filaments (protonema) can be directly screened for the target, either by treatment with antibiotics or with PCR. Unique among plants, this procedure for reverse genetics is as efficient as in yeast. Gene targeting has been successfully applied to cattle, sheep, swine and many fungi. The frequency of gene targeting can be significantly enhanced through the use of engineered endonucleases such as zinc finger nucleases, engineered homing endonucleases, and nucleases based on engineered TAL effectors. This method has been applied to species including Drosophila melanogaster, tobacco, corn, human cells, mice and rats.

Gene targeting has been widely used to study human genetic diseases by removing ("knocking out"), or adding ("knocking in"), specific mutations of interest. Previously used to engineer rat cell models, advances in gene targeting technologies enable a new wave of isogenic human disease models. These models are the most accurate in vitro models available to researchers and facilitate the development of personalized drugs and diagnostics, particularly in oncology.

Random mutagenesis:

Random mutagenesis is an incredibly powerful tool for altering the properties of enzymes. Imagine, for example, you were studying a G-protein coupled receptor (GPCR) and wanted to create a temperature-sensitive version of the receptor or one that was activated by a different ligand than the wild-type.

Firstly, you would clone the gene encoding the receptor, then randomly introduce mutations into the gene sequence to create a "library" containing thousands of versions of the gene. Each version (or "variant") of the gene in the library would contain different mutations and so encode receptors with slightly altered amino acid sequences giving them slightly different enzymatic properties than the wild-type.

Next, you could transform the library into a strain where the receptor would be expressed and apply a high throughput screen to pick out variants in the library that have the properties you are looking for. Using a high throughput screen for GPCR activity you could pick out the variants from the library that were temperature-sensitive or were activated by different ligands. Creating a random mutant library that contains enough variants to give you a good chance of obtaining the altered enzyme you desire is a challenge in itself. There are many ways to create random mutant libraries, each with it's own pros and cons. Here are some of them:

1. Error-prone PCR. This approach uses a "sloppy" version of PCR, in which the polymerase has a fairly high error rate (up to 2%), to amplify the wild-type sequence. The PCR can be made error-prone in various ways including increasing the MgCl2 in the reaction, adding MnCl2 or using unequal concentrations of each nucleotide. After amplification, the library of mutant coding sequences must be cloned into a suitable plasmid. The drawback of this approach is that size of the library is limited by the efficiency of the cloning step. Although point mutations are the most common types of mutation in error prone PCR, deletions and frameshift mutations are also possible. There are a number of commercial error-prone PCR kits available, including those from Stratagene and Clontech.

2. Rolling circle error-prone PCR is a variant of error-prone PCR in which wild-type sequence is first cloned into a plasmid, then the whole plasmid is amplified under error-prone conditions. This eliminates the ligation step that limits library size in conventional error-prone PCR but of course the amplification of the whole plasmid is less efficient than amplifying the coding sequence alone.

3. Mutator strains. In this approach the wild-type sequence is cloned into a plasmid and transformed into a mutator strain, such as Stratagene'sXL1-Red. XL1-red is an *E.coli* strain whose deficiency in three of the primary DNA repair pathways (*mutS*, *mutD* and *mutT*) causes it to make errors during replicate of it's DNA, including the cloned plasmid. As a result each copy of the plasmid replicated in this strain has the potential to be different from the wild-type. One advantage of mutator strains is that a wide variety of mutations can be incorporated including substitutions, deletions and frame-shifts. The drawback with this method is that the strain becomes progressively sick as it accumulates more and more mutations in it's own genome so several steps of growth, plasmid isolation, transformation and re-growth are normally required to obtain a meaningful library.

4. Temporary mutator strains. Temporary mutator strains can be built by over-expressing a mutator allele such as *mutD5* (a dominant negative version of *mutD*) which limits the cell's ability to repair DNA lesions. By expressing *mutD5* from an inducible promoter it is possible to allow the cells to cycle between mutagenic (*mutD5* expression on) and normal (*mutD5* expression off) periods of growth. The periods of normal growth allow the cells to recover from the mutagenesis, which allows these strains to grow for longer than conventional mutator strains. If a plasmid with a temperature-sensitive origin of replication is used, the mutagenic plasmid can easily be removed restore normal DNA repair, allowing the mutants to

be grown up for analysis/screening.

5. Insertion mutagenesis. Finnzymes have a kit that uses a transposon-based system to randomly insert a 15-base pair sequence throughout a sequence of interest, be it an isolated insert or plasmid. This inserts 5 codons into the sequence, allowing any gene with an insertion to be expressed (i.e. no frame-shifts or stop codons are cause). Since the insertion is random, each copy of the sequence will have different insertions, thus creating a library.

6. Ethyl methane sulfonate (EMS) is a chemical mutagen. EMS aklylates guanidine residues, causing them to be incorrectly copied during DNA replication. Since EMS directly chemically modifies DNA, EMS mutagenesis can be carried out either in vivo (i.e. whole-cell mutagenesis) or invitro.

7. Nitrous acid is another chemical mutagen. It acts by de-aminating adenine and cytosine residues causing transversion point mutations (A/T to G/C and vice versa).

8. DNA Shuffling is a very powerful method in which members of a library (i.e. copies of same gene each with different types of mutation) are randomly shuffled. This is done by randomly digesting the library with DNase-I then randomly re-joining the fragments using self-priming PCR.

Insertional mutagenesis:

In molecular biology, insertional mutagenesis is the creation of mutations of DNA by the addition of one or more base pairs. Such insertional mutations can occur naturally, mediated by viruses or transposons, or can be artificially created for research purposes in the lab. This is a technique used to study the function of genes. A transposon such as the P element of Drosophila melanogaster is allowed to integrate at random locations in the genome of the organism being studied. Mutants generated by this method are then screened for any unusual phenotypes. If such a phenotype is found then it can be assumed that the insertion has caused the gene relating to the usual phenotype to be inactivated. Because the sequence of the transposon is known, the gene can be identified, either by sequencing the whole genome and searching for the sequence, or using the polymerase chain reaction to amplify specifically that gene.

Virus insertional mutagenesis

Because many viruses integrate their own genomes into the genomes of their host cells in order to replicate, mutagenesis caused by viral infections is a fairly common occurrence. Not all integrating viruses cause insertional mutagenesis, however some DNA insertions will lead to no noticeable mutation. In recent gene therapy trials, the lentiviral vectors used to insert therapeutic DNA showed no tendency to disrupt gene function or promote oncogenic development.[1][2] Because of these advances, it is now considered safe to use such integrating vectors for gene therapy. An advantage is that the lentiviral vectors integrate the DNA permanently, whereas other, non-integrating, viruses' effect is transient. For those viruses such as gamma retroviruses that tend to integrate their DNA in genetically unfavorable locations, the severity of any ensuing mutation depends entirely on the location within the host's genome wherein the viral DNA is inserted. If the DNA is inserted into the middle of an essential gene, the effects on the cell will be drastic. Additionally, insertion into a gene's promoter region can have equally drastic effects. Likewise, if the viral DNA is inserted into a repressor, the promoter's corresponding gene may be over-expressed – leading to an overabundance of its product and altered cellular activity. If the DNA is inserted into a gene's enhancer region, the gene may be under-expressed – leading to relative absence of its product, which can significantly interrupt the activity of the cell.

Alteration of different genes will have varying effects on the cell. Not all mutations will significantly affect the proliferation of the cell. However, if the insertion occurs in an essential gene or a gene that is involved in cellular replication or programmed cell death, the insertion may compromise the viability of

the cell or even cause the cell to replicate interminably – leading to the formation of a tumor, which may become cancerous.

Insertional mutagenesis is possible whether the virus is of the self-inactivating types commonly used in gene therapy or competent to replicate. The virus inserts a gene (known as a viral onocogene) normally near the cellular myc (c-myc)gene. The c-myc gene is normally turned off in the cell; however when it is turned on it is able to push the cell into the G1 phase of the cell cycle and cause the cell to begin replication, causing unchecked cell proliferation while allowing the viral gene to be replicated. After many replications where the viral gene stays latent tumours begin to grow. These tumours are normally derived from one mutated/transformed cell (clonal in origin). Avian leukosis virus is an example of a virus that causes a disease by insertional mutagenesis. Newly hatched chicks infected with Avian leukosis virus will begin to form tumours that will begin to appear in their Bursa of Fabricus (like the human thymus). This viral geneinsertionisalsoknownasapromoterinsertionasitdrivestheexpressionofthec-myc gene. There is an example of an insertional mutagenesis event caused by a retrotransposon in the human genome where it causes Fukuyama-type muscular dystrophy.

Insertional inactivation

Insertional inactivation is a technique used in recombinant DNA engineering where a plasmid (such as pBR322) is used to disable expression of a gene. The inactivation of a gene by inserting a fragment of DNA into the middle of its coding sequence. Any future products from the inactivated gene will not work because of the extra codes added to it. An example is the use of pBR322, which has genes that respectively encode polypeptides that confer resistance to ampicillin and tetracycline antibiotics. Hence, when a genetic region is interrupted by integration of pBR322, the gene function is lost but new gene function (resistance to specific antibiotics) is gained.

An alternative strategy for insertional mutagenesis has been used in vertebrate animals to find genes that cause cancer. In this case a transposon, e.g. Sleeping Beauty, is designed to interrupt a gene in such a way that it causes maximal genetic havoc. Specifically, the transposon contains signals to truncate expression of an interrupted gene at the site of the insertion and then restart expression of a second truncated gene. This method has been used to identify oncogenes.

Transcriptome analysis:

The transcriptome is the set of all RNA molecules in one cell or a population of cells. It is sometimes used to refer to all RNAs, or just mRNA, depending on the particular experiment. It differs from the exome in that it includes only those RNA molecules found in a specified cell population, and usually includes the amount or concentration of each RNA molecule in addition to the molecular identities.

he term can be applied to the total set of transcripts in a given organism, or to the specific subset of transcripts present in a particular cell type. Unlike the genome, which is roughly fixed for a given cell line (excluding mutations), the transcriptome can vary with external environmental conditions. Because it includes all mRNA transcripts in the cell, the transcriptome reflects the genes that are being actively expressed at any given time, with the exception of mRNA degradation phenomena such as transcriptional attenuation.

The study of transcriptomics, (which includes expression profiling, splice variant analysis etc), examines the expression level of RNAs in a given cell population, often focusing on mRNA, but sometimes including others such as tRNAs, sRNAs.

Transcriptomics techniques include DNA microarrays and next-generation sequencing technologies called RNA-Seq. Transcription can also be studied at the level of individual cells by single-cell transcriptomics.

There are two general methods of inferring transcriptome sequences. One approach maps sequence reads onto a reference genome, either of the organism itself (whose transcriptome is being studied) or of a closely related species. The other approach, *de novo* transcriptome assembly, uses software to infer transcripts directly from short sequence reads.

The transcriptomes of stem cells and cancer cells are of particular interest to researchers who seek to understand the processes of cellular differentiation and carcinogenesis. Analysis of the transcriptomes of human oocytes and embryos is used to understand the molecular mechanisms and signaling pathways controlling early embryonic development, and could theoretically be a powerful tool in making proper embryo selection in in vitro fertilization.
Transcriptomics is an emerging and continually growing field in biomarker discovery for use in assessing the safety of drugs or chemical risk assessment. Transcriptomes may also be used to infer phylogenetic relationships among individuals.

Analysis methods:

Transcriptomics technologies are the techniques used to study an organism's transcriptome, the sum of all of its RNA transcripts. The information content of an organism is recorded in the DNA of its genome and expressed through transcription. Here, mRNA serves as a transient intermediary molecule in the information network, whilst non-coding RNAs perform additional diverse functions. A transcriptome captures a snapshot in time of the total transcripts present in a cell. Transcriptomics technologies provide a broad account of which cellular processes are active and which are dormant.

The first attempts to study whole transcriptomes began in the early 1990s. Subsequent technological advances since the late 1990s have repeatedly transformed the field, and made transcriptomics a widespread discipline in biological sciences. There are two key contemporary techniques in the field: microarrays, which quantify a set of predetermined sequences, and RNA- Seq, which uses high-throughput sequencing to record all transcripts. As the technology improved, the volume of data produced by each transcriptome experiment increased. As a result, data analysis methods have steadily been adapted to more accurately and efficiently analyse increasingly large volumes of data. Transcriptome databases have grown and increased in utility as more transcriptomes are collected and shared by researchers. It would be almost impossible to interpret the information contained in a transcriptome without the context of previous experiments.

Measuring the expression of an organism's genes in different tissues or conditions, or at different times, gives information on how genes are regulated and reveal details of an organism's biology. It can also be used to infer the functions of previously unannotated genes. Transcriptome analysis has enabled the study of how gene expression changes in different organisms and has been instrumental in the understanding of human disease. An analysis of gene expression in its entirety allows detection of broad coordinated trends which cannot be discerned by more targeted assays.

The dominant contemporary techniques, microarrays and RNA-Seq, were developed in the mid- 1990s and 2000s. Microarrays that measure the abundances of a defined set of transcripts via their hybridization to an array of complementary probes were first published in 1995. Microarray technology allowed the assay of thousands of transcripts simultaneously and at a greatly reduced cost per gene and labour saving. Both spotted oligonucleotide arrays and Affymetrix high- density arrays were the method of choice for transcriptional profiling until the late 2000s. Over this period, a range of microarrays were produced to cover known genes in model or economically important organisms. Advances in design and manufacture

of arrays improved the specificity of probes and allowed more genes to be tested on a single array. Advances in fluorescence detection increased the sensitivity and measurement accuracy for low abundance transcripts.

RNA-Seq refers to the sequencing of transcript cDNAs, where abundance is derived from the number of counts from each transcript. The technique has therefore been heavily influenced by the development of high-throughput sequencing technologies. Massively parallel signature sequencing (MPSS) was an early example based on generating 16–20 bp sequences via a complex series of hybridizations, and was used in 2004 to validate the expression of ten thousand genes in *Arabidopsis thaliana*. The earliest RNA-Seq work was published in 2006 with one hundred thousand transcripts sequenced using 454 technology.[This was sufficient coverage to quantify relative transcript abundance. RNA-Seq began to increase in popularity after 2008 when new Solexa/Illumina technologies allowed one billion transcript sequences to be recorded. This yield now allows for the quantification and comparison of human transcriptomes.

Data gathering

Generating data on RNA transcripts can be achieved via either of two main principles: sequencing of individual transcripts (ESTs, or RNA-Seq) or hybridization of transcripts to an ordered array of nucleotide probes (microarrays).

a. Isolation of RNA

All transcriptomic methods require RNA to first be isolated from the experimental organism before transcripts can be recorded. Although biological systems are incredibly diverse, RNA extraction techniques are broadly similar and involve mechanical disruption of cells or tissues, disruption of RNase with chaotropic salts, disruption of macromolecules and nucleotide complexes, separation of RNA from undesired biomolecules including DNA, and concentration of the RNA via precipitation from solution or elution from a solid matrix. Isolated RNA may additionally be treated with DNase to digest any traces of DNA. It is necessary to enrich messenger RNA as total RNA extracts are typically 98% ribosomal RNA. Enrichment for transcripts can be performed by poly-A affinity methods or by depletion of ribosomal RNA using sequence-specific probes. Degraded RNA may affect downstream results; for example, mRNA enrichment from degraded samples will result in the depletion of 5’ mRNA ends and an uneven signal across the length of a transcript. Snap-freezing of tissue prior to RNA isolation is typical, and care is taken to reduce exposure to RNase enzymes once isolation is complete.

b. Serial and cap analysis of gene expression(SAGE/CAGE).

Serial analysis of gene expression (SAGE) was a development of EST methodology to increase the throughput of the tags generated and allow some quantitation of transcript abundance. cDNA is generated from the RNA but is then digested into 11 bp "tag" fragments using restriction enzymes that cut DNA at a specific sequence, and 11 base pairs along from that sequence. These cDNA tags are then joined head-to-tail into long strands (>500 bp) and sequenced using low- throughput, but long read-length methods such as Sanger sequencing. The sequences are then divided back into their original 11 bp tags using computer software in a process called deconvolution. If a reference genome is available, these tags may be matched to their corresponding gene in the genome. If a reference genome is unavailable, the tags can be directly used as diagnostic markers if found to be differentially expressed in a disease state.

The cap analysis gene expression (CAGE) method is a variant of SAGE that sequences tags from the 5’ end of an mRNA transcript only. Therefore, the transcriptional start site of genes can be identified when

the tags are aligned to a reference genome. Identifying gene start sites is of use for promoter analysis and for the cloning of full-length cDNAs. SAGE and CAGE methods produce information on more genes than was possible when sequencing single ESTs, but sample preparation and data analysis are typically more labour-intensive.

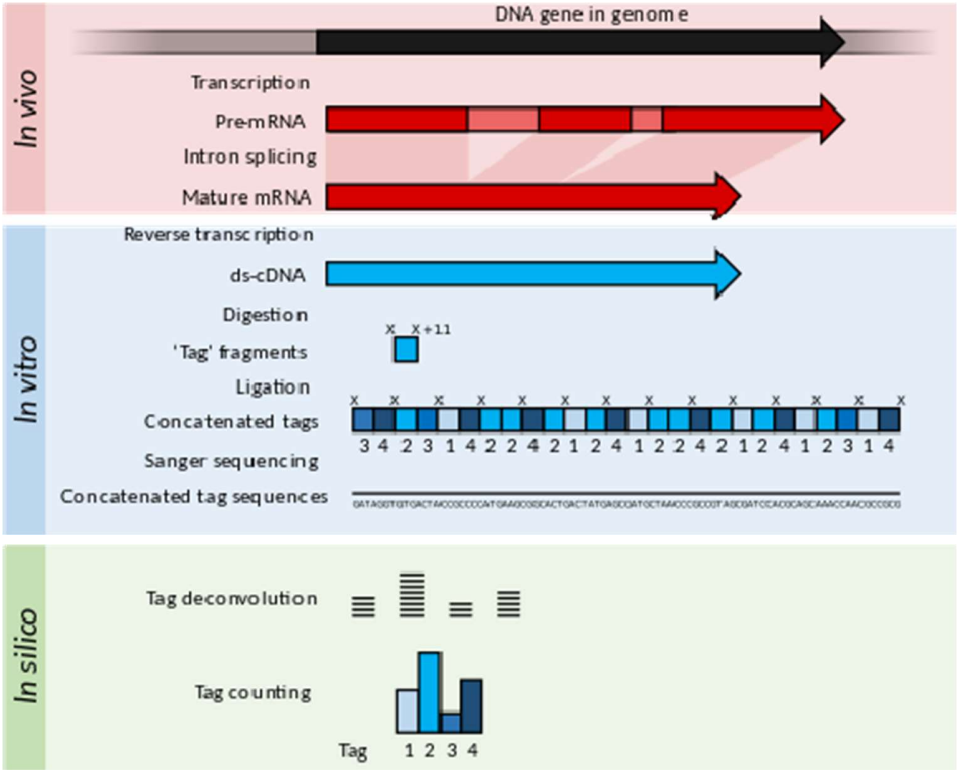


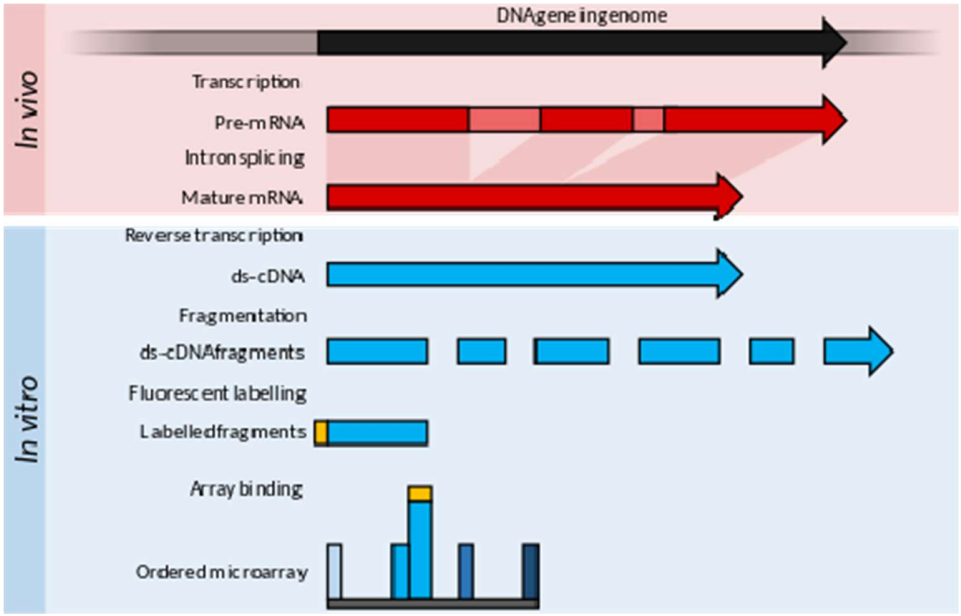
Figure: SAGE. Within the organisms, genes are transcribed and spliced (in eukaryotes) to produce mature mRNA transcripts (red). The mRNA is extracted from the organism, and reverse transcriptase is used to copy the mRNA into stable double-stranded–cDNA (ds-cDNA; blue). In SAGE, the ds-cDNA is digested by restriction enzymes (at location 'X' and 'X'+11) to produce 11-nucleotide "tag"fragments. These tags are concatenated and sequenced using long-read

Sanger sequencing (different shades of blue indicate tags from different genes). The sequences are deconvoluted to find the frequency of each tag. The tag frequency can be used to report on transcription of the gene that the tag came from.

DNA Microarrays :

Microarrays consist of short nucleotide oligomers, known as "probes", which are typically arrayed in a grid on a glass slide. Transcript abundance is determined by hybridization of fluorescently labelled transcripts to these probes. The fluorescence intensity at each probe location on the array indicates the transcript abundance for that probe sequence .Microarrays require some genomic knowledge from the organism of interest, for example, in the form of an annotated genome sequence, or a library of ESTs that can be used to generate the probes for the array.Microarrays for transcriptomics typically fall into one of two broad categories: low-density spotted arrays or high-density short probe arrays. Transcript abundance is inferred from the intensity of fluorescence derived from fluorophore-tagged transcripts that bind to the array.

Spotted low-density arrays typically feature picolitre drops of a range of purified cDNAs arrayed on the surface of a glass slide. These probes are longer than those of high-density arrays and cannot identify alternative splicing events. Spotted arrays use two different fluorophores to label the test and control samples, and the ratio of fluorescence is used to calculate a relative measure of abundance. High-density arrays use a single fluorescent label, and each sample is hybridized and detected individually. High-density arrays were popularized by the Affymetrix GeneChip array, where each transcript is quantified by several short 25-mer probes that together assay one gene. NimbleGen arrays were a high-density array produced by a maskless-photochemistry method, which permitted flexible manufacture of arrays in small or large numbers. These arrays had 100,000s of 45 to 85-mer probes and were hybridized with a one-colour labelled sample for expression analysis. Some designs incorporated up to 12 independent arrays per slide.



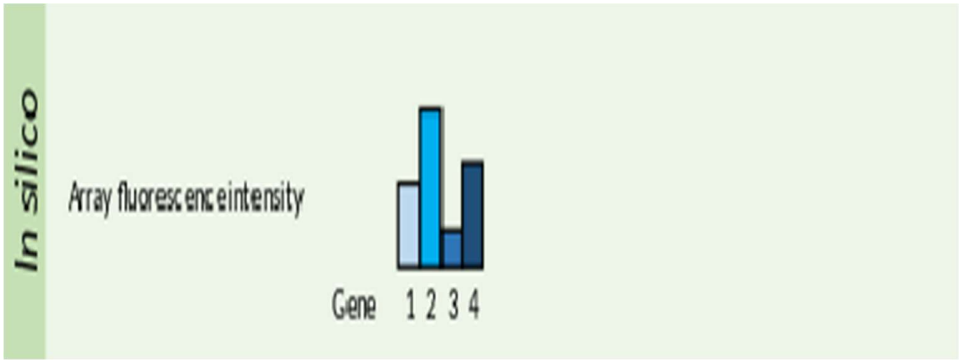


Figure: DNA Microarrays. Within the organisms, genes are transcribed and spliced (in eukaryotes) to produce mature mRNA transcripts (red). The mRNA is extracted from the Organism and reverse transcriptase is used to copy them RNA into stable ds-cDNA(blue). In microarrays, the ds-cDNA is fragmented and fluorescently labelled (orange). The labelled fragments bind to an ordered array of complementary oligonucleotides, and measurement of fluorescent intensity across the array indicates the abundance of a predetermined set of sequences. These sequences are typically specifically chosen to report on genes of interest within the organism's genome.

Data Analysis:

Transcriptomics methods are highly parallel and require significant computation to produce meaningful data for both microarray and RNA-Seq experiments. Microarray data is recorded as high-resolution images, requiring feature detection and spectral analysis. Microarray raw image files are each about 750 MB in size, while the processed intensities are around 60 MB in size. Multiple short probes matching a single transcript can reveal details about the intron-exon structure, requiring statistical models to determine the authenticity of the resulting signal. RNA- Seq studies produce billions of short DNA sequences, which must be aligned to reference genomes composed of millions to billions of base pairs. *De novo* assembly of reads within a dataset requires the construction of highly complex sequence graphs. RNA-Seq operations are highly repetitious and benefit from parallelized computation but modern algorithms mean consumer computing hardware is sufficient for simple transcriptomics experiments that do not require *de novo* assembly of reads. A human transcriptome could be accurately captured using RNA-Seq with 30 million 100 bp sequences per sample. This example would require approximately 1.8 gigabytes of disk space per sample when stored in a compressed fastq format. Processed count data for each gene would be much smaller, equivalent to processed microarray intensities. Sequence data may be stored in public repositories, such as the Sequence Read Archive (SRA). RNA-Seq datasets can be uploaded via the Gene Expression Omnibus

Image processing:

Microarray image processing must correctly identify the regular grid of features within an image and independently quantify the fluorescence intensity for each feature. Image artefacts must be additionally identified and removed from the overall analysis. Fluorescence intensities directly indicate the abundance of each sequence, since the sequence of each probe on the array is already known.

The first steps of RNA-seq also include similar image processing; however, conversion of images to sequence data is typically handled automatically by the instrument software. The Illumina sequencing-by-synthesis method results in an array of clusters distributed over the surface of a flow cell. The flow cell is imaged up to four times during each sequencing cycle, with tens to hundreds of cycles in total. Flow cell clusters are analogous to microarray spots and must be correctly identified during the early stages of the sequencing process. In Roche's pyrosequencing method, the intensity of emitted light determines the number of consecutive nucleotides in a homopolymer repeat. There are many variants on these methods, each with a different error profile for the resulting data.

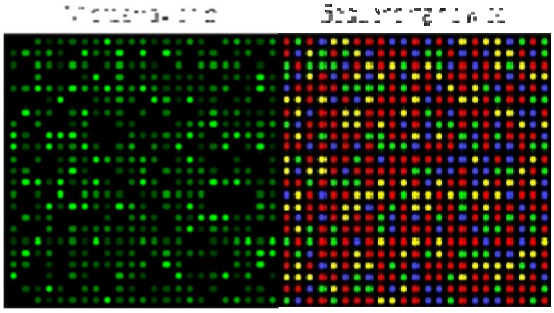


Figure: Microarray and sequencing flow cell. Microarrays and RNA-seq rely on image analysis in different ways. In a microarray chip, each spot on a chip is a defined oligonucleotide probe, and fluorescence intensity directly detects the abundance of a specific sequence (Affymetrix).

Differential expression : Once quantitative counts of each transcript are available, differential gene expression is measured by normalizing, modelling, and statistically analysing the data. Most tools will read a table of genes and read counts as their input, but some programs, such as cuffdiff, will accept binary alignment map format read alignments as input. The final outputs of these analyses are gene lists with associated pair-wise tests for differential expression between treatments and the probability estimates of those differences.

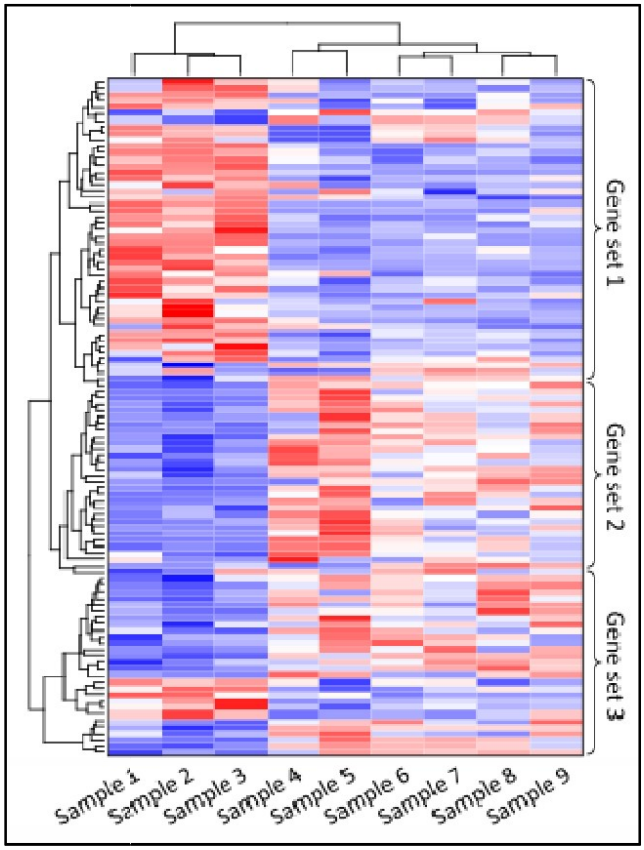


Figure: Heatmap identification of gene co expression patterns across different samples. Each column contains the measurements for gene expression change for a single sample. Relative gene expression is indicated by colour. High expression (red), medium expression (white and low expression (blue). Genes and samples with similar expression profiles can be automatically grouped (left and top trees). Samples may be different individuals, environments and health conditions. In this example, expression of gene set 1 is high and expression of gene set 2 is low in samples 1, 2, and3.

Proteomics:

The gene transcripts that an individual can make in a lifetime—termed as transcriptome (by analogy with the term genome)—refers to the haploid set of chromosomes carrying all the functional genes. Similarly, all the proteins made by an organism are now grouped under the shade of proteomics. Proteomics involves the systematic study of proteins in order to provide a comprehensive view of the structure, function and role in the regulation of a biological system.

These include protein-protein interaction, protein modification, protein function and its localization studies. The aim of proteomics is not only to identify all the proteins in a cell but also to create a complete three-dimensional map of the cell indicating where proteins are located. Coupled with advances in bioinformatics, this approach to comprehensively describing biological systems will undoubtedly have a major impact on our understanding of the phenotype of both normal and diseased cells. The proteome (term coined by Mark Wilkins in 1995) of a given cell is the total number of proteins at any given instant and it is highly dynamic in response to internal and external cues. Proteins can be modified by post-translational modifications, undergo translocations within the cell or be synthesized or degraded.

Therefore, the examination of proteins of a cell at a particular time reflects the immediate protein environment in which it is studied. A cellular proteome is the collection of proteins found in a particular cell type under the influence of a particular set of environmental conditions like exposure to hormone stimulation. A complete set of proteins from all of the various cellular proteomes will form an organism's complete proteome. An interesting finding of the Human Genome Project is that there are far more proteins in the human proteome (~ 400,000 proteins) than there are protein-coding genes in the human genome(~ 22,000 genes). The large increase in protein diversity is thought to be due to alternative splicing and post-translational modification of proteins. This indicates that protein diversity cannot be fully characterized by gene expression analysis alone. Proteomics, thus is a useful tool for characterizing cells and tissues of interest.

The first protein studies that can be called proteomics began with the introduction of two dimensional gel electrophoresis of E. coli proteins (O'Ferrall, 1975) followed by mouse and guinea pig protein studies (Ksole, 1975). Although 2-dimensional electrophoresis (2-DE) was a major step forward and many proteins could be separated and visualized by this technique but it was not enough for the protein identification through any sensitive protein sequencing technology.

After certain efforts the first major technology for the identification of protein was protein sequencing by Edman degradation (Edman, 1949). This technology was used for the identification of proteins from 2-D gels to create first 2D database (Celis et al. 1987). Another most important development in protein identification was Mass Spectrometry (MS) technology (Andersen et al. 2000). Protein sequencing by MS technology has been increased due to its sensitivity of analysis, tolerate protein complexes and amenable to high throughput operations. Although several advancements have been made in protein identification (by MS or Edman sequencing) without having the database of large scale DNA sequencing of expressed sequences and genomic DNA, proteins could not be characterized because different protein isoforms can be generated from a single gene through several modifications. And the majority of DNA and protein sequences have been accumulated within a short period of time.

In 1995, the sequencing of the genome of an organism was done for the first time in *Haemophilus influenzae* (Fleischmann et al. 1995). Till date, sequencing of several other eukaryotic genomes have been completed viz. Arabidopsis thaliana (Tabata, 2000), *Sachcharomyces cerevisiae* (Goffeau, 1996), *Caenorhabditis elegans* (Abbott, 1998), *Oryza sativa*(Matsumoto, 2001) and human (Venter,2001). For protein expression profiling, a common procedure is the analysis of mRNA by different methods including serial analysis of gene expression (SAGE) (Velculescu et al. 1995) and DNAmicroarray technology (Shalon, 1996). However, the level of transcription of a gene gives only a rough idea of the real level of expression of that gene. An mRNA may be produced in abundance, but at the same time degraded rapidly, or translated inefficiently keeping the amount of protein minimum. Proteins having been formed are subjected to post-translational modifications also. Different post-translational modifications or proteolysis and compartmentalization regulate the protein functions in the cell.

The average number of proteins formed per gene was predicted to be one or two in bacterium, three in yeast and three or more in humans (Wilkins et al. 1996). In response to extra-cellular responses, a number of proteins undergo post-translational modifications. Protein phosphorylation is an important signaling mechanism and dis-regulation of protein kinase and phosphatase can result oncogenesis (Hunter, 1995).

Through proteome analysis, changes in the modifications of many proteins expressed by a cell can be analyzed after translation. Another important feature of a protein is its localization in the cell. The mis-localization of proteins is known to have an adverse effect on cellular function (cystic fibrosis) (Drumm and Collins, 1993). The cell growth, programmed cell death and the decision to proceed through the cell cycle are all regulated by signal transduction through protein complexes (Pippin et al. 1993). The protein interaction can be detected by using yeast two-hybrid system (Rain et al. 2001).

To Understand a Proteome, Three Distinct Type of Analysis must be Carried Out:

(1) Protein-expression proteomics is the quantitative study of the protein expression of the entire proteome or sub-proteome of two samples that differ by some variable. Identification of novel proteins in signal transduction and disease specific proteins are major outcome of thisapproach.

(2) Structural proteomics attempts to identify all the proteins within a complex or organelle, determine their localization, and characterize all protein-protein interactions. The major goal of these studies is to map out the structure of protein complexes or cellular organelle proteins (Blackstock and Weir,1999).

(3)Functional proteomics allows the study of a selected group of proteins responsible in signaling pathways, diseases and protein-protein interactions. This may be possible by isolating the specific sub-proteomes by affinity-chromatography.

Technology of Proteomics:

Measurement of the level of a gene transcript does not necessarily give clear picture of protein products formed. Therefore, for the measurement of real gene expression, the proteins should be analyzed. Before the identification and measurement of the activity, all the proteins in a proteome for any instant should be separated from each other.

A Typical Proteomics Experiment (e. g. Protein Expression Profiling) can be Divided into the following Categories:

(i) Separation and isolation of protein

(ii) The acquisition of protein structural information for protein identification and characterization

(iii) Database utilization.

(i) Protein Separation and Isolation:

An essential component of proteomics is the protein electrophoresis, the most effective way to resolve a complex mixture of proteins. Two types of electrophoresis are available as one and two-dimensional electrophoresis. In one dimensional gel electrophoresis (1-DE), proteins are resolved on the basis of their molecular masses. Proteins are stable enough during 1-DE due to their solubility in sodium dodycyl sulphate (SDS). Proteins with molecular mass of 10-300 kDa can be easily separated through 1-DE.

But with complex protein mixtures, results with 1-DE are limited, so for more complex protein mixture such as crude cell lysate, the best separation tool available is two dimensional gel electrophoresis (2-DE) (O'Ferrall, 1975). Here, proteins are separated according to their net charges in first dimension and according to their molecular masses in second dimension.
As a single 2-DE gel can resolve thousands of proteins, it remains a powerful tool for the cataloging of proteins. Two-dimensional electrophoresis has the ability to resolve proteins that have gone under some post-translational modifications as well as protein expression of any two samples can be compared quantitatively and qualitatively. Recently pH gradients have been introduced to 2-DE which greatly improved the reproducibility of this technique (Bjellqvist et al. 1993).

However, few problems with 2-DE still remain to be solved. Despite efforts to automate protein analysis by 2-DE, it is still a labour-intensive and time-consuming process. Another major limitation of 2-DE is the inability to detect low copy number proteins when a total cell lysate is analyzed (Link et al. 1997; Shevchenko et al. 1996) as well as inefficiency to speed up the in-gel digestion process also. Therefore, alternatives have been searched to bypass protein gel electrophoresis. One approach is proteolytic digestion of protein mixture to convert them into peptides and then purify the peptides before subjecting them to analysis by mass spectrometry (MS). Peptide purification has been simplified through liquid chromatography (Link et al. 1999; McCormack et al. 1997), capillary electrophoresis (Figeys et al. 1999; Tong et al. 1999) and reverse phase chromatography (Opiteck et al. 1997).

Recently, Juan et al. (2005) have developed a new approach to speed up the protein identification process utilizing 'microwave' technology. Proteins excised from the gels are subjected to trypsin digestion by microwave irradiation, which rapidly produces peptides fragments. These fragments could be analyzed by MALDI (Matrix Assisted Laser Desorption/Ionization). Despite much downstream research on certain alternatives to 2-DE, this is the most widely utilized technique for proteome studies.

(ii) Acquisition of Protein Structures: Protein Identification:

Edman Sequencing (ES):

One of the earliest methods used for protein identification was micro sequencing by Edman chemistry to obtain N-terminal amino acid sequences. This technique was introduced by Edman in 1949. In Edman sequencing, N-terminal of a protein is sequenced to determine its true start site. Edman sequencing is more applicable sequencing method for the identification of proteins separated by SDS-Polyacrylamide gel electrophoresis. This method has been used extensively in the starting years of proteomics but certain limitations have emerged in recent time. One of the major limitations is the N-terminal modification of proteins. If any protein is blocked on N-terminal before sequencing, then it is very difficult to identify the protein. To overcome this problem a novel

approach of mixed peptide sequencing (Damer et al. 1998) has been employed recently. In this approach, a protein is converted into peptides by cleavage with cyanogen bromide (CNBr) or skatole followed by the Edman sequencing of peptides.

Mass Spectrometry (MS):

The most significant breakthrough in proteomics has been the mass spectrometric identification of gel-separated proteins. Due to its high sensitivity levels, identification of proteins in protein complexes/mixtures and high throughput, this technique has been proved far better than ES.

In mass spectrometry, proteins are digested into peptides in the gel itself by suitable protease such as trypsin, because proteins, as such, are difficult to elute out from the gels. Moreover, molecular weight of proteins is not usually suitable for database identification. In contrast, peptides can be eluted from the gels easily and matching of even a small set of peptides to the database is quite sufficient to identify aprotein.

There are Two Main Approaches to Mass Spectrometric Protein Identification:

(i) "Electrospray ionization" (ESI) involves the fragmentation of individual peptides followed by direct ionization through electrospray in a tandem mass spectrometer. In ESI, a liquid sample flows from a microcapillary tube into the orifice of the mass spectrometer, where a potential difference between the capillary and the inlet to the mass spectrometer results in the generation of a fine mist of charged droplets (Fenn et al. 1989; Hunt et al.1981). It has the ability to resolve peptides in a mixture, isolate one species at a time and dissociate it into amino or carboxy-terminal containing fragments designated 'b' and 'y', respectively.

(ii) In "Peptide mass mapping" approach (Henzel et al. 1993) the mass spectrum of the eluted peptide mixture is acquired, which result in a peptide mass fingerprint of the protein being studied. The mass spectrum is obtained by a relatively simple 'mass spectrometric method- matrix assisted laser desorption/ ionization'(MALDI).

In this approach, tryptic peptide mixture is analyzed because trypsin cleaves proteins at the amino acid arginine and lysine. As the tryptic peptides can be predicted theoretically for any protein, the predicted peptide masses can be compared with those obtained experimentally by MALDI analysis. If the sufficient number of peptide matches with the existing protein sequence in database, the accuracy for protein identification is high.

After the protease cleavages of the proteins, they are analyzed by mass analysis also. Mass analysis follows the conversion of proteins or peptides into molecular ions. These ions got separated in a mass spectrometer based on their mass/charge (m/z) ratio. It is determined by the time it takes for the ions to reach the detector. Hence the instrument is called a time of flight (TOF) instrument.

The relationship that allows the m/z ratio to be determined is $E = 1/2\ (m/z)v^2$. In this equation. E is the energy imparted on the charged ions as a result of the voltage that is applied by the instrument and V is the velocity of the ions down the flight path. As peptide ions are introduced into the collision chamber, they interact with collision gas and undergo fragmentation along the peptide backbone (Fig. 18.4). Because all the ions are exposed to the same electric field, all similarly charged ions will have similar energies. Therefore, based on the above equation, ions that have larger mass must have lower velocities and hence will require longer times to reach the detector.

(iii) Database Utilization:

Initially, sequencing of some proteins or peptides followed by the submission of sequences together created an assembly of proteins called protein database. Proteolytic digestion of many proteins are also predicted theoretically and deposited in database. Hence, at present, so much information has been accumulated that we can search for a homology between a new peptide sequence and the existing sequences in the database to identify the protein.

The major goal of database searching is to identify a large number of proteins—quickly and accurately. All the information accumulated through Edman sequencing or mass spectrometry are used to identify the proteins. In peptide mass fingerprinting database searching, the mass of a unknown peptide after proteolytic digestion is compared to the predicted mass of peptide from theoretical digestion of proteins in database. In amino acid sequence database searching, the sequence of amino acids from a peptide is identified and can be used to search databases to find the protein from which it was derived.

Collection of protein sequence databases are thus designed to represent a partial list of an organism's genome, that is, the genes and all of the proteins they encode. The protein families are usually classified according to their evolutionary history inferred from sequence homology.

These databases are excellent tools for gene discovery, comparative genomics and molecular evolution. The purpose of database similarity searching is the sensitive detection of sequence homologues, regardless of the species relationship in order to infer similarity of function from similarity of sequence. Recently, Chromatography-based proteomics is used to measure the concentration of low molecular weight peptides in complex mixtures such as plasma or sera. These technologies use time-of-flight (TOF) spectroscopy with matrix-assisted or surface- enhanced laser desorption/ionization to produce a spectrum of mass-to-charge (m/z) ratios that can be analysed in order to identify unique signatures from its chromatography pattern.

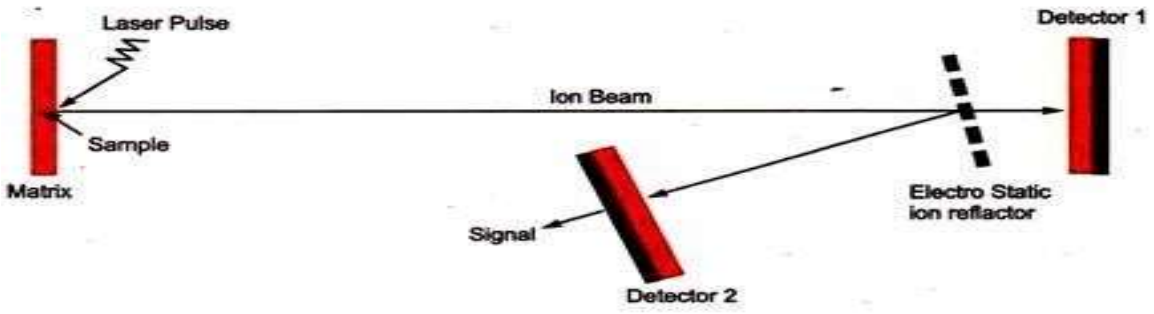


Fig. 18-4 : *Principle behind MALDI-TOF mass spectrometry. A sample is placed on the matrix and ionize by the laser beam. Due to the potential developed between the matrix and the sample, ions start moving towards the detector and get reflected by a reflector in the mid-way. Again after a flight in the tube the ions are detected by another detector. The time taken by these ions in the flight tubes depends on their masses. Therefore, we can calculate the ratio between the mass of an ion and the time of flight in the tube taken by that particular ion*

Applications of Proteomics:

1. Post-Translational Modifications:

Proteomics studies involve certain unique features as the ability to analyze post- translational modifications of proteins. These modifications can be phosphorylation, glycosylation and sulphation as well as some other modifications involved in the maintenance of the structure of a protein.

These modifications are very important for the activity, solubility and localization of proteins in the cell. Determination of protein modification is much more difficult rather than the identification of proteins. As for identification purpose, only few peptides are required for protease cleavages followed by database alignment of a known sequence of a peptide. But for determination of modification in a protein, much more material is needed as all the peptides do not have the expected molecular mass need to be analyzed further.

For example, during protein phosphorylation events, phosphopeptides are 80 Da heavier than their unmodified counterparts. Therefore, it gives, rise to a specific fragment (PO^{3-} mass 79) bind to metal resins, get recognized by specific antibodies and later phosphate group can be removed by phosphatases (Clauser et al. 1999; Colledge and Scott, 1999). So protein of interest (post-translationally modified protein) can be detected by Western blotting with the help of antibodies or ^{32}P-labelling that recognize only the active state of molecules. Later, these spots can be identified by mass spectrometry.

The major attribution of proteomics towards the development of protein interactions map of a cell is of immense value to understand the biology of a cell. The knowledge about the time of expression of a particular protein, its level of expression, and, finally, its interaction with another protein to form an intermediate for the performance of a specific biological function is currently available. These intermediates can be exploited for therapeutic purposes also. An attractive way to study the protein-protein interactions is to purify the entire multi-protein complex by affinity based methods using GST-fusion proteins, antibodies, peptides etc.

The yeast two-hybrid system has emerged as a powerful tool to study protein-protein interactions (Haynes and Yates, 2000). According to Pandey and Mann (2000) it is a genetic method based on the modular structure of transcription factors in the close proximity of DNA binding domain to the activation domain induces increased transcription of a set of genes. The yeast hybrid system uses ORFs fused to the DNA binding or activation domain of GAL4 such that increased transcription of a reporter gene results when the proteins encoded by two ORFs interact in the nucleus of the yeast cell. One of the main consequences of this is that once a positive interaction is detected, simply sequencing the relevant clones identifies the ORF. For this reason it is a generic method that is simple and amenable to high throughput screening of protein-protein interactions.

Phage display is a method where bacteriophage particles are made to express either a peptide or protein of interest fused to a capsid or coat protein. It can be used to screen for peptide epitopes, peptide ligands, enzyme substrate or single chain antibody fragments. Another important method to detect protein-protein interactions involves the use of fluorescence resonance energy transfer (FRET) between fluorescent tags on interacting proteins. FRET is a non-radioactive process whereby energy from an excited donor fluorophore is transferred to an acceptor fluorophore. After excitation of the first fluorophore, FRET is detected either by emission from the second fluorophore using appropriate filters or by alteration of the fluorescence lifetime of the donor.

A proteomics strategy of increasing importance involves the localization of proteins in cells as a necessary first step towards understanding protein function in complex cellular networks. The discovery of GFP (green fluorescent protein) and the development of its spectral variants has opened the door to analysis of proteins in living cells by use of the light microscope. Large-scale approaches of localizing GFP-tagged proteins in cells have been performed in the genetically amenable yeast S. pombe (Ding et al. 2000) and in Drosophila (Morin et al. 2001). To localize proteins in mammalian cells, a strategy was developed that enables the systematic GFP tagging of ORFs from novel full-length cDNAs that are identified in genome projects.

2. Protein Expression Profiling:

The largest application of proteomics continues to be protein expression profiling. The expression levels of a protein sample could be measured by 2-DE or other novel technique such as isotope coded affinity tag (ICAT). Using these approaches the varying levels of expression of two different protein samples can also be analyzed.

This application of proteomics would be helpful in identifying the signaling mechanisms as well as disease specific proteins. With the help of 2-DE several proteins have been identified that are responsible for heart diseases and cancer (Celis et al. 1999). Proteomics helps in identifying the cancer cells from the non-cancerous cells due to the presence of differentially expressed proteins.

The technique of Isotope Coded Affinity Tag has developed new horizons in the field of proteomics. This involves the labeling of two different proteins from two different sources with two chemically identical reagents that differ in their masses due to isotope composition (Gygietal. 1999). The biggest advantage of this technique is the elimination of protein quantitation by 2- DE. Therefore, high amount of protein sample can be used to enrich low abundance proteins.

Different methods have been used to probe genomic sets of proteins for biochemical activity. One method is called a biochemical genomics approach, which uses parallel biochemical analysis of a proteome comprised of pools of purified proteins in order to identify proteins and the corresponding ORFs responsible for a biochemical activity.

The second approach for analyzing genomic sets of proteins is the use of functional protein microarrays, in which individually purified proteins are separately spotted on a surface such as a glass slide and then analyzed for activity. This approach has huge potential for rapid high-throughput analysis of proteomes and other large collections of proteins, and promises to transform the field of biochemical analysis.

3. Molecular Medicine:

With the help of the information available through clinical proteomics, several drugs have been designed. This aims to discover the proteins with medical relevance to identify a potential target for pharmaceutical development, a marker(s) for disease diagnosis or staging, and risk assessment—both for medical and environmental studies. Proteomic technologies will play an important role in drug discovery, diagnostics and molecular medicine because of the link between genes, proteins and disease.

As researchers study defective proteins that cause particular diseases, their findings will help

develop new drugs that either alter the shape of a defective protein or mimic a missing one. Already, many of the best-selling drugs today either act by targeting proteins or are proteins themselves. Advances in proteomics may help scientists eventually create medications that are "personalized" for different individuals to be more effective and have fewer side effects. Current research is looking at protein families linked to disease including cancer, diabetes and heart disease.

Protein-Protein Interaction:

Proteins are the workhorses that facilitate most biological processes in a cell, including gene expression, cell growth, proliferation, nutrient uptake, morphology, motility, intercellular communication and apoptosis. But cells respond to a myriad of stimuli, and therefore protein expression is a dynamic process; the proteins that are used to complete specific tasks may not always be expressed or activated. Additionally, all cells are not equal, and many proteins are expressed in a cell type–dependent manner. These basic characteristics of proteins suggest a complexity that can be difficult to investigate, especially when trying to understand protein function in the proper biological context.

Critical aspects required to understand the function of a protein include:

- **Protein sequence and structure**—used to discover motifs that predict protein function
- **Evolutionary history and conserved sequences**—identifies key regulatory residues
- **Expression profile**—reveals cell-type specificity and how expression is regulated
- **Post-translational modifications**—phosphorylation, acylation, glycosylation and ubiquitination suggest localization, activation and/or function
- **Interactions with other proteins**—function may be extrapolated by knowing the function of binding partners
- **Intracellular localization**—may allude to the function of the protein

Until the late 1990s, protein function analyses mainly focused on single proteins. However, because the majority of proteins interact with other proteins for proper function, they should be studied in the context of their interacting partners to fully understand their function. With the publication of the human genome and the development of the field of proteomics, understanding how proteins interact with each other and identifying biological networks has become vital to understanding how proteins function within the cell.

Types of protein–protein interactions

Protein interactions are fundamentally characterized as stable or transient, and both types of interactions can be either strong or weak. Stable interactions are those associated with proteins that are purified as multi-subunit complexes, and the subunits of these complexes can be identical or different. Hemoglobin and core RNA polymerase are examples of multi-subunit interactions that form stable complexes.

Transient interactions are expected to control the majority of cellular processes. As the name implies, transient interactions are temporary in nature and typically require a set of conditions that promote the interaction, such as phosphorylation, conformational changes or localization to discrete areas of the cell. Transient interactions can be strong or weak, and fast or slow. While in contact with their binding partners, transiently interacting proteins are involved in a wide range of cellular processes, including protein modification, transport, folding, signaling, apoptosis and cell cycling. The following example provides an illustration of protein interactions that regulate apoptotic and anti-apoptotic processes.

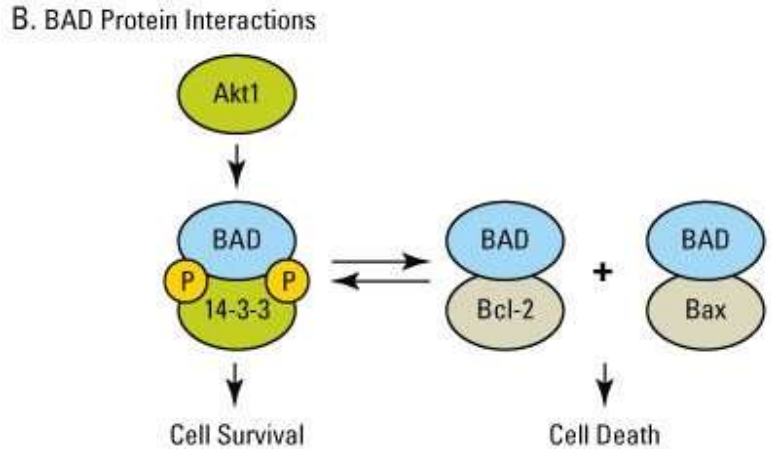


Figure ; Heavy BAD protein–protein interaction. Panel A: Coomassie-stained SDS-PAGE gel of recombinant light and heavy BAD-GST-HA-6xHIS purified from HeLa IVT lysates (L), using glutathione resin (E1) and cobalt resin (E2) tandem affinity. The flow-through (FT) from each column is indicated. Panel B: Schematic of BAD phosphorylation and protein interactions during cell survival and cell death (i.e., apoptosis). Panel C: BAD protein sequence coverage showing identified Akt consensus phosphorylation sites (red box). Panel D: MS spectra of stable isotope-labeled BAD peptide HSSYPAGTEDDEGmGEEPSPFr.

Proteins bind to each other through a combination of hydrophobic bonding, van der Waals forces, and salt bridges at specific binding domains on each protein. These domains can be small binding clefts or large surfaces and can be just a few peptides long or span hundreds of amino acids. The strength of the binding is influenced by the size of the binding domain. One example of a common surface domain that facilitates stable protein–protein interactions is the leucine zipper, which consists of α-helices on each protein that bind to each other in a parallel fashion through the hydrophobic bonding of regularly-spaced leucine residues on each α-helix that project between the adjacent helical peptide chains. Because of the tight molecular packing, leucine zippers provide stable binding for multi-protein complexes, although all leucine zippers do not bind identically due to non-leucine amino acids in the α-helix that can reduce the molecular packing and therefore the strength of the interaction.

Two Src homology (SH) domains, SH2 and SH3, are examples of common transient binding domains that bind short peptide sequences and are commonly found in signaling proteins. The SH2 domain recognizes peptide sequences with phosphorylated tyrosine residues, which are often indicative of protein activation. SH2 domains play a key role in growth factor receptor signaling,

during which ligand-mediated receptor phosphorylation at tyrosine residues recruits downstream effectors that recognize these residues via their SH2 domains. The SH3 domain usually recognizes proline-rich peptide sequences and is commonly used by kinases, phospholipases and GTPases to identify target proteins. Although both SH2 and SH3 domains generally bind to these motifs, specificity for distinct protein interactions is dictated by neighboring amino acid residues in the respective motif.

Biological effects of protein–protein interactions

The result of two or more proteins that interact with a specific functional objective can be demonstrated in several different ways. The measurable effects of protein interactions have been outlined as follows:

- Alter the kinetic properties of enzymes, which may be the result of subtle changes in substrate binding or allosteric effects
- Allow for substrate channeling by moving a substrate between domains or subunits, resulting ultimately in an intended end product
- Create a new binding site, typically for small effector molecules
- Inactivate or destroy aprotein
- Change the specificity of a protein for its substrate through the interaction with different binding partners, e.g., demonstrate a new function that neither protein can exhibit alone
- Serve a regulatory role in either an upstream or a downstream event

Common methods to analyze protein–protein interactions

Usually a combination of techniques is necessary to validate, characterize and confirm protein interactions. Previously unknown proteins may be discovered by their association with one or more proteins that are known. Protein interaction analysis may also uncover unique, unforeseen functional roles for well-known proteins. The discovery or verification of an interaction is the first step on the road to understanding where, how and under what conditions these proteins interact *in vivo* and the functional implications of these interactions.

While the various methods and approaches to studying protein–protein interactions are too numerous to describe here, the table below and the remainder of this section focuses on common methods to analyze protein–protein interactions and the types of interactions that can be studies using each method. In summary, stable protein–protein interactions are easiest to isolate by physical methods like co-immunoprecipitation and pull-down assays because the protein complex does not disassemble over time. Weak or transient interactions can be identified using these methods by first covalently crosslinking the proteins to freeze the interaction during the co- IP or pull-down. Alternatively, crosslinking, along with label transfer and far–western blot analysis, can be performed independent of other methods to identify protein–protein interactions.

a. Co-immunoprecipitation (co-IP)

Co-immunoprecipitation (co-IP) is a popular technique for protein interaction discovery. Co-IP is conducted in essentially the same manner as an immunoprecipitation (IP) of a single protein, except that the target protein precipitated by the antibody, also called the "bait", is used to co-precipitate a

binding partner/protein complex, or "prey", from a lysate. Essentially, the interacting protein is bound to the target antigen, which is bound by the antibody that is immobilized to the support. Immunoprecipitated proteins and their binding partners are commonly detected by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis. The assumption that is usually made when associated proteins are co- precipitated is that these proteins are related to the function of the target antigen at the cellular level. This is only an assumption, however, that is subject to further verification.

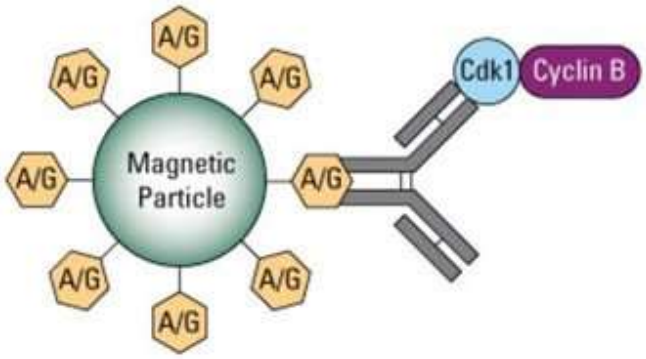


Fig : Co-immunoprecipitation of cyclin B and Cdk1. The Thermo Scientific Pierce Protein A/G Magnetic Beads bind to Cdk1 antibody complexed with Cdk1. Cyclin B is bound to the Cdk1, and is captured along with its binding partner.

b. Pull-down assays

Pull-down assays are similar in methodology to co-immunoprecipitation because of the use of beaded support to purify interacting proteins. The difference between these two approaches, though, is that while co-IP uses antibodies to capture protein complexes, pull-down assays use a "bait" protein to purify any proteins in a lysate that bind to the bait. Pull-down assays are ideal for studying strong or stable interactions or those for which no antibody is available for co-immunoprecipitation.

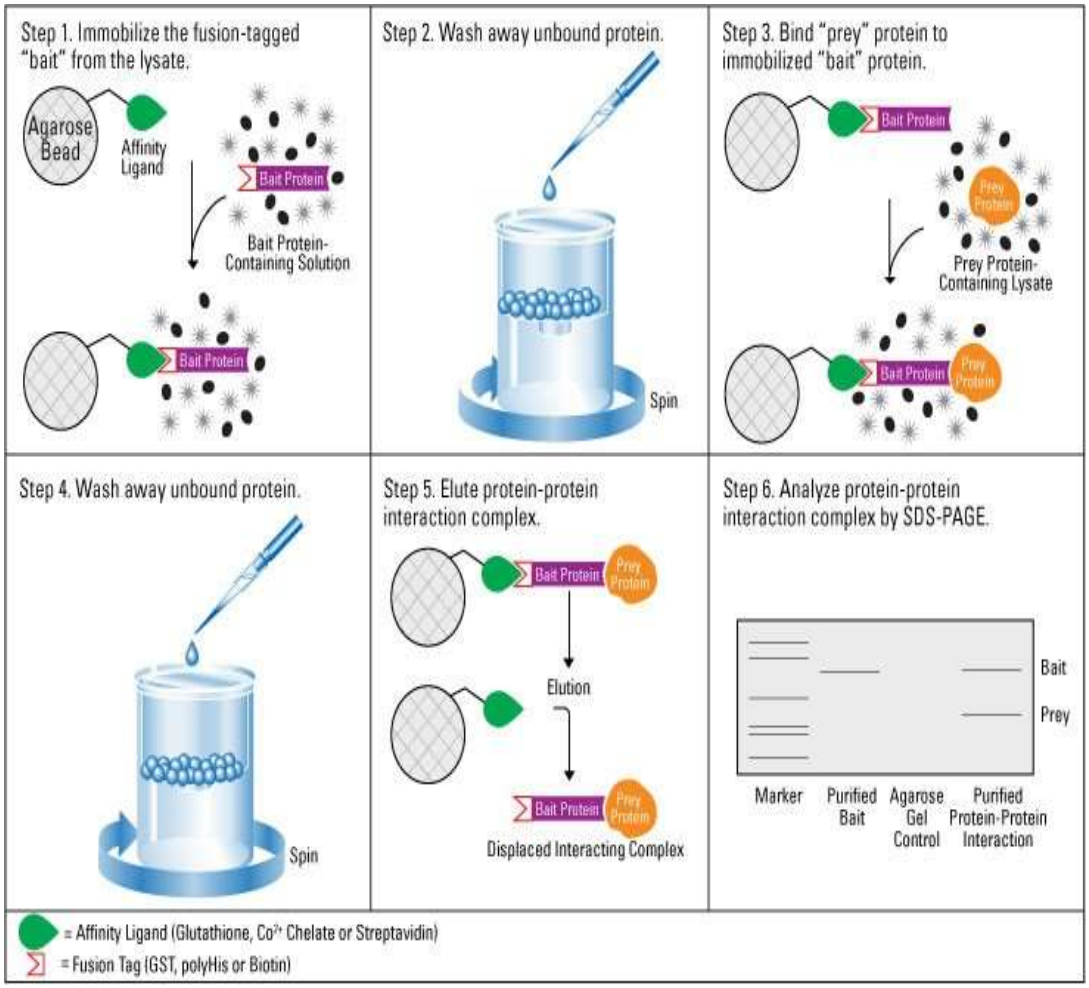


Figure: General schematic of a pull-down assay**.** A pull-down assay is a small-scale affinity purification technique similar to immunoprecipitation, except that the antibody is replaced by some other affinity system. In this case, the affinity system consists of a glutathione S-transferase (GST)–, poly His- or streptavidin-tagged protein or binding domain that is captured by glutathione-, metal chelate (cobalt or nickel) – or biotin-coated agarose beads, respectively. The immobilized fusion-tagged protein acts as the "bait" to capture a putative binding partner (i.e., the "prey"). In a typical pull-down assay, the immobilized bait protein is incubated with a cell lysate, and after the prescribed washing steps, the complexes are selectively eluted using competitive analytes or low pH or reducing buffers for in-gel or western blot analysis.

c. Crosslinking protein interaction analysis

Most protein–protein interactions are transient, occurring only briefly as part of a single cascade or other metabolic function within cells. Crosslinking interacting proteins is an approach to stabilize or permanently adjoin the components of interaction complexes. Once the components of an interaction are covalently crosslinked, other steps (e.g., cell lysis, affinity purification, electrophoresis or mass spectrometry) can be used to analyze the protein–protein interaction while maintaining the original interacting complex.
Homobifunctional, amine-reactive crosslinkers can be added to cells to crosslink potentially interacting proteins together, which can then be analyzed after lysis by western blotting.

Crosslinkers can be membrane permeable, such as DSS, for crosslinking intracellular proteins, or they can be non–membrane permeable, such as BS3, for crosslinking cell-surface proteins. Furthermore, some crosslinkers can be cleaved by reducing agents, such as DSP or DTSSP, to reverse the crosslinks.

Alternatively, heterobifunctional crosslinkers that contain a photoactivatable group, such as SDA product or Sulfo-SDA, can be used to capture transient interactions that may occur, such as after a particular stimulus. Photoactivation can also be also be after metabolic labeling with photoactivatable amino acids such as L-Photo-Leucine or L-Photo-Methionine.

Crosslinking sites between proteins can be mapped by high precision using mass spectrometry, especially if a MS-cleavable crosslinker such as DSSO or DSBU is used.

d. Label transfer protein interaction analysis

Label transfer involves crosslinking interacting molecules (i.e., bait and prey proteins) with a labeled crosslinking agent and then cleaving the linkage between the bait and prey so that the label remains attached to the prey. This method is particularly valuable because of its ability to identify proteins that interact weakly or transiently with the protein of interest. New non-isotopic reagents and methods continue to make this technique more accessible and simple to perform by any researcher.

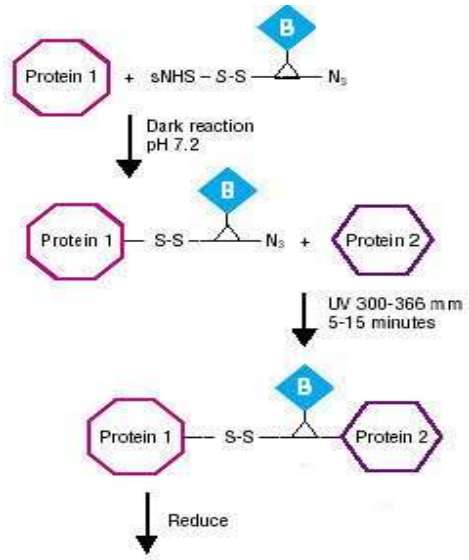


Fig: Experimental strategy for Sulfo-SBED biotin label transfer and analysis by western blotting.

e. Far–Western blot analysis

Just as pull-down assays differ from co-IP in the detection of protein–protein interactions by using tagged proteins instead of antibodies, so is far–western blot analysis different from western blot analysis, as protein–protein interactions are detected by incubating electrophoresed proteins with a purified, tagged bait protein instead of a target protein-specific antibody, respectively. The term "far" was adopted to emphasize this distinction.

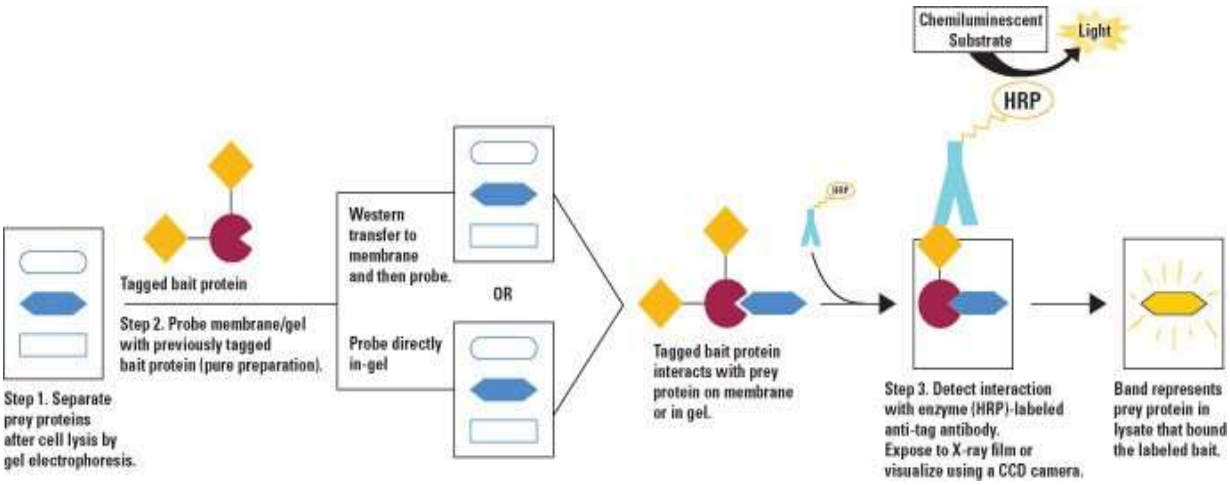


Fig : Diagram of far–western blot to analyze protein–protein interactions. In this example, a tagged bait protein is used to probe either the transfer membrane or a gel for the prey protein. Once bound, enzyme (horseradish peroxidase; HRP)-conjugated antibody that targets the bait tag is used to label the interaction, which is then detected by enzymatic chemiluminescence. This general approach can be adjusted by using untagged bait protein that is detected by antibody, biotinylated bait protein that is detected by enzyme-conjugated streptavidin, or radiolabeled bait protein that is detected by exposure to film.

Probable Questions:

1. What is gene targeting?
2. What is random mutagenesis? How random mutant libraries can be created?
3. What do you mean by insertional inactivation?
4. How transcriptome analysis is done?
5. What is DNA microarrays? Describe the procedure.
6. How image processing and data analysis is done in microarray?
7. What is Proteomics? Describe briefly different steps of proteome analysis.
8. What are the applications of proteomics?
9. How protein protein interaction are studied?
10. What is Biological effects of protein–protein interactions ?

Suggested readings

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7th Edition.
6. Gene cloning and DNA Analysis by T. A. Brown, Sixth Edition.
7. Genetics . Verma and Agarwal.

Unit-IV

Protein folding and processing: Chaperones and folding; enzymes and protein folding, protein cleavage, glycosylation, attachment of lipids.

Objective: In this unit you will learn about different aspects of protein folding.

Introduction:

Translation completes the flow of genetic information within the cell. The sequence of nucleotides in DNA has now been converted to the sequence of amino acids in a polypeptide chain. The synthesis of a polypeptide, however, is not equivalent to the production of a functional protein. To be useful, polypeptides must fold into distinct three-dimensional conformations, and in many cases multiple polypeptide chains must assemble into a functional complex. In addition, many proteins undergo further modifications, including cleavage and the covalent attachment of carbohydrates and lipids, that are critical for the function and correct localization of proteins within the cell.

Chaperones and Protein Folding

The three-dimensional conformations of proteins result from interactions between the side chains of their constituent amino acids. The classic principle of protein folding is that all the information required for a protein to adopt the correct three-dimensional conformation is provided by its amino acid sequence. This was initially established by Christian Anfinsen's experiments demonstrating that denatured RNase can spontaneously refold in vitro to its active conformation. Protein folding thus appeared to be a self-assembly process that did not require additional cellular factors. More recent studies, however, have shown that this is not an adequate description of protein folding within the cell. The proper folding of proteins within cells is mediated by the activities of other proteins.

Proteins that facilitate the folding of other proteins are called molecular chaperones. The term "chaperone" was first used by Ron Laskey and his colleagues to describe a protein (nucleoplasmin) that is required for the assembly of nucleosomes from histones and DNA. Nucleoplasmin binds to histones and mediates their assembly into nucleosomes, but nucleoplasmin itself is not incorporated into the final nucleosome structure. Chaperones thus act as catalysts that facilitate assembly without being part of the assembled complex. Subsequent studies have extended the concept to include proteins that mediate a variety of other assembly processes, particularly protein folding.

It is important to note that chaperones do not convey additional information required for the folding of polypeptides into their correct three-dimensional conformations; the folded conformation of a protein is determined solely by its amino acid sequence. Rather, chaperones catalyze protein folding by assisting the self-assembly process. They appear to function by binding to and stabilizing unfolded or partially folded polypeptides that are intermediates along the pathway leading to the final correctly folded state. In the absence of chaperones, unfolded or partially folded polypeptide chains would be unstable within the cell, frequently folding incorrectly or aggregating into insoluble complexes. The binding of chaperones stabilizes these unfolded polypeptides, thereby preventing incorrect folding or aggregation and allowing the polypeptide chain to fold into its correct conformation.

A good example is provided by chaperones that bind to nascent polypeptide chains that are still

being translated on ribosomes, thereby preventing incorrect folding or aggregation of the amino-terminal portion of the polypeptide before synthesis of the chain is finished (Figure 1). Presumably, this interaction is particularly important for proteins in which the carboxy terminus (the last to be synthesized) is required for correct folding of the amino terminus. In such cases, chaperone binding stabilizes the amino-terminal portion in an unfolded conformation until the rest of the polypeptide chain is synthesized and the completed protein can fold correctly. Chaperones also stabilize unfolded polypeptide chains during their transport into subcellular organelles—for example, during the transfer of proteins into mitochondria from the cytosol (Figure 2). Proteins are transported across the mitochondrial membrane in partially unfolded conformations that are stabilized by chaperones in the cytosol. Chaperones within the mitochondrion then facilitate transfer of the polypeptide chain across the membrane and its subsequent folding within the organelle. In addition, chaperones are involved in the assembly of proteins that consist of multiple polypeptide chains, in the assembly of macromolecular structures (e.g., nucleoplasmin), and (as discussed later in this chapter) in the regulation of protein degradation.

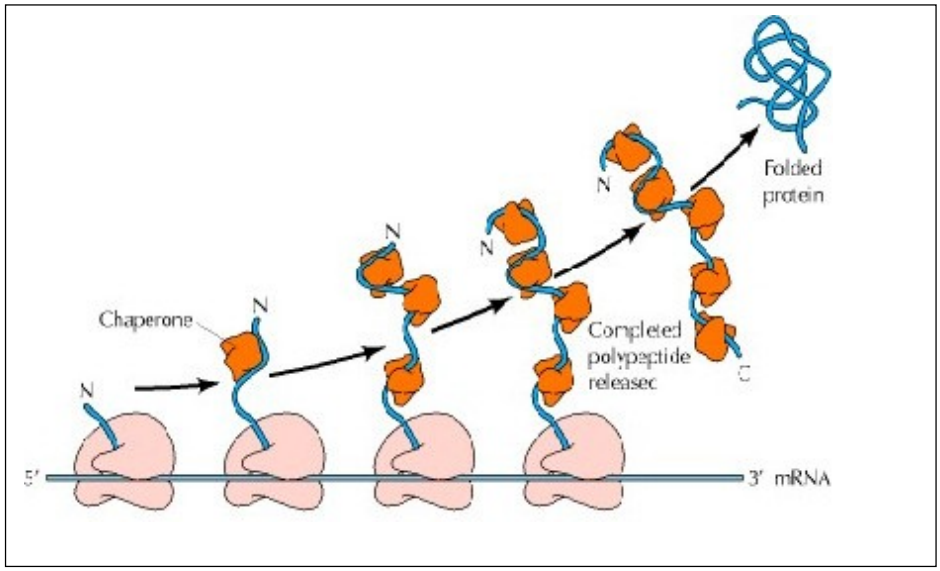


Figure 1. Action of chaperones during translation. Action of chaperones during translation. Chaperones bind to the amino (N) terminus of the growing polypeptide chain, stabilizing it in an unfolded configuration until synthesis of the polypeptide is completed. The completed protein is then released from the ribosome and is able to fold into its correct three- dimensional conformation.

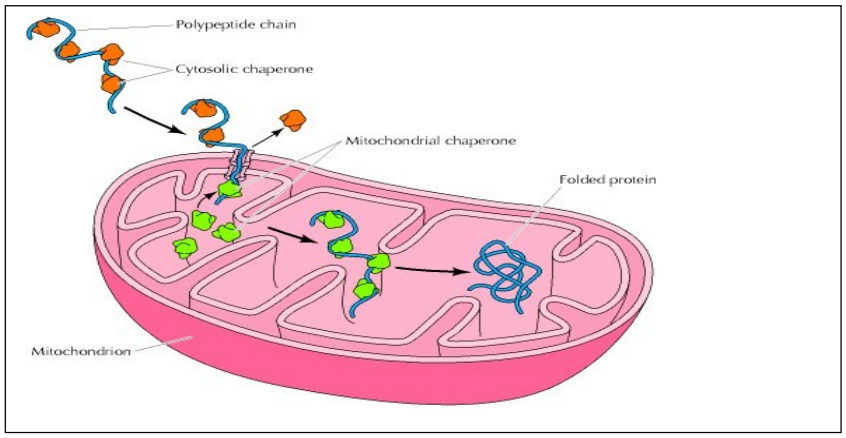


Figure 2. Action of chaperones during protein transport. A partially unfolded polypeptide is transported from the cytosol to a mitochondrion. Cytosolic chaperones stabilize the unfolded configuration. Mitochondrial chaperones facilitate transport and subsequent folding of the polypeptide chain within the organelle.

Many of the proteins now known to function as molecular chaperones were initially identified as heat-shock proteins, a group of proteins expressed in cells that have been subjected to elevated temperatures or other forms of environmental stress. The heat-shock proteins (abbreviated Hsp), which are highly conserved in both prokaryotic and eukaryotic.

cells, are thought to stabilize and facilitate the refolding of proteins that have been partially denatured as a result of exposure to elevated temperature. However, many members of the heat-shock protein family are expressed and have essential cellular functions under normal growth conditions. These proteins serve as molecular chaperones, which are needed for polypeptide folding and transport under normal conditions as well as in cells subjected to environmental stress.

The Hsp70 and Hsp60 families of heat-shock proteins appear to be particularly important in the general pathways of protein folding in both prokaryotic and eukaryotic cells. The proteins of both families function by binding to unfolded regions of polypeptide chains. Members of the Hsp70 family stabilize unfolded polypeptide chains during translation as well as during the transport of polypeptides into a variety of subcellular compartments, such as mitochondria and the endoplasmic reticulum. These proteins bind to short segments (seven or eight amino acid residues) of unfolded polypeptides, maintaining the polypeptide chain in an unfolded configuration and preventing aggregation.

Members of the Hsp60 family (also called chaperonins) facilitate the folding of proteins into their native conformations. Each chaperonin consists of 14 subunits of approximately 60 kilodaltons (kd) each, arranged in two stacked rings to form a “double doughnut” structure (Figure-3). Unfolded polypeptide chains are shielded from the cytosol by being bound within the central cavity of the chaperonin cylinder. In this isolated environment protein folding can proceed while aggregation of unfolded segments of the polypeptide chain is prevented by their binding to the chaperonin. The binding of unfolded polypeptides to the chaperonin is a reversible reaction that is coupled to the hydrolysis of ATP as a source of energy. ATP hydrolysis thus drives multiple rounds of release and rebinding of unfolded regions of the polypeptide chain to the chaperonin, allowing the polypeptide to fold gradually into the correct conformation.

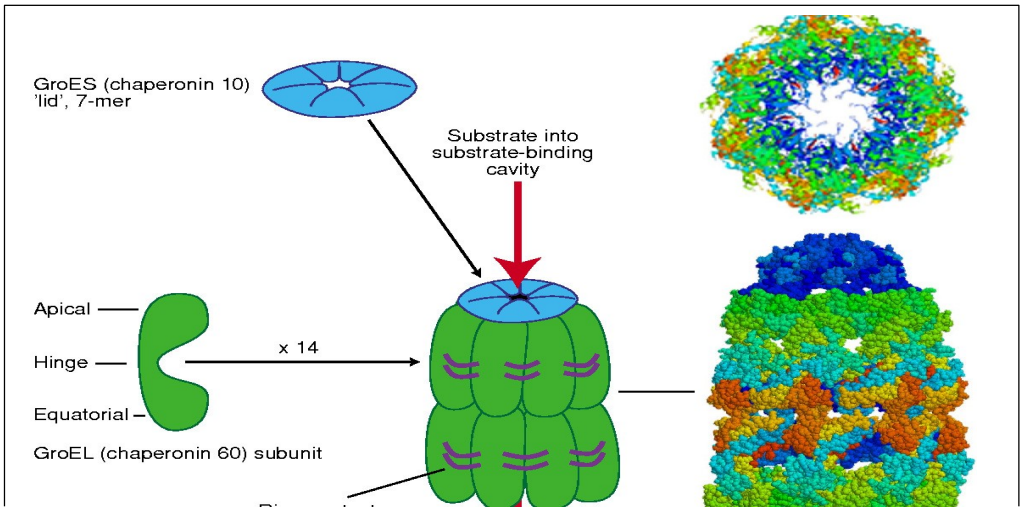


Figure 3.Structure of a chaperonin. Structure of a chaperonin. GroEL, a member of the Hsp60 family, is a porous cylinder composed of two stacked rings. Each ring consists of seven subunits. (Courtesy of Paul B. Sigler, Yale University.)

In some cases, members of the Hsp70 and Hsp60 families have been found to act together in a sequential fashion. For example, Hsp70 and Hsp60 family members act sequentially during the transport of proteins into mitochondria and during the folding of newly synthesized proteins in E. coli (Figure 4). First, an Hsp70 chaperone stabilizes nascent polypeptide chains until protein synthesis is completed. The unfolded polypeptide chain is then transferred to an Hsp60 chaperonin, within which protein folding takes place, yielding a protein correctly folded into its functional three-dimensional conformation. Members of the Hsp70 and Hsp60 families are found in the cytosol and in subcellular organelles (e.g., mitochondria) of eukaryotic cells, as well as in bacteria, so the sequential action of Hsp70 and Hsp60 appears to represent a general pathway of protein folding. An alternative pathway for the folding of some proteins in the cytosol and endoplasmic reticulum may involve the sequential actions of Hsp70 and Hsp90 family members, although the function of Hsp90 is not yet well understood.

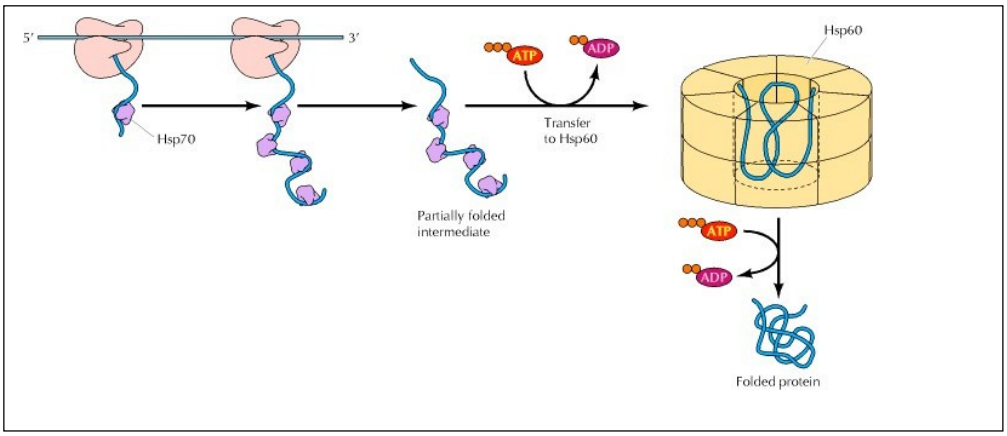


Figure 4.Sequential actions of Hsp70 and Hsp60 chaperones. Chaperones of the Hsp70 family bind to and stabilize unfolded polypeptide chains during translation. The unfolded polypeptide is then transferred to chaperones of the Hsp60 family, within which protein folding takes place. ATP hydrolysis is required for release of the unfolded polypeptide from Hsp70 as well as for folding within Hsp60.

Enzymes and Protein Folding

In addition to chaperones, which facilitate protein folding by binding to and stabilizing partially folded intermediates, cells contain at least two types of enzymes that catalyze protein folding by breaking and re-forming covalent bonds. The formation of disulfide bonds between cysteine residues is important in stabilizing the folded structures of many proteins . Protein disulfide isomerase, which was discovered by Christian Anfinsen in 1963, catalyzes the breakage and re-formation of these bonds (Figure 5). For proteins that contain multiple cysteine residues, protein disulfide isomerase (PDI) plays an important role by promoting rapid exchanges between paired disulfides, thereby allowing the protein to attain the pattern of disulfide bonds that is compatible with its stably folded conformation. Disulfide bonds are generally restricted to secreted proteins and some membrane proteins because the cytosol contains reducing agents that maintain cysteine residues in their reduced (—SH form), thereby preventing the formation of disulfide (S—S) linkages. In eukaryotic cells, disulfide bonds form in the endoplasmic reticulum, in which an oxidizing environment is maintained. Consistent with the role of disulfide bonds in stabilizing secreted proteins, the activity of PDI in the endoplasmic reticulum is correlated with the level of protein secretion in different types of cells.

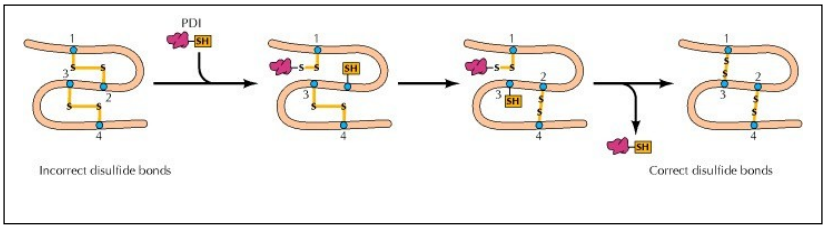


Figure 5. The action of protein disulfide isomerase. Protein disulfide isomerase (PDI) catalyzes the breakage and rejoining of disulfide bonds, resulting in exchanges between paired disulfides in a polypeptide chain. The enzyme forms a disulfide bond with a cysteine residue of the polypeptide and then exchanges its paired disulfide with another cysteine residue. In this example, PDI catalyzes the conversion of two incorrect disulfide bonds (1-2 and 3-4) to the correct pairing (1-3 and2-4).

The second enzyme that plays a role in protein folding catalyzes the isomerization of peptide bonds that involve proline residues (Figure 6). Proline is an unusual amino acid in that the equilibrium between the cis and trans conformations of peptide bonds that precede proline residues is only slightly in favor of the trans form. In contrast, peptide bonds between other amino acids are almost always in the trans form. Isomerization between the cis and trans configurations of prolyl peptide bonds, which could otherwise represent a rate-limiting step in protein folding, is catalyzed by the enzyme peptidylprolyl isomerase. This enzyme is widely distributed in both prokaryotic and eukaryotic cells and can catalyze the refolding of at least some proteins. However, its physiologically important substrates and role within cells have not yet been determined.

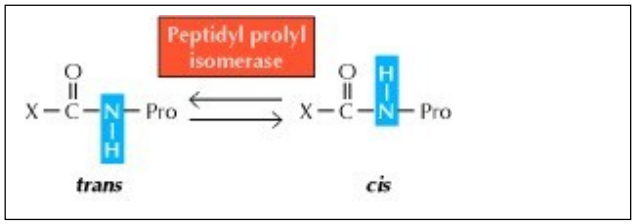


Figure-6.The action of peptidylprolyl isomerase. Peptidylprolyl isomerase catalyzes the isomerization of peptide bonds that involve proline between the cis and transconformations.

Protein Cleavage

Cleavage of the polypeptide chain (proteolysis) is an important step in the maturation of many proteins. A simple example is removal of the initiator methionine from the amino terminus of many polypeptides, which occurs soon after the amino terminus of the growing polypeptide chain emerges from the ribosome. Additional chemical groups, such as acetyl groups or fatty acid chains (discussed shortly), are then frequently added to the amino-terminal residues.

Proteolytic modifications of the amino terminus also play a part in the translocation of many proteins across membranes, including secreted proteins in both bacteria and eukaryotes as well as proteins destined for incorporation into the plasma membrane, lysosomes, mitochondria, and chloroplasts of eukaryotic cells. These proteins are targeted for transport to their destinations by amino-terminal sequences that are removed by proteolytic cleavage as the protein crosses the membrane. For example, amino-terminal signal sequences, usually about 20 amino acids long,
target secreted proteins to the plasma membrane of bacteria or to the endoplasmic reticulum of eukaryotic cells while translation is still in progress (Figure 7). The signal sequence, which consists predominantly of hydrophobic amino acids, is inserted into the membrane as it emerges from the ribosome. The remainder of the polypeptide chain passes through a channel in the membrane as translation proceeds. The signal sequence is then cleaved by a specific membrane protease (signal peptidase), and the mature protein is released. In eukaryotic cells, the translocation of growing polypeptide chains into the endoplasmic reticulum is the first step in targeting proteins for secretion, incorporation into the plasma membrane, or incorporation into lysosomes.

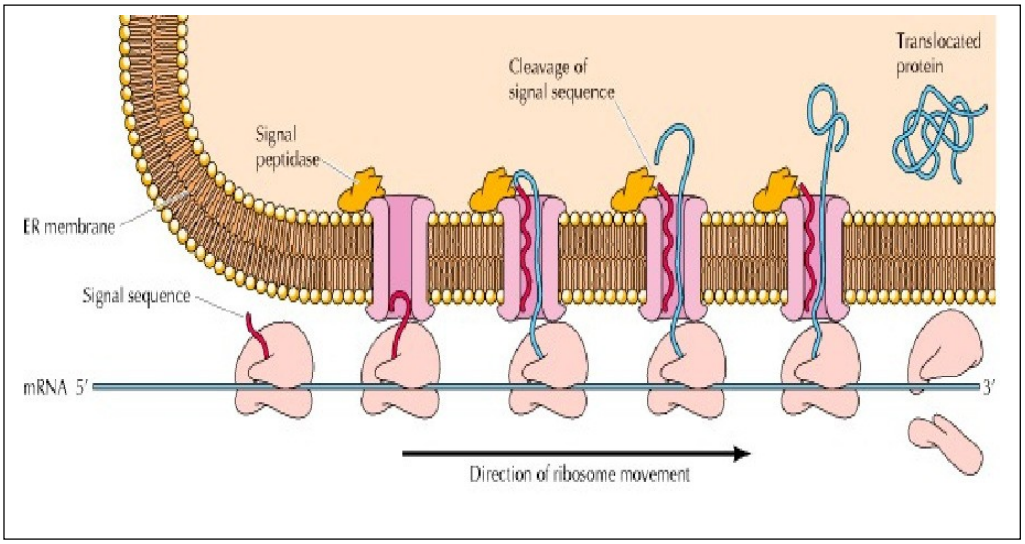


Figure 7. The role of signal sequences in membrane translocation. Signal sequences target the translocation of polypeptide chains across the plasma membrane of bacteria or into the endoplasmic reticulum of eukaryotic cells (shown here). The signal sequence, a stretch of hydrophobic amino acids at the amino terminus of the polypeptide chain, inserts into a membrane channel as it emerges from the ribosome. The rest of the polypeptide is then translocated through the channel and the signal sequence is cleaved by the action of signal peptidase, releasing the mature translocated protein.

In other important instances of proteolytic processing, active enzymes or hormones form via cleavage of larger precursors. Insulin, which is synthesized as a longer precursor polypeptide, is a

good example. Insulin forms by two cleavages. The initial precursor (preproinsulin) contains an amino-terminal signal sequence that targets the polypeptide chain to the endoplasmic reticulum . Removal of the signal sequence during transfer to the endoplasmic reticulum yields a second precursor, called proinsulin. This precursor is then converted to insulin, which consists of two chains held together by disulfide bonds, by proteolytic removal of an internal peptide. Other proteins activated by similar cleavage processes include digestive enzymes and the proteins involved in blood clotting.

It is interesting to note that the proteins of many animal viruses are derived from the cleavage of larger precursors. One particularly important example of the role of proteolysis in virus replication is provided by HIV. In the replication of HIV, a virus-encoded protease cleaves precursor polypeptides to form the viral structural proteins. Because of its central role in virus replication, the HIV protease (in addition to reverse transcriptase) is an important target for the development of drugs used for treating AIDS. Indeed, such protease inhibitors are now among the most effective agents available for combating this disease.

Glycosylation

Many proteins, particularly in eukaryotic cells, are modified by the addition of carbohydrates, a process called glycosylation. The proteins to which carbohydrate chains have been added (called glycoproteins) are usually secreted or localized to the cell surface, although some nuclear and cytosolic proteins are also glycosylated. The carbohydrate moieties of glycoproteins play important roles in protein folding in the endoplasmic reticulum, in the targeting of proteins for delivery to the appropriate intracellular compartments, and as recognition sites in cell-cell interactions.

Glycoproteins are classified as either N-linked or O-linked, depending on the site of attachment of the carbohydrate side chain. In N-linked glycoproteins, the carbohydrate is attached to the nitrogen atom in the side chain of asparagine. In O-linked glycoproteins, the oxygen atom in the side chain of serine or threonine is the site of carbohydrate attachment. The sugars directly attached to these positions are usually either N-acetylglucosamine or N-acetylgalactosamine, respectively.
Most glycoproteins in eukaryotic cells are destined either for secretion or for incorporation into the plasma membrane. These proteins are usually transferred into the endoplasmic reticulum (with the cleavage of a signal sequence) while their translation is still in progress. Glycosylation is also initiated in the endoplasmic reticulum before translation is complete. The first step is the transfer of a common oligosaccharide consisting of 14 sugar residues (2 N-acetylglucosamine, 3 glucose, and 9 mannose) to an asparagine residue of the growing polypeptide chain (Figure 9). The oligosaccharide is assembled within the endoplasmic reticulum on a lipid carrier (dolichol phosphate). It is then transferred as an intact unit to an acceptor asparagine (Asn) residue within the sequence Asn-X-Ser or Asn-X-Thr (where X is any amino acid other than proline).

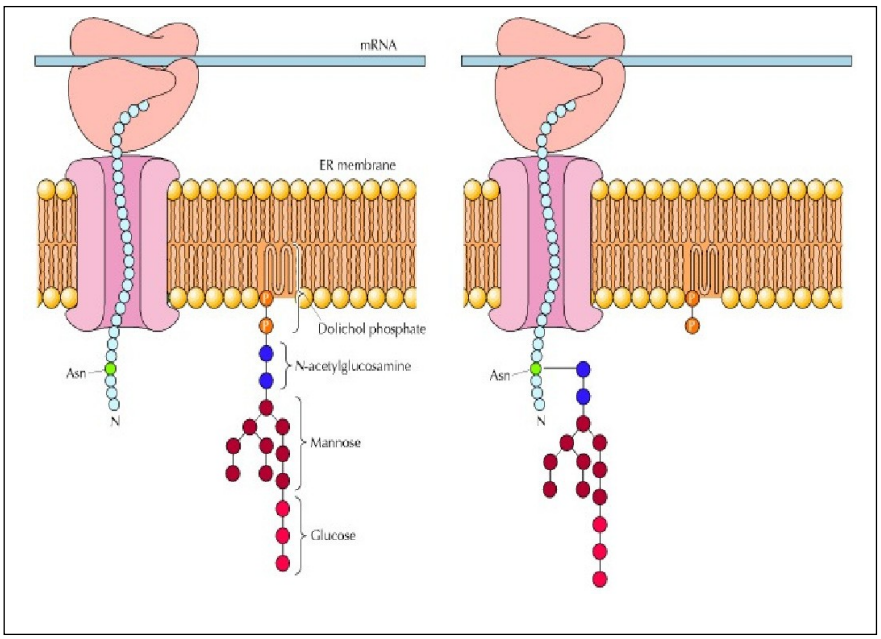


Figure 9. Synthesis of N-linked glycoproteins. The first step in glycosylation is the addition of an oligosaccharide consisting of 14 sugar residues to a growing polypeptide chain in the endoplasmic reticulum (ER). The oligosaccharide (which consists of two N-acetylglucosamine, nine mannose, and three glucose residues) is assembled on a lipid carrier (dolichol phosphate) in the ER membrane. It is then transferred as a unit to asparagine residue of the polypeptide.

In further processing, the common N-linked oligosaccharide is modified. Three glucose residues and one mannose are removed while the glycoprotein is in the endoplasmic reticulum. The oligosaccharide is then further modified in the Golgi apparatus, to which glycoproteins are transferred from the endoplasmic reticulum. These modifications include both the removal and addition of carbohydrate residues as the glycoprotein is transported through the compartments of the Golgi . The N- linked oligosaccharides of different glycoproteins are processed to different extents, depending on both the enzymes present in different cells and on the accessibility of the oligosaccharide to the enzymes that catalyze its modification. Glycoproteins with inaccessible oligosaccharides do not have new sugars added to them in the Golgi. The relatively simple oligosaccharides of these glycoproteins are called high- mannose oligosaccharides because they contain a high proportion of mannose residues, similar to the common oligosaccharide originally added in the endoplasmic reticulum. In contrast, glycoproteins with accessible oligosaccharides are processed more extensively, resulting in the formation of a variety of complex oligosaccharides.

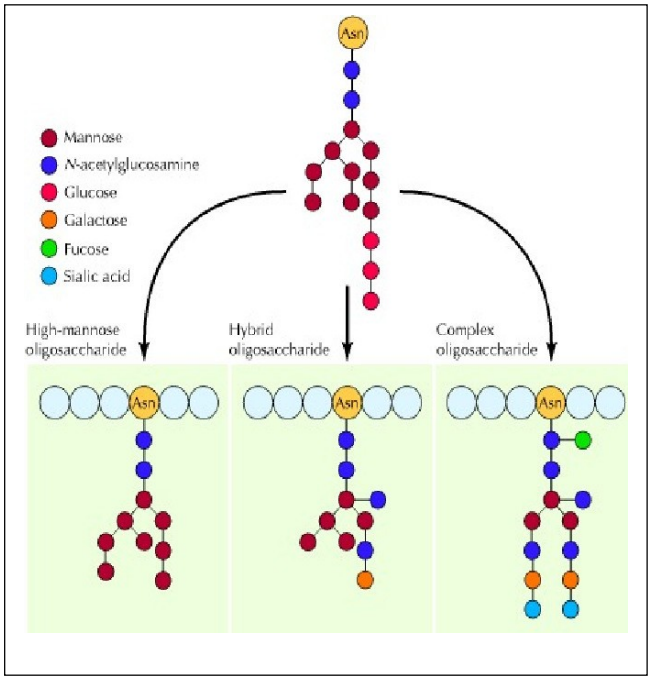

Asn
Mannose
N-acetylglucosamine
Glucose
Galactose
Fucose
Sialic acid
High-mannose oligosaccharide
Hybrid oligosaccharide
Complex oligosaccharide
Asn
Asn
Asn

Figure 10. Examples of N-linked oligosaccharides. Various oligosaccharides form from further modifications of the common 14-sugar unit initially added in the endoplasmic reticulum (see Figure 7.26). In high-mannose oligosaccharides, the glucose residues and some mannose residues are removed, but no other sugars are added. In the synthesis of complex oligosaccharides, more mannose residues are removed and other sugars are added. Hybrid oligosaccharides are intermediate between high-mannose and complex oligosaccharides. The structures shown are representative examples.

O-linked oligosaccharides are also added within the Golgi apparatus. In contrast to the N-linked oligosaccharides, O-linked oligosaccharides are formed by the addition of one sugar at a time and usually consist of only a few residues (Figure 11). Many cytoplasmic and nuclear proteins, including a variety of transcription factors, are also modified by the addition of single O-linked N-acetylglucosamine residues, catalyzed by a different enzyme system. However, the roles of carbohydrates in the function of these cytoplasmic and nuclear glycoproteins are not yet understood.

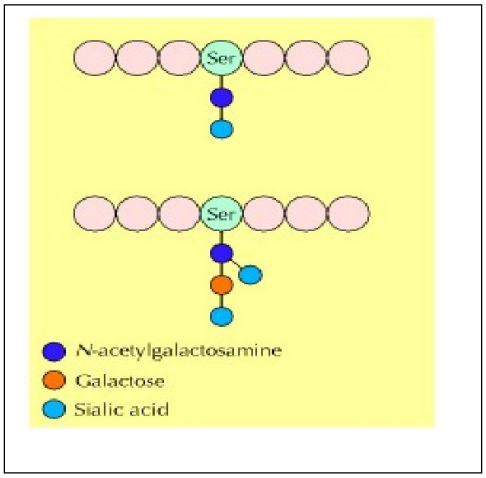


Figure 11.Examples of O-linked oligosaccharides

Attachment of Lipids

Some proteins in eukaryotic cells are modified by the attachment of lipids to the polypeptide chain. Such modifications frequently target and anchor these proteins to the plasma membrane, with which the hydrophobic lipid is able to interact . Three general types of lipid additions—N-myristoylation, prenylation, and palmitoylation—are common in eukaryotic proteins associated with the cytosolic face of the plasma membrane. A fourth type of modification, the addition of glycolipids, plays an important role in anchoring some cell surface proteins to the extracellular face of the plasma membrane.

In some proteins, a fatty acid is attached to the amino terminus of the growing polypeptide chain during translation. In this process, called N-myristoylation, myristic acid (a 14-carbon fatty acid) is attached to an N-terminal glycine residue (Figure 12). The glycine is usually the second amino acid incorporated into the polypeptide chain; the initiator methionine is removed by proteolysis before fatty acid addition. Many proteins that are modified by N-myristoylation are associated with the inner face of the plasma membrane, and the role of the fatty acid in this association has been clearly demonstrated by analysis of mutant proteins in which the N-terminal glycine is

changed to an alanine. This substitution prevents myristoylation and blocks the function of the mutant proteins by inhibiting their membrane association.

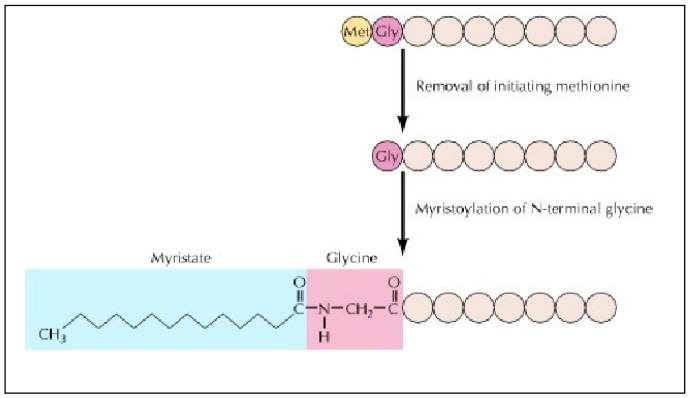


Figure 12.Addition of a fatty acid by N-myristoylation. The initiating methionine is removed, leaving glycine at the N terminus of the polypeptide chain. Myristic acid (a 14-carbon fatty acid) is then added.

Lipids can also be attached to the side chains of cysteine, serine, and threonine residues. One important example of this type of modification is prenylation, in which specific types of lipids (prenyl groups) are attached to the sulfur atoms in the side chains of cysteine residues located near the C terminus of the polypeptide chain (Figure 7.3130). Many plasma membrane– associated proteins involved in the control of cell growth and differentiation are modified in this way, including the Ras oncogene proteins, which are responsible for the uncontrolled growth of many human cancers. Prenylation of these proteins proceeds by three steps. First, the prenyl group is added to a cysteine located three amino acids from the carboxy terminus of the polypeptide chain. The prenyl groups added in this reaction are either farnesyl (15 carbons, as shown in Figure 7.30) or geranylgeranyl (20 carbons). The amino acids following the cysteine residue are then removed, leaving cysteine at the carboxy terminus. Finally, a methyl group is added to the carboxyl group of the C-terminal cysteine residue.

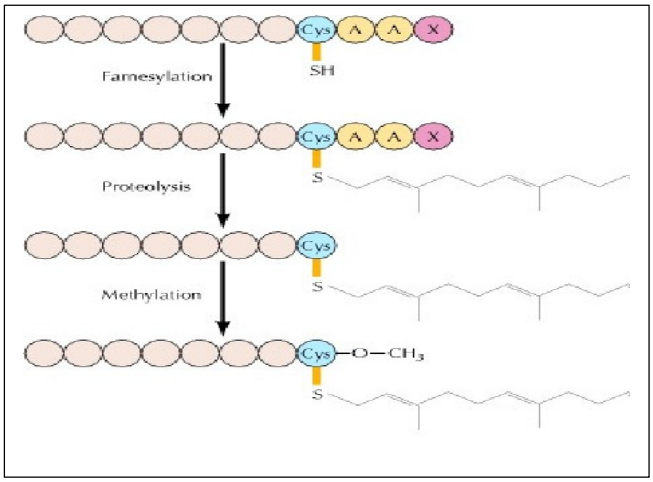


Figure 13.Prenylation of a C-terminal cysteine residue. The type of prenylation shown affects Ras proteins and proteins of the nuclear envelope (nuclear lamins). These proteins terminate with a

cysteine residue (Cys) followed by two aliphatic amino acids (A) and any other amino acid (X) at the C terminus. The first step in their modification is addition of the 15-carbon farnesyl group to the side chain of cysteine (farnesylation). This step is followed by proteolytic removal of the three C-terminal amino acids and methylation of the cysteine, which is now at the C terminus

The biological significance of prenylation is indicated by the fact that mutations of the critical cysteine block the membrane association and function of Ras proteins. Because farnesylation is a relatively rare modification of cellular proteins, interest in this reaction has been stimulated by the possibility that inhibitors of the key enzyme (farnesyltransferase) might prove useful as drugs for the treatment of cancers that involve Ras proteins. Such inhibitors of farnesylation have been found to interfere with the growth of cancer cells in experimental models and are undergoing evaluation of their efficacy against human tumors in clinical trials.

In the third type of fatty acid modification, palmitoylation, palmitic acid (a 16-carbon fatty acid) is added to sulfur atoms of the side chains of internal cysteine residues . Like N-myristoylation and prenylation, palmitoylation plays an important role in the association of some proteins with the cytosolic face of the plasma membrane.

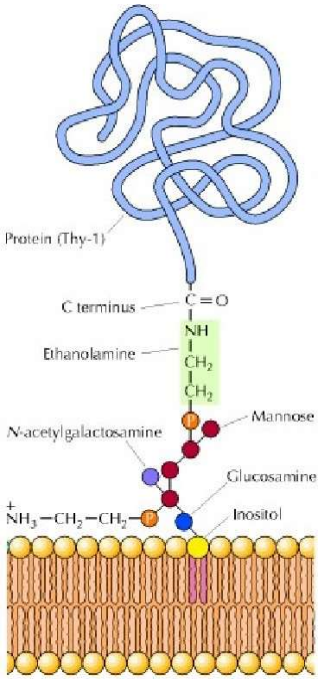


Figure 14.Structure of a GPI anchor.

Finally, lipids linked to oligosaccharides (glycolipids) are added to the C-terminal carboxyl groups of some proteins, where they serve as anchors that attach the proteins to the external face of the plasma membrane. Because the glycolipids attached to these proteins contain phosphatidylinositol, they are usually called glycosylphosphatidylinositol, or GPI, anchors (Figure 14). The oligosaccharide portions of GPI anchors are attached to the terminal carboxyl group of polypeptide chains. The inositol head group of phosphatidylinositol is in turn attached to the oligosaccharide, so the carbohydrate serves as a bridge between the protein and the fatty acid chains of the phospholipid. The GPI anchors are synthesized and added to proteins as a preassembled unit within the endoplasmic reticulum. Their addition is accompanied by cleavage

of a peptide consisting of about 20 amino acids from the C terminus of the polypeptide chain. The modified protein is then transported to the cell surface, where the fatty acid chains of the GPI anchor mediate its attachment to the plasma membrane.

Probable questions:

1. How Chaperones helps in protein folding?
2. Describe the role of disulfideisomerase in protein folding.
3. What is protein cleavage how it occurs in the cell?
4. How proteins are glycosylated?
5. How lipids get attached to protein?

Suggested readings:

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7th Edition.
6. Gene cloning and DNA Analysis by T. A. Brown, Sixth Edition.
7. Genetics . Verma and Agarwal.

Unit-V

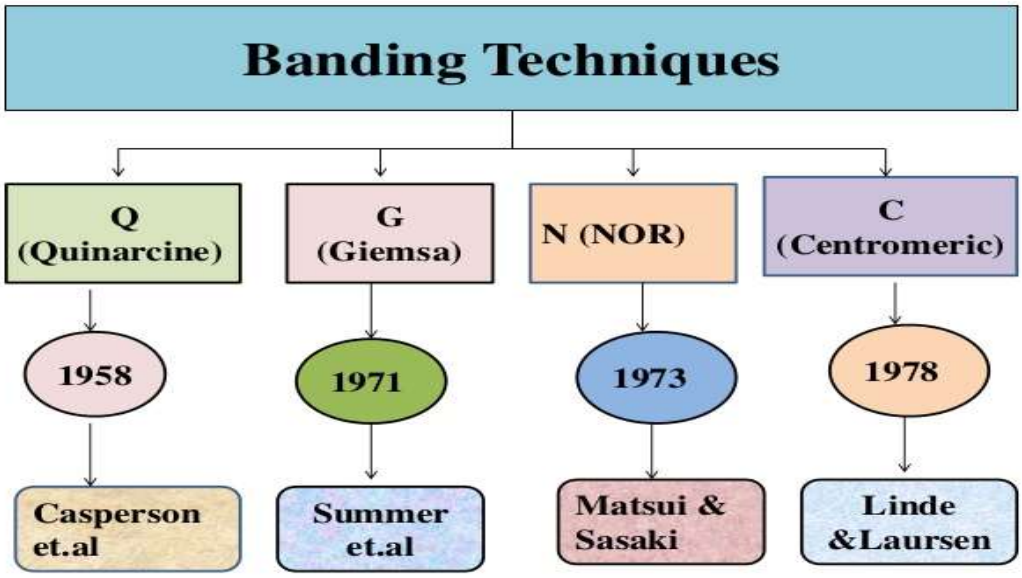
Current development of chromosome banding techniques and SCE

Objective: In this unit you will learn about different banding techniques as well as some recent development of banding technique.

Chromosome Banding:

Chromosome banding is the "lengthwise variation in staining properties along a chromosome…normally independent of any immediately obvious structural variation," and thus excludes patterns such as those seen on polytene chromosomes of Drosophila, which have a morphological component. Although the first observations of what could be called chromosome banding were made at the end of the nineteenth century, modern chromosome banding methods date from 1968 and can be applied to chromosomes of a wide variety of species with no more than slight modifications. Following the introduction of Q-banding by Caspersson and his colleagues in 1968, Pardue and Gall inadvertently produced differential staining of heterochromatin in their pioneering in situ hybridization studies, leading directly to C-banding, and in 1971 G-banding was discovered by several authors. R-banding was also introduced in 1971. Over the next few years, many other banding techniques, too numerous to mention individually, were introduced, many of them using fluorochromes. Silver staining for nucleolus organizing regions(NORs) was introduced in 1975, methods to show chromosome replication were invented, and the use of autoimmune sera to label kinetochores immunocytochemically was discovered.

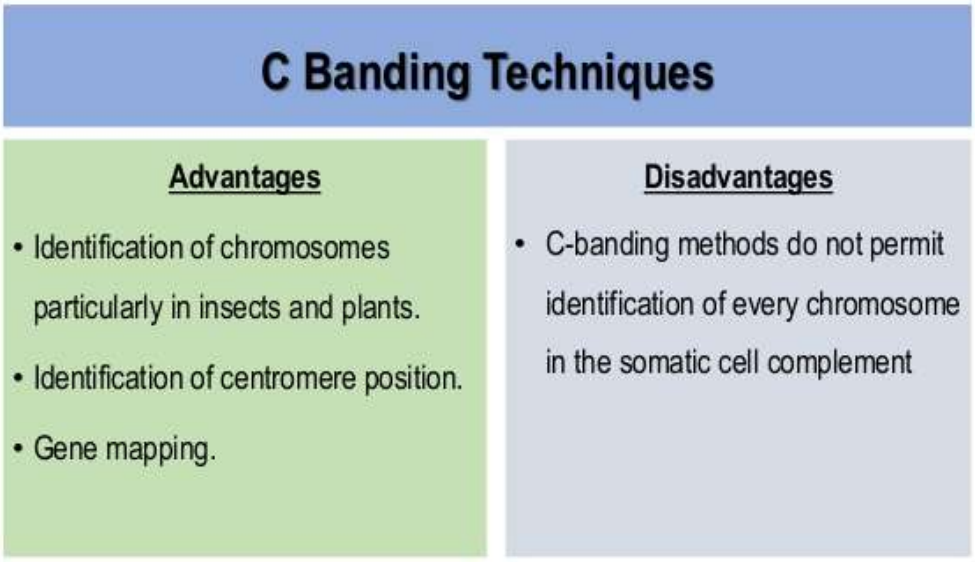
Chromosome Banding Techniques:



a. C-Banding:

The technique of C-banding originated after the work of Pardue and Gall who reported that constitutive heterochromatin can be stained specifically by Giemsa-solution. Each chromosome possesses a different degree of constitutive heterochromatin which enables the identification of individual chromosomes.

Constitutive heterochromatin is located near the centromere, at telomeres and in the nucleolar organizer regions; it is composed of highly repetitive DNA. C-banding represents the constitutive heterochromatin, and the banding is caused by differential staining reactions of the DNA of heterochromatin and euchromatin. The banding method is a complex technique that involves several treatments with acid, alkali or increased temperature. Denaturation of DNA is caused by these treatments. Subsequently, DNA renaturation occurs in treatments with sodium-citrate at 60°C. By these treatments, the repetitive DNA (heterochromatin) re-natures but low repetitive and unique DNAs do not re-nature. This results in differential staining of the specific chromosome regions. Giemsa-C-banding technique has been used to identify chromosomes of various plant and animal species including human. The Y chromosome of mammals is mostly heterochromatic and therefore, the technique of C-banding is quite useful for its identification. The banding pattern reveals the AT-rich centromere, which consists of constitutive heterochromatin. This technique involves acid treatment, hot saline incubation, and alkali treatment of the chromosomes. These treatments depurinate the DNA and break the DNA backbone, which then cause the extraction of the DNA from certain regions of the chromosomes. C-bands are produced due to this differential extraction of the DNA. It was observed that the DNA in the C-bands is more resistant to extraction than the DNA in the other regions of the chromosomes. This is due to the stronger interaction of the proteins, which protects the DNA from extraction, with the DNA in the C-bands than in the other regions of the chromosomes.



Gupta ,P. K., 2012. Cytogenetics an advanced study, *Chapter 1*: 3-16.

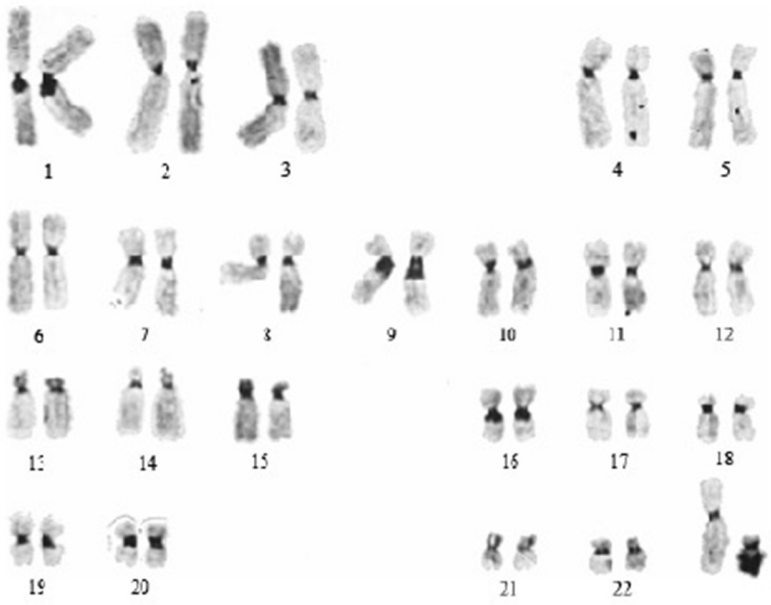
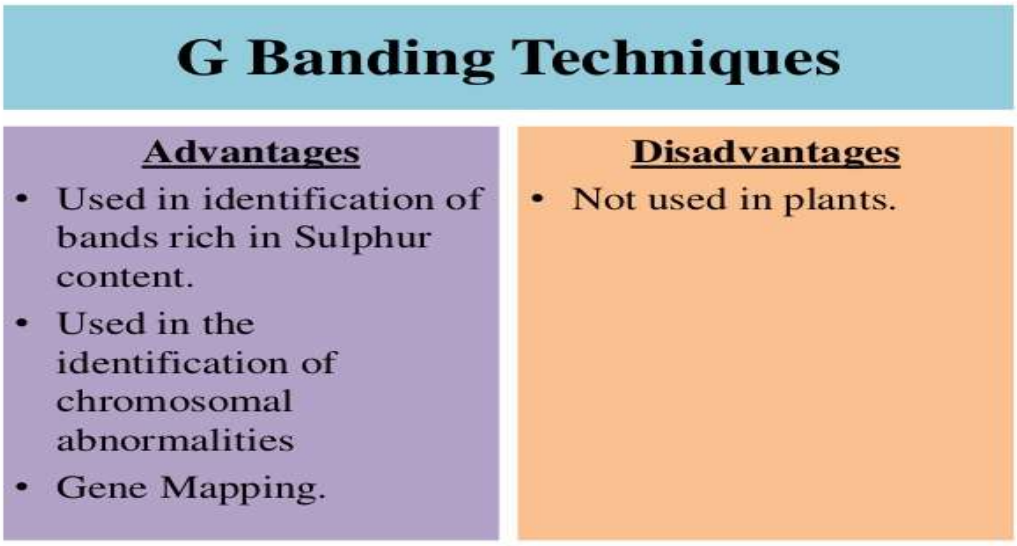


Fig 1: C-banding of human chromnosome

b. G-Banding:

The technique of G-banding involves Giemsa staining following pre-treatment with weak trypsin solution, urea or protease. It provides greater detail than C-banding. It was first used for human chromosomes by Summer et al. in 1971. G-bands may reflect a stronger chromatin condensation. However, this technique is not suitable for plant chromosomes.



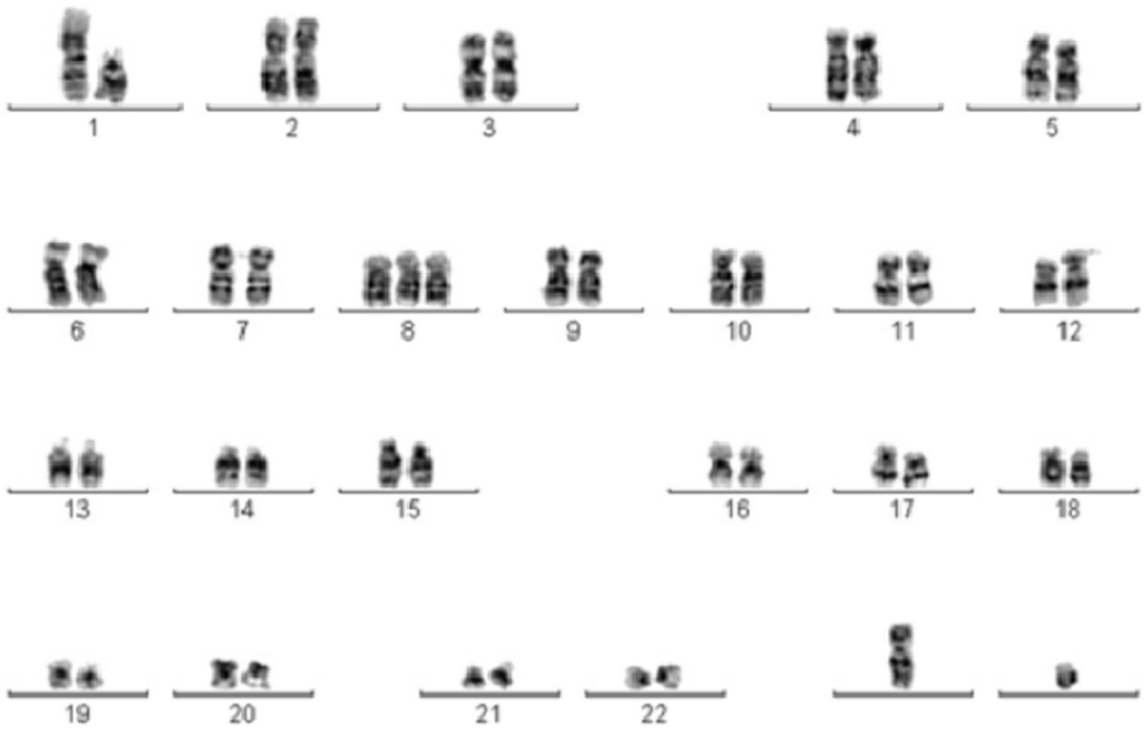
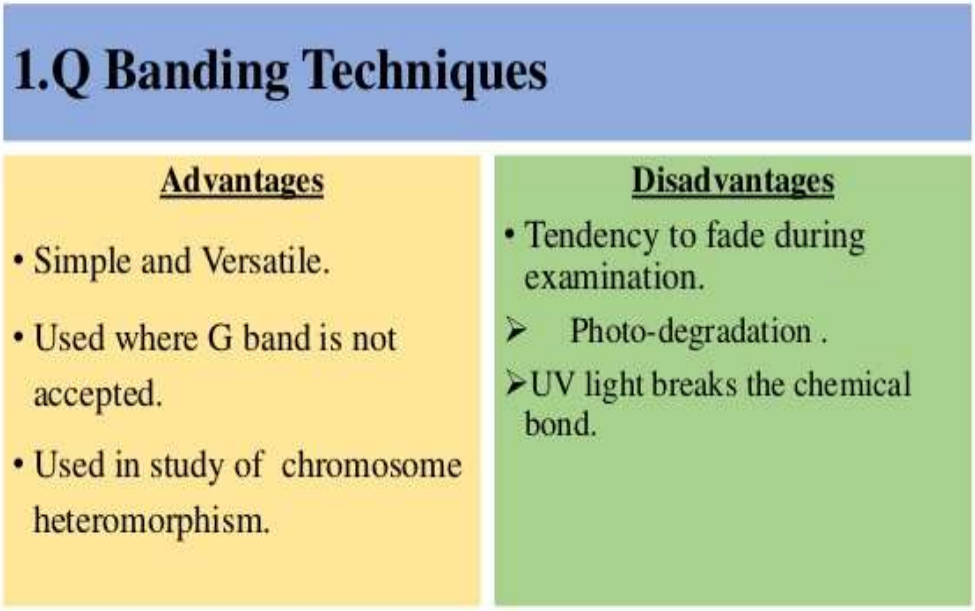


Fig. 2: Karyotype of human chromosome using G-banding technique

Giemsa is a visible light dye that binds to DNA through intercalation and thus, is used for chromosome staining. It is a mixture of cationic thiazine dyes, most importantly azure B, and anionic eosin dyes such as eosin Y. Staining of the chromosomes involves the formation of a thiazine-eosin precipitate in a 2:1 molar ratio. Two molecules of the small, fast diffusing thiazine dye first intercalate between the base pairs of the DNA in a configuration that favors the binding of the large, slow diffusing eosin molecule. The chromosomes stain blue as a result of this. The eosin molecule then forms a precipitate with the thiazine molecules thus causing the chromosomes to stain purple. The formation of this precipitate is favored on a hydrophobic environment.

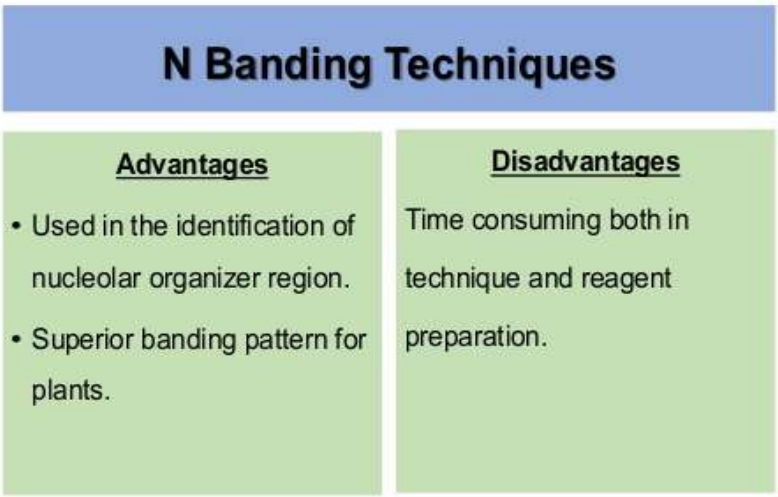
G-banding is the most widely used banding method for cytogenetic analysis that was first developed by Seabright in 1971. This technique, which is nonfluorescent, is advantageous in the aspect of stability and resolution of the bands produced. Visible light dyes are more stable and capable of producing clearer bands than fluorochromes. In order for banding to occur, the chromosomes must first undergo a pretreatment process before staining with Giemsa. The most common pretreatment method used is the digestion of the chromosomes with a protease such as trypsin. Other pre-treatment methods include incubation of the chromosomes in hot-saline citrate or subjecting the chromosomes into a detergent or urea solution. These pretreatment methods are known to extract a characteristic subset of proteins from the chromosomes. This differential extraction of the proteins throughout the length of the chromosome is responsible for the banding and is a reflection of the difference in the structure of the various chromosomal regions. Giemsa stains preferentially regions rich in adenine and thymine. Standard G band staining techniques allow between 400 and 600 bands to be seen on metaphase chromosomes. With high resolution G banding techniques, as many as two thousand different bands have been catalogued on the twenty four human chromosomes.

c. Q-Banding:
The method of Q-banding was developed by Caspersson et al. in 1968. The chromosomes stained with Quinacrine mustard show bright and dark zones under UV light. This technique is used to identify human and mice chromosomes.



d. N-Banding:
The technique of N-banding was originally described by Matsui and Sasaki in 1973. Briefly, air-dried chromosomes slides are stained for 90 minutes with Giemsa (diluted 1 : 10 in 1/15 M phosphate buffer at pH 7.0) following extraction with 5% trichloroacetic acid at 95°C for 30 minutes and then 0.1 NHCl at 60″C for 30 minutes. The N-bands are generally located at the secondary constriction, satellites, centromeres, telomeres and heterochromatic segments. It is suggested that the N-bands represent certain structural non-histone proteins specifically linked to the nucleolar organizer region of the eukaryotic chromosomes.

The N- banding patterns have been used for the location of nucleolar regions in the different organisms, such as, mammals, birds, amphibians, fishes, insects and plants. N-banding patterns differ in the chromosomes of different species. In 1980, Islam used this method to identify the barley chromosomes from those of wheat in the reciprocal wheat-barely F, hybrids, and to detect translocations between the wheat and barley chromosomes. He also used this technique to isolate lines possessing a pair of barely chromosomes substituted for particular pair of wheat chromosomes. A modified Giemsa-N-banding technique was developed by Singh and Tsuchiya in 1982 for the identification of barley chromosomes. This method is a combination of acetocarmine staining and Giemsa-N-banding. After processing according to this method, the centromeric region looks like a **"**diamond-shaped" structure; this is not seen in other techniques. Early metaphase or prometaphase chromosomes are more suitable for this staining as they show better banding pattern than the chromosomes at mid-metaphase in somatic cells.



e. R-Banding:

R-banding reveals the GC-rich euchromatin and produces positive bands that correspond to the negative G-bands. Banding is produced by incubating the chromosomes in an ionic solution at a high temperature (~ 87ºC) followed by staining with Giemsa. The incubation process causes the denaturation of the AT regions of the chromosomes because of the low melting point of these regions (~ 65ºC) as compared to that of the GC regions (~ 105ºC)

Other Techniques of Chromosome Banding:

Besides the above, there are other techniques for chromosome banding, e.g., R-banding (Reverse Giemsa banding). H-banding, and T-banding (Terminal banding). Chromosome banding patterns can be used not only for the identification of individual chromosomes of an organism but also to establish evolutionary relationships between different species. Banding patterns in human, chimpanzee, gorilla and orangutan have indicated that the evolutionary relationship between human and chimpanzee is closer than that between human and gorilla. It has further indicated that humans have a more distant evolutionary relationship with orangutans.

Uses of Chromosome Banding

G-and R-banding are the most commonly used techniques for chromosome identification (karyotyping) and for identifying abnormalities of chromosome number, translocations of material from one chromosome to another, and deletions, inversions or amplifications of chromosome segments. This has had an invaluable impact on human genetics and medicine and the power of this approach has been augmented by combining cytogenetics with fluorescence in situ hybridization (FISH). The detection of chromosome deletions associated with disorders, very often contiguous gene syndromes, provided some of the

first disease gene localizations in humans. Similarly, translocations have been important in pinpointing the location of disease-associated genes and the characteristic translocations associated with some leukaemia is important ,not only for understanding the molecular basis of these cancers, but also for their diagnosis and prognosis. One of the best examples of this is the translocation between human chromosomes 9 and 22 – t(9;22)(q34:q11)– or the Philadelphia chromosome diagnostic of chronic myelogenous leukaemia (CML).

Comparisons of chromosome banding patterns can confirm evolutionary relationships between species and also reveal changes in karyotype that may have been important in speciation. The banding patterns of human, gorilla and chimpanzee chromosomes are almost identical, though human chromosome 2 is the result of a fusion between two great ape chromosomes. There are also extensive similarities between human chromosome bands and those of lower primates.

Evolution of chromosome bands

Whereas Q-, G- and R-banding patterns have only been observed in some eukaryotes, replication banding is almost universal among living organisms possessing chromosomes

large enough to see by microscopy, suggesting that itis a fundamental consequence of, or requirement for, the compartmentalization of complex genomes. Chromosomes from most mammals and birds can be G and R-banded. With amphibia, fish and plants, some species band whereas others do not. The lowest vertebrates with reported good G-banding are the bony fish. Evolutionary analysis of chromosome banding patterns suggests that the first cytogenetically detectable compartmentalization that arose in the genomes of eukaryotes was the temporal control of replication and differences in chromatin packaging and the segregation of some chromosomal domains into heterochromatin. Ability to be G banded(and we will assume here that this is a reflection of differences in chromatin structure on mitotic chromosomes) followed later. Fluorochrome banding seems to have appeared on the scene last of all.

Fluorochromes for Chromosome Staining: Fluorochromes are organic molecules that are capable of undergoing fluorescence. These molecules contain large conjugated systems such as aromatic or heterocyclic groups and are characterized by rigid and planar structures. There are several parameters that are important in describing the fluorescence of a fluorochrome. These include the excitation and emission wavelengths, and quantum efficiency or yield. The excitation wavelength shows how much energy is required to excite the fluorochrome while the emission wavelength shows the energy of the photon emitted by the fluorochrome. The emission wavelength is usually longer than the excitation wavelength. In chromosome staining, fluorochromes are used when the chromosomes are to be studied with a fluorescent microscope. They are also capable of producing bands in the chromosomes. Although fluorochromes are less stable than visible light dyes such as Giemsa, as discussed earlier, they offer several advantages inbanding.Giemsarequirestheuseoftrypsin,whichremovesproteinsfromthechromosomes, in order for banding to occur. On the other hand, fluorochromes do not require this pretreatment process**.** Hence, the extraction of proteins from the chromosomes is avoided. There are various fluorochromes that can be used in chromosome banding. These fluorochromes produce bands either through differential quenching or fluorescence, or differential binding depending on the structure of the fluorochrome. Differential fluorescence or quenching is responsible for banding using fluorochromes that uniformly bind throughout the length of the chromosome. These fluorochromes usually bind to the chromosomes through intercalation and hence, do not produce a binding specificity. Banding using these fluorochromes occur due to the quenching of their fluorescence at certain regions of the chromosomes.

Fluorochrome	Binding Mode	Mechanism of Banding	Selectivity
Quinacrine	Intercalation	Differential fluorescence	AT
Daunomycin	Intercalation		AT
DAPI	Minor groove		AT
Hoechst 33258	Minor groove	Differential binding	AT
Chromomycin A3	Minor groove		GC

Table 1: Some fluorochromes used in chromosome banding techniques.

DAPI: 4',6-diamidino-2-phenylindole, DAPI, is a fluorochrome that is widely used in chromosome staining. The reason for this is that DAPI has a very high quantum yield (~ 0.92). At low DAPI/DNA ratio, DAPI binds to the minor groove of consecutive (3 to 4 base pairs) AT- rich sequences of the DNA. It forms H-bonds with the nitrogen atoms at position 3 of the adenine bases and/or with the oxygen atoms at position 2 of the thymine bases. As a result of this sequence selectivity in the binding of DAPI, bands are produced. The banding pattern obtained from DAPI is similar to that obtained from G-banding. Studies show that DAPI binds to the major groove of the GC-rich regions of the DNA. As a result of the possibility of DAPI binding to the GC rich regions of the DNA, DAPI does not produce very clear bands as compared to those obtained from other fluorochromes such as quinacrine.

Quanacrine: Chromosome banding using fluorochromes was first achieved by Caspersson in 1970 with the use of quinacrine mustard, an aminoacridine dye. Quinacrine mustard was then replaced by a less toxic quinacrine compound called quinacrine dihydrochloride. At low dye/DNA ratio and high ionic strength, quinacrine binds to DNA through intercalation. Meanwhile, at high dye/DNA ratio and low ionic strength, quinacrine binds to DNA through an external ionic interaction. Quinacrine has a positive charge that is capable of interacting with the negatively charged phosphate groups of the DNA. The amino group at position 2 of the guanine bases of the DNA quenches the fluorescence of quinacrine thus causing the AT-rich regions of the chromosomes to fluoresce more brightly than the GC rich regions. The bands produced are called Q-bands. The bright yellow-green positive Q bands correspond to the positive G-bands minus the G-bands representing the centromeric constitutive heterochromatin. Thus, Q-bands represent the facultative heterochromatin. Hoechst 33258 : Hoechst 33258 is a bi-benzimidazole derivative. It binds to the minor groove of the DNA, specifically to the AT-rich regions. The mechanism of binding of Hoechst 33258 with DNA is very similar to that of DAPI at low DAPI/DNA ratio. Hence, the banding pattern produced with Hoechst 33258 is similar to that of DAPI. In addition to this differential binding of Hoechst 33258 with DNA, the fluorescence of Hoechst 33258 is enhanced to a greater degree in the AT-rich regions of the DNA as compared to the GC-rich regions thus further improving the resolution of the bands produced.

Silver Staining (AgNOR): Silver staining is a method to stain the nucleolar organizer regions (NORs) on the human acrocentric chromosomes. NORs, which contain the genes for ribosomal RNA or proteins, were known early to stain with silver. Using this information, Howell et al showed that NORs on chromosomes could be stained with silver nitrate and called their technique

"Ag-SAT". Howell and Black subsequently developed a simplified technique using a colloidal developer for better results. Many laboratories use this method with various modifications. There is still controversy as to the nature or exact location of this silver staining. Miller et al. showed that the activity of NOR regions appeared to be responsible for the staining. Goodpasture et al. showed the actual location of the staining was in the satellite stalks of acrocentric chromosomes and not the satellites themselves, although the silver stained mass may appear to cover or extend into the satellite region if the stalk or satellite is small. Subsequent experiments by Verma et al. showed that Ag-NOR positive chromosomes are those that are found frequently in satellite associations while the Ag-NOR negative chromosomes are not seen in such associations. Silver staining is an important banding method to study heteromorphic variations in the size and number of NORs, and to characterize marker chromosomes or other structural rearrangements involving the acrocentric chromosomes.

Sister Chromatid Exchange (SCE)

Sister chromatid exchange (SCE) is a phenomenon that occurs widely in nature; this suggests that it represents or is the expression of a biologically relevant process that has been preserved through evolution. However, its biological meaning and the mechanism of formation have not been fully elucidated. Evidence has been obtained that the occurrence of SCE requires the cell to pass through DNA synthesis; there are also some data suggesting that the replication fork is the site at which this event occurs. It has been reported that SCE could be caused by the presence of DNA lesions at the moment of replication or by the alteration of this latter process. DNA synthesis, and particularly formation of the replication fork, is the moment at which SCE most probably occurs, because this is when the homologous double strands are close together and a homologous recombinational event could take place more easily. Clear evidence has recently been reported relating SCE to homologous recombination.

Homologous recombination (HR) is an important pathway for genomic repair of many forms of DNA damage including chromosomal double-strand breaks (DSBs), interstrand cross-linking damage, and collapsed replication forks. HR is usually considered to be "error-free" repair because it uses the available, identical sequence from the sister chromatid to repair the DSB, thereby preserving DNA sequence information. Mitotic HR is a complex, varied, and tightly regulated process, and defects in several of the components of HR have long been associated withcancer.

Symmetrical exchanges between newly replicated chromatids and their sisters can be visualized cytologically in vertebrate cells if the DNA of one chromatid is labelled with 5-bromodeoxyuridine (BUdR) during synthesis. The two double-strand break (DSB) repair pathways of HR and NHEJ are highly conserved between yeast and vertebrate cells. HR uses a homologous chromosome or a sister chromatid as a template to effect precise repair of a DNA lesion, while the NHEJ pathway carries out repair with lower fidelity and no requirement for homology.

The protocol described below utilizes 5-bromo-2′-deoxyuridine (BrdU) incorporation and fluorescence plus Giemsa (FPG) staining to make exchanges between sister chromatids visible. BrdU is a nucleoside analog that resembles thymidine and is efficiently incorporated into replicating DNA. Since DNA replication is semiconservative, after BrdU has been made available to cells, it is incorporated as the nascent strand is elongated. After two rounds of replication, paired metaphase sister chromatids differ in the amount of BrdU each contains. One sister has one strand of non-BrdU substituted DNA and one strand with BrdU substitution. In the other sister, both DNA strands contain BrdU substitutions. Subsequent incorporation of the intercalating ultraviolet (UV) light absorbing Hoechst 33258 dye into the DNA, followed by UV light exposure, causes "bleaching" of the DNA in proportion to amount of incorporated BrdU in the double-stranded molecule, likely due to free-radical mediated damage. Subsequent staining of the

UV-treated chromosomes with Giemsa makes this differential bleaching apparent by light microscopy, where doubly substituted chromosomes stain much more faintly than hemi-substituted chromosomes. Fig. 3 shows schematic of the procedure and expected results. SCEs following either the first or second round of DNA replication in the presence of BrdU lead to visible exchanges after the staining procedure.

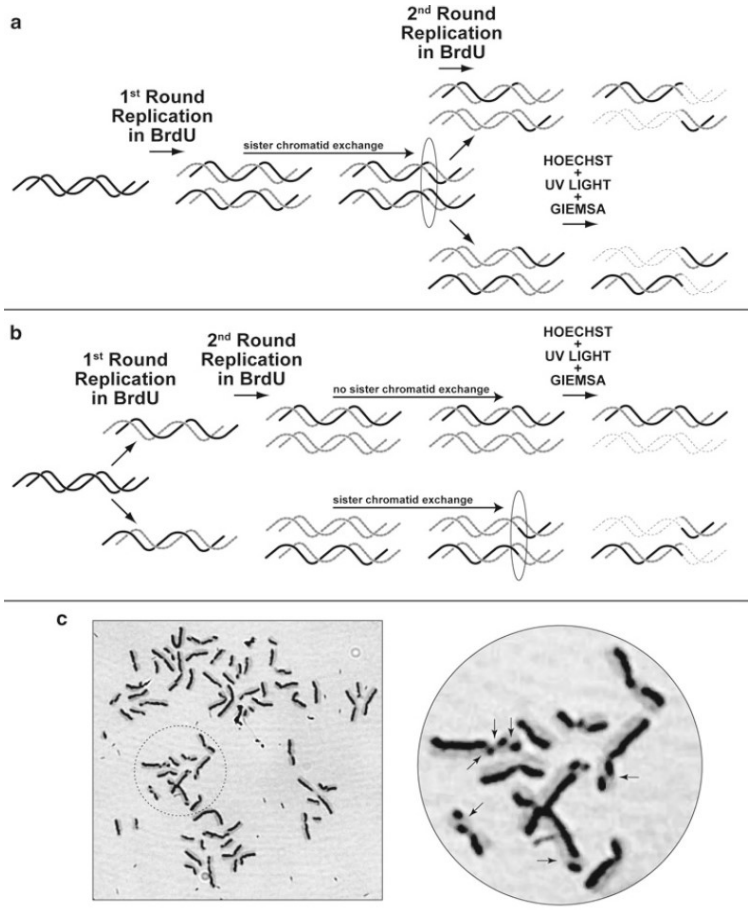


Fig: 3: Schematic of sister chromatid staining using two rounds of BrdU incorporation followed by Hoechst 33258 staining, exposure to UV light, and staining with Giemsa. **Solid black lines** : unsubstituted DNA single strand; **dotted gray lines** : BrdU substituted DNA single strand; **ellipse** : point of physical SCE

Probable Questions:

1. write a note on chromosome banding. Name 4 types of chromosome banding.
2. What do you mean by C banding? state its advantages and disadvantages.
3. What do you mean by Q banding? state its advantages and disadvantages.
4. What do you mean by N banding? state its advantages and disadvantages.
5. What do you mean by G banding? state its advantages and disadvantages.
6. what are the uses of Chromosome banding ?
7. How Chromosomes bands have evolved?
8. Describe silver staining procedure of chromosome banding.
9. Describe sister chromatid exchange procedure.
10. How fluorochrome are used in chromosome painting?

Suggested readings:

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7th Edition.
6. Gene cloning and DNA Analysis by T. A. Brown, Sixth Edition.
7. Genetics . Verma and Agarwal.

Unit-VI

Microbial genetics: organization of prokaryotic genome; single stranded DNA phages; RNA phages; cycle and gene expression in SV40 virus; Lytic and lysogenic phage morphogenesis; bacterial conjugation, transduction and transformation

Objective: In this unit you will learn about organization of prokaryotic genome, single stranded DNA phages, RNA phages, cycle and gene expression in SV40 virus; Lytic and lysogenic phage morphogenesis, bacterial conjugation, transduction and transformation

Genome organization in prokaryotes: Much of what is known about prokaryotic chromosome structure was derived from studies of *Escherichia coli*, a bacterium that lives in the human colon and is commonly used in laboratory cloning experiments. In the 1950s and 1960s, this bacterium became the model organism of choice for prokaryotic research when a group of scientists used phase-contrast microscopy and autoradiography to show that the essential genes of *E. coli* are encoded on a single circular chromosome packaged within the cell nucleoid.

Prokaryotic cells do not contain nuclei or other membrane-bound organelles. Most of the well- characterized prokaryotic genomes consist of double-stranded DNA organized as a single circular chromosome 0.6-10 Mb in length and one or more circular plasmid species of 2 kb-1.7 Mb. In fact, the word "prokaryote" literally means "before the nucleus." The nucleoid is simply the area of a prokaryotic cell in which the chromosomal DNA is located. This arrangement is not as simple as it sounds, however, especially considering that the *E. coli* chromosome is several orders of magnitude larger than the cell itself. So, if bacterial chromosomes are so huge, how can they fit comfortably inside a cell-much less in one small corner of the cell? The answer to this question lies in DNA packaging. Whereas eukaryotes wrap their DNA around proteins called histones to help package the DNA into smaller spaces, most prokaryotes do not have histones (with the exception of those species in the domain Archaea). Thus, one way prokaryotes compress their DNA into smaller spaces is through supercoiling (Figure1).

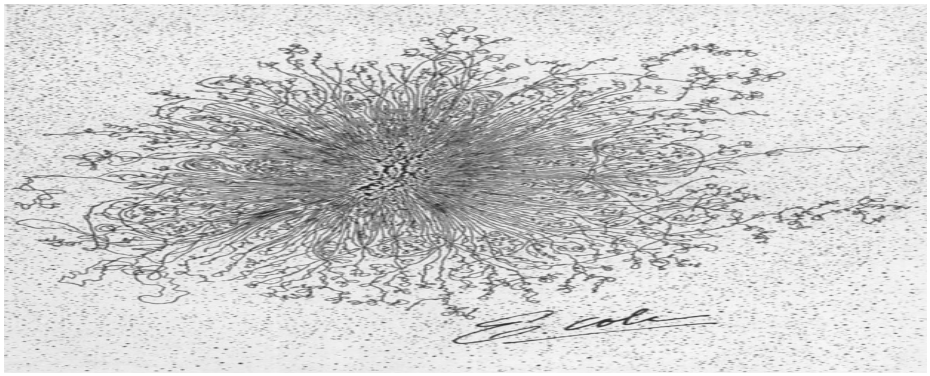


Fig. 1: Supercoiled DNA in prokaryotes (*E. coli)*

Imagine twisting a rubber band so that it forms tiny coils. Now twist it even further, so that the original coils fold over one another and form a condensed ball. When this type of twisting happens to a bacterial genome, it is known as supercoiling (Fig.2: a and b).

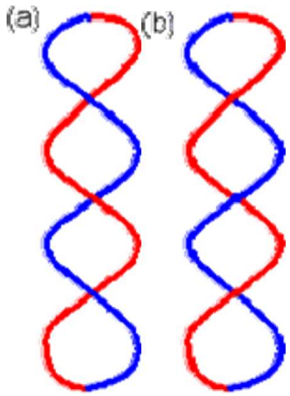


Fig.2: (a) Positive supercoils (the front segment of a DNA molecule cross over the back segment from left to right). (b) Negative supercoils.

Genomes can be negatively supercoiled, meaning that the DNA is twisted in the opposite direction of the double helix, or positively supercoiled, meaning that the DNA is twisted in the same direction as the double helix. Most bacterial genomes are negatively supercoiled during normal growth.

Proteins Involved in Supercoiling: During the 1980s and 1990s, researchers discovered that multiple proteins act together to fold and condense prokaryotic DNA. In particular, one protein called HU, which is the most abundant protein in the nucleoid, works with an enzyme called topoisomerase I to bind DNA and introduce sharp bends in the chromosome, generating the tension necessary for negative supercoiling. Recent studies have also shown that other proteins, including integration host factor (IHF), can bind to specific sequences within the genome and introduce additional bends. The folded DNA is then organized into a variety of conformations that are supercoiled and wound around tetramers of the HU protein, much like eukaryotic chromosomes are wrapped around histones.
Once the prokaryotic genome has been condensed, DNA topoisomerase I, DNA gyrase, and other proteins help maintain the supercoils. One of these maintenance proteins, H-NS, plays an active role in transcription by modulating the expression of the genes involved in the response to environmental stimuli. Another maintenance protein, factor for inversion stimulation (FIS), is abundant during exponential growth and regulates the expression of more than 231 genes, including DNA topoisomerase-I.

Accessing Supercoiled Genes:
Supercoiling explains how chromosomes fit into a small corner of the cell, but how do the proteins involved in replication and transcription access the thousands of genes in prokaryotic chromosomes when everything is packaged together so tightly? It has been determined that prokaryotic DNA replication occurs at a rate of 1,000 nucleotides per second, and prokaryotic transcription occurs at a rate of about 40 nucleotides per second, so bacteria must have highly efficient methods of accessing their DNA strands. But how?
Researchers have noted that the nucleoid usually appears as an irregularly shaped mass within the prokaryotic cell, but it becomes spherical when the cell is treated with chemicals to inhibit transcription or translation. Moreover, during transcription, small regions of the chromosome can be seen to project from the nucleoid into the cytoplasm (i.e., the interior of the cell), where they unwind and associate with ribosomes, thus allowing easy access by various transcriptional proteins. These projections are thought to explain the mysterious shape of nucleoids during active growth.

When transcription is inhibited, however, the projections retreat into the nucleoid, forming the aforementioned spherical shape.
Because there is no nuclear membrane to separate prokaryotic DNA from the ribosomes within the cytoplasm, transcription and translation occur simultaneously in these organisms. This is strikingly different from eukaryotic chromosomes, which are confined to the membrane-bound nucleus during most of the cell cycle. In eukaryotes, transcription must be completed in the nucleus before the newly synthesized mRNA molecules can be transported to the cytoplasm to undergo translation into proteins.

Variations in Prokaryotic Genome Structure: Recently, it has become apparent that one size does not fit all when it comes to prokaryotic chromosome structure. While most prokaryotes, like *E. coli*, contain a single circular DNA molecule that makes up their entire genome, recent studies have indicated that some prokaryotes contain as many as four linear or circular chromosomes. For example, *Vibrio cholerae*, the bacteria that causes cholera, contains two circular chromosomes. One of these chromosomes contains the genes involved in metabolism and virulence, while the other contains the remaining essential genes. An even more extreme example is provided by *Borrelia burgdorferi*, the bacterium that causes Lyme disease. This organism is transmitted through the bite of deer ticks, and it contains up to 11 copies of a single linear chromosome. Unlike *E. coli*, *Borrelia* cannot supercoil its linear chromosomes into a tight ball within the nucleoid; rather, these strands are diffused throughout the cell. Other organisms, such as *Bacillus subtilis*, form nucleoids that closely resemble those of *E. coli*, but they use different architectural proteins to do so. Furthermore, the DNA molecules of Archaea, a taxonomic domain composed of single-celled, non-bacterial prokaryotes that share many similarities with eukaryotes, can be negatively supercoiled, positively supercoiled, or not supercoiled at all. It is important to note that archaea are the only group of prokaryotes that use eukaryote like histones, rather than the architectural proteins described above, to condense their DNA molecules. The acquisition of histones by archaea is thought to have paved the way for the evolution of larger and more complex eukaryotic cells. Nobel Prize winner Arthur Kornberg used ΦX174 as a model to first prove that DNA synthesized in a test tube by purified enzymes could produce all the features of a natural virus. In 2003, it was reported by Craig Venter's group that the genome of ΦX174 was the first to be completely assembled *in vitro* from synthesized oligonucleotides. The ΦX174 virus particle has also been successfully assembled invitro.

This bacteriophage has a [+] circular single-stranded DNA genome of 5386 nucleotides encoding 11 proteins. Of these 11 genes, only 8 are essential to viral morphogenesis. The GC-content is 44% and 95% of nucleotides belong to coding genes. Infection begins when G protein binds to lipopolysaccharides on the bacterial host cell surface. H protein (or the DNA Pilot Protein) pilots the viral genome through the bacterial membrane of *E.coli* bacteria most likely via a predicted N-terminal transmembrane domain helix. Additionally, H protein induces lysis of the bacterial host at high concentrations as the predicted N-terminal transmembrane helix easily pokes holes through the bacterial wall. The DNA is ejected through a hydrophilic channel at the 5-fold vertex. It is understood that H protein resides in this area but experimental evidence has not verified its exact location. Once inside the host bacterium, replication of the [+] ssDNA genome proceeds via negative sense DNA intermediate. This is done as the phage genome supercoils and the secondary structure formed by such supercoiling attracts a primosome protein complex. This translocates once around the genome and synthesizes a [-]ssDNA from the positive original genome. [+] ssDNA genomes to package into viruses are created from this by a rolling circle mechanism. This is the mechanism by which the double stranded supercoiled genome is nicked on the positive strand by a virus-encoded A protein, also attracting a bacterial DNA polymerase (DNAP) to the site of cleavage. DNAP will use the negative strand as a template to make positive sense DNA. As it translocates around the genome it displaces the outer strand of already-synthesised DNA, which is

immediately coated by SSBP proteins. The A protein will cleave the complete genome every time it recognizes the origin sequence.
As D protein is the most abundant gene transcript, it is the most protein in the viral procapsid. Similarly, gene transcripts for F, J, and G are more abundant than for H as the stoichiometry for these structural proteins is 5:5:5:1. The primosomes are protein complexes which attach/bind the enzyme helicase on the template. Primosomes gives RNA primers for DNA synthesis to strands. Phi X is regularly used as a positive control in DNA sequencing due to its relatively small genome size in comparison to other organisms, its relatively balanced nucleotide content- about23% G, 22% C, 24% A, and 31% T, i.e., 45% G+C and 55% A+T, for its 5,386 nucleotide long sequence.

RNA phages: Bacteriophages occur abundantly in the biosphere, with different genomes, and lifestyles. Phages are classified by the International Committee on Taxonomy of Viruses (ICTV) according to morphology and nucleic acid. Nineteen families are currently recognized by the ICTV that infect bacteria and archaea. Of these, only two families have RNA genomes, and only five families are surrounded by an envelope.

Family	**Morphology**	**Nucleic acid**	**Examples**
Cystoviridae	Enveloped, spherical	Segmented dsRNA	
Leviviridae	Nonenveloped, isometric	Linear ssRNA	MS2, Q

Table 1: ICTV classification of prokaryotic (bacterial and archaeal) viruses

Bacteriophage MS2

The bacteriophage MS2 is an icosahedral, positive-sense single-stranded RNA virus that infects the bacterium Escherichia coli and other members of the Enterobacteriaceae. MS2 is a member of a family of closely related bacterial viruses that includes bacteriophage f2, bacteriophage Qβ, R17, and GA. The MS2 genome is one of the smallest known, consisting of 3569 nucleotides of single-stranded RNA. It encodes just four proteins: the maturation protein (A-protein), the lysis protein, the coat protein, and the replicase protein. The gene encoding lysis protein (lys) overlaps both the 3'-end of the upstream gene (cp) and the 5'-end of the downstream gene (rep), and was one of the first known examples of overlapping genes. The positive-stranded RNA genome serves as messenger RNA, and is translated upon viral uncoating within the host cell. Although the four proteins are encoded by the same messenger/viral RNA, they are not all expressed at the same levels; expression of these proteins is regulated by a complex interplay between translation and RNA secondary structure.

Fig.4: Location of protein-coding genes within bacteriophage MS2 RNA. Note that the lys gene overlaps segments of both the cp and rep genes. Scale is approximate.

Capsid structure: An MS2 virion (viral particle) is about 27 nm in diameter, as determined by electron microscopy. It consists of one copy of the maturation protein and 180 copies of the coat protein (organized as 90 dimers) arranged into an icosahedral shell with triangulation number T=3, protecting the genomic RNA inside. The structure of the coat protein is a five-stranded β- sheet with two α-helices and a hairpin. When the capsid is assembled, the helices and hairpin face the exterior of the particle, while the β-sheet faces the interior.

Life cycle: Once the viral RNA has entered the cell, it begins to function as a messenger RNA for the production of phage proteins. The gene for the most abundant protein, the coat protein, can be immediately translated. The translation start of the replicase gene is normally hidden within RNA secondary structure, but can be transiently opened as ribosomes pass through the coat protein gene. Replicase translation is also shut down once large amounts of coat protein have been made; coat protein dimers bind and stabilize the RNA "operator hairpin", blocking the replicase start. The start of the maturation protein gene is accessible in RNA being replicated but hidden within RNA secondary structure in the completed MS2 RNA; this ensures translation of only a very few copies of maturation protein per RNA. Finally, the lysis protein gene can only be initiated by ribosomes that have completed translation of the coat protein gene and "slip back" to the start of the lysis protein gene, at about a 5%frequency.

Replication of the plus-strand MS2 genome requires synthesis of the complementary minus strand RNA, which can then be used as a template for synthesis of a new plus strand RNA. MS2 replication has been much less well studied than replication of the highly related bacteriophage Qβ, partly because the MS2 replicase has been difficult to isolate, but is likely to be similar.
The formation of the virion is thought to be initiated by binding of maturation protein to the MS2 RNA; in fact, the complex of maturation protein and RNA is infectious. The assembly of the icosahedral shell or capsid from coat proteins can occur in the absence of RNA; however, capsid assembly is nucleated by coat protein dimer binding to the operator hairpin, and assembly occurs at much lower concentrations of coat protein when MS2 RNA is present. Bacterial lysis and release of newly formed virions occurs when sufficient lysis protein has accumulated. Lysis protein forms pores in the cytoplasmic membrane, which leads to loss of membrane potential and breakdown of the cell wall, while the β-sheet faces the interior.

Applications: Since 1998, the MS2 operator hairpin and coat protein have found utility in the detection of RNA in living cells. MS2 and other viral capsids are also currently under investigation as agents in drug delivery, tumor imaging, and light harvesting applications.MS-2, due to its structural similarities to noroviruses, its similar optimum proliferation conditions, and non-pathogenicity to humans, has been used as substitute for noroviruses in studies of disease transmission.

Cycle and gene expression in SV 40 virus: In 1960, Sweet and Hilleman first described an agent, which they named SV40, induced cytopathic effects and vacuole formation in monkey cells. Since its discovery, simian virus 40 (SV40) has been one of the most intensely studied animal viruses. The molecular biology of SV40 has led to seminal discoveries in the fields of transcription, DNA replication, and oncogenic transformation. Over the last decade, provocative evidence has

accumulated that suggests SV40 may be a human pathogen. Does SV40 infect humans? If so, when did this monkey polyomavirus enter the human population and where is the reservoir? What is the behavior of SV40 in human cells? Does it cause or contribute to acute or chronic disease?
SV40 was isolated from normal monkey kidney cells, stocks of the Sabin poliovirus vaccine, and an adenovirus vaccine. The last two reagents were prepared in primary kidney cell cultures derived from rhesus monkeys. Subsequent analyses found that the Salk poliovirus vaccine administered from 1955 to 1963 in the United States was also contaminated with SV40, potentially exposing an estimated 100 million people. Although poliovirus in the Salk vaccine was inactivated by formalin treatment, the conditions were insufficient to completely inactivate SV40. Soon thereafter, it was demonstrated that SV40 could infect humans and also induce tumors in experimental animals. These observations raised concerns that vaccinated people worldwide may have been inadvertently exposed to an oncogenic virus. Early epidemiological studies allayed these fears, revealing no increased incidence of cancers directly related to immunization status. However, these initial analyses were necessarily limited in that it was unknown whether (i) the virus could be transmitted, either horizontally or vertically; (ii) vaccinated, immunocompetent individuals would be at equal risk for development of cancer with others having defective immunity or a cancer predisposition; (iii) the power of the analysis was sufficient to detect increases in rare cancers; and (iv) SV40 normally circulated in humans before development of the poliovirus vaccine. A recent review of all epidemiological data by the Institute of Medicine concluded that evidence to date was "inadequate to accept or reject a causal relationship between SV40-containing poliovirus vaccines and cancer". Criticisms included misclassification bias, lack of confidence intervals for the data, and "ecological" study design, which are unlikely to be remedied by further follow-up of the study populations.

A brief overview of the biology of SV40 is relevant to understand the concerns raised by these initial analyses. When SV40 infects its natural host, it initially undergoes a lytic replication cycle. The early viral genes encode the tumor (T) antigens: large T antigen (LT), small t antigen (ST), and 17K T antigen (also tiny T or T′). LT plays a dominant role in infection, repressing early viral gene transcription and stimulating late viral gene transcription. LT is also an initiation factor for viral DNA replication, recruiting the DNA polymerase α-primase complex to the origin of replication and acting as a helicase. Following the strategy of other DNA viruses, the SV40 early proteins dysregulate the cell cycle and impede cell apoptosis in order to maximize virus production. LT binds the members of the retinoblastoma protein family, pRb, p107, and p130, resulting in release and activation of E2F transcription factors, which stimulate expression of genes involved in S-phase progression and DNA synthesis. LT also binds p53 and inactivates its function, preventing the infected cell from undergoing apoptotic cell death. After viral DNA replication is under way, the infection enters the late phase, when viral structural proteins are synthesized and new virions are produced. Ultimately, the infected cell releases progeny virions, frequently but not always by cell lysis. The immune system is critical for controlling the initial lytic phase in vivo, quenching the initial infection to a state of persistent low-level or nonreplicating genomes (i.e., in the proximal renal tubular epithelium for SV40), with detectable lytic viral reactivation coincident only with host immune suppression.
Some data on the infectivity of SV40 in humans were obtained from volunteers and individuals receiving contaminated vaccines. However, antibody data from many surveys must be viewed with the knowledge that the human BK virus (BKV) and JC virus (JCV) (closely related human polyomavirus family members) might give an indistinguishable response in these assays due to the high degree of cross-reactivity between capsid protein antigens. Melnick and Stinebaugh found SV40 (by cytopathic effects in monkey cells) in the stools of children 3 to 4 weeks after ingestion of 100 to 1,000 PFU of SV40 with oral poliovirus vaccine. Morris et al. gave SV40 intranasally to volunteers and found subclinical infections. They were able to isolate virus 7 to 11 days after administration from 3 of 8 subjects, and they detected antibody responses of various amplitudes. Horváth and Fornosi found SV40 excreted in the stools of 10 of 35 children 1 to 2 weeks after being given contaminated oral poliovirus vaccines. Thus, SV40 may replicate in humans after oral administration, but the efficiency and duration of the replication may be low in these immunocompetent subjects who were

given small inocula.

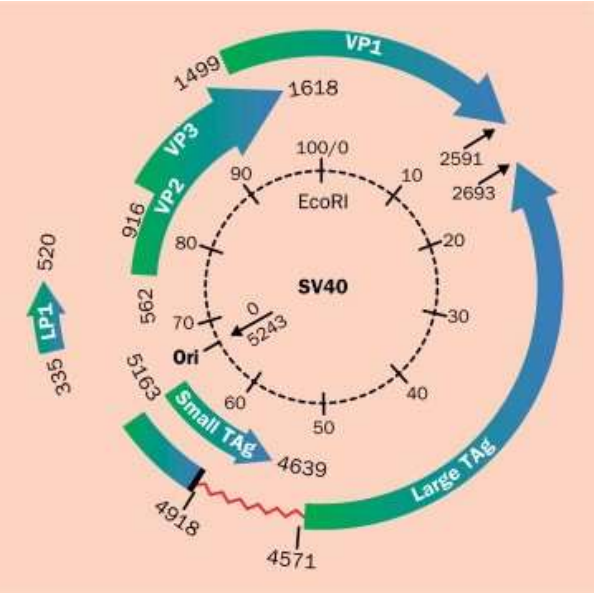


Fig 5: Genome organization of SV40 virus

The biology of SV40 in human cells was first studied in the 1960s with fibroblast cell lines or primary human fibroblast cell cultures. Whereas uninfected primary human fibroblasts can only be passaged a finite number of times before ceasing to divide and undergoing senescence, cell cultures infected with SV40 undergo a "crisis" at this same stage, followed by the outgrowth of a small number of cells that are phenotypically transformed. During the initial phase of the infection, generally the first 4 weeks, approximately 0.1% of the cells produce 500 to 1,000 virions per cell. Virus output from the culture then remains at a constant, very low level but with 100% of the cells producing virus at a rate of approximately 1 to 2 virions/cell. Once the cell culture progresses through crisis, virus production generally decreases, accompanied by a concomitant decrease in production of viral capsid proteins and an increase in the production of LT. One interpretation of these data is that the cells producing large amounts of virus are killed, but the cells that produce low levels of virus (as assayed by infectious center assays) survive. Finally, as the culture reaches its passage limit, most cells die, but those expressing a threshold level of LT overgrow the culture. Interestingly, the onset of transformation varies quite significantly in cells isolated from different individuals, ranging from 20 to almost 50 weeks in culture. Based on these early studies, human cells were termed semi permissive for SV40 growth. This nomenclature is confusing since the virus can clearly replicate in some human cell types more efficiently than in others, although the development of cytopathic effect is more rapid in African green monkey kidney cells.

SV40 is highly oncogenic in experimental animals and readily transforms rodent cells in culture. Hamsters inoculated with SV40 develop lymphomas, brain tumors, osteosarcomas, and mesotheliomas. SV40 is likely oncogenic in rodents because LT is unable to interact functionally with the rodent DNA polymerase α-primase complex. In this setting, the oncogenic functions of the T antigens are engaged but the productive cycle is not completed, resulting in uncontrolled cell division rather than cell lysis. LT is both necessary and sufficient for initiation and maintenance of transformation of rodent cells in tissue culture in most instances. Under certain conditions, however, usually involving primary cells in the absence of growth factors, ST is also required. ST functions by inhibiting the activity of the cellular phosphatase PP2A, resulting in activation of cell growth signal transduction pathways. Mice that are transgenic for LT transcriptionally regulated by

tissue-specific promoters develop tumors in those tissues (for an example, see reference 105). Transgenic mice in which LT expression is regulated by the native viral promoter elements specifically develop tumors of the choroid plexus (6), the specialized epithelial structure of the brain ependymal lining that produces cerebrospinal fluid. This finding is interesting in view of the discovery of SV40 DNA in certain brain tumors, as discussed below. After the discovery of SV40's tumorigenic and cell transformation properties, a wave of studies in the 1960s and 1970s pursued the identification of viral oncogenic agents in humans. SV40 DNA was detected on rare occasions, usually in brain tumors, using relatively low-sensitivity Southern hybridization techniques, immunostaining for LT, and electron microscopy. Also duringthisperiod,thedistinctlyhumanpolyomavirusesBKVandJCVwereidentified,andthedestructive brain white matter disease progressive multifocal leukoencephalopathy was attributed to JCV infection. These human viruses were also shown to induce tumors in animals and to transform rodent and human cells in culture. However, virtually all investigations failed to reveal any significant associations between human malignancy and these suspected oncogenic viruses. With the discovery of oncogenes, the emphasis in cancer research shifted from viruses to genomic mutations.

Lytic and Lysogenic cycles - phage multiplication cycle: A Lytic or virulent phages are phages which can only multiply on bacteria and kill the cell by lysis at the end of the life cycle.

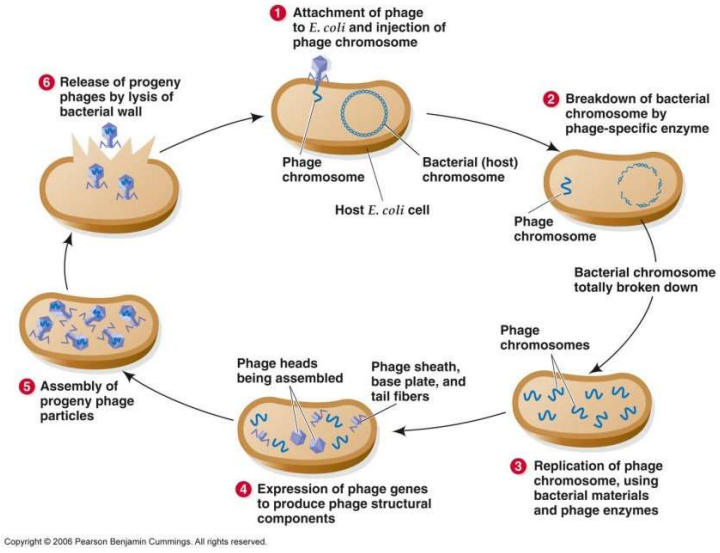


Fig 6: Lytic cycle of Phage

Steps: This cycle consists of the following steps

a. Eclipse period - During the eclipse phase, no infectious phage particles can be found either inside or outside the bacterial cell. The phage nucleic acid takes over the host biosynthetic machinery and phage specified m-RNA's and proteins are made. There is an orderly expression of phage directed macromolecular synthesis, just as one sees in animal virus infections. Early m-RNA's code for early proteins which are needed for phage DNA synthesis and for shutting off host DNA, RNA and protein biosynthesis. In some cases the early proteins actually degrade the host chromosome. After phage DNA is made late m-RNA's and late proteins are made. The late proteins are the structural proteins that comprise the phage as well as the proteins needed for lysis of the bacterial cell.

b. Intracellular Accumulation Phase - In this phase the nucleic acid and structural proteins that have been made are assembled and infectious phage particles accumulate within the cell. c. Lysis and Release Phase - After a while the bacteria begin to lyse due to the accumulation of the phage lysis protein and intracellular phage are released into the medium. The number of particles released per infected bacteria may be as high as1000.

B. Lysogenic or Temperate Phage

Lysogenic or temperate phages are those that can either multiply via the lytic cycle or enter a quiescent state in the cell. In this quiescent state most of the phage genes are not transcribed; the phage genome exists in a repressed state. The phage DNA in this repressed state is called a prophage because it is not a phage but it has the potential to produce phage. In most cases the phage DNA actually integrates into the host chromosome and is replicated along with the host chromosome and passed on to the daughter cells. The cell harboring a prophage is not adversely affected by the presence of the prophage and the lysogenic state may persist indefinitely.The cell harboring a prophage is termed a lysogen.

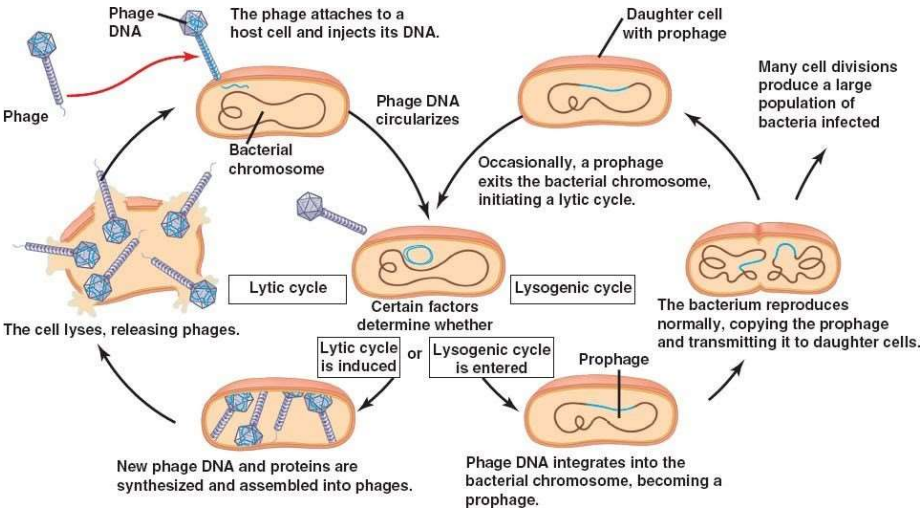


Fig 7: Lytic and Lysogenic cycle of phages

Significance of Lysogeny:

(a) Model for animal virus transformation -Lysogeny is a model system for virus transformation of animal cells.

(b) Lysogenic conversion-When a cell becomes lysogenized, occasionally extra genes carried by the phage get expressed in the cell. These genes can change the properties of the bacterial cell. Thisprocessiscalledlysogenicorphageconversion.Thiscanbeofsignificanceclinically.e.g.Lysogenic phages have been shown to carry genes that can modify the Salmonella O antigen, which is one of the major antigens to which the immune response is directed. Toxin production by *Corynebacterium diphtheriae* is mediated by a gene carried by a phage. Only those strain that have been converted by lysogeny are pathogenic.

Conjugation: Conjugation is one of three mechanism of DNA exchange between bacteria, the other being transformation and transduction. It is the process by which one bacterium transfers genetic material to another through direct contact. During conjugation, one bacterium serves as the donor of the genetic material, and the other serves as the recipient and the direction of transfer of

genetic material is one way; DNA is transferred from a donor to a recipient. The donor bacterium carries a DNA sequence called the fertility factor (Fig.1), or F-factor. Bacteria that have F plasmid are referred to as F+ or male. Those that do not have F plasmid are called F- or female. The F-factor allows the donor to produce a thin, tube like structure called a pilus, which the donor uses to contact the recipient. The pilus then draws the two bacteria together, at which time the donor bacterium transfers genetic material to the recipient bacterium. Typically, the genetic material is in the form of a plasmid, or a small, circular piece of DNA. The genetic material transferred during conjugation often provides the recipient bacterium with some sort of genetic advantage. For instance, in many cases, conjugation serves to transfer plasmids that carry antibiotic resistance genes. The transfer of these conjugative genes requires a sophisticated machinery that ensures DNA mobilization brought about by MOB genes and mating pair formation (organized by the MPF genes). These genes can be encoded by an autonomous replicating plasmid or by integrative conjugative elements (ICE) inserted in the chromosome. Conjugation in Gram-negative bacteria is mediated by the Type IV secretion system (T4SS), a large macromolecular complex involved in substrate transport and pilus biogenesis. T4SSs are implicated not only in bacterial conjugation, but also in the secretion of virulence factors to eukaryotic cells.

Steps of plasmid mobilization by conjugation

1. Conjugative plasmids initiate gene transfer by altering the cell surface to allow contact between the plasmid-containing donor cell (F+ or male) and a plasmid lacking recipients (F- or female). Sex pilus originates from the donor and establishes conjugative bridge (temporary cytoplasmic bridge) that serves as the conduit for DNA transfer from donor to recipient bacterial cell
2. Intercellular contact established
3. A copy of DNA from donor cell (F+ Cell) is transferred to recipient cell (F-cell).
4. Complementary DNA strand is synthesized in both donor cell and recipient cell
5. As the recipient cell now contains F plasmid it behaves as a donor cell.

If F- cells and F+ cells are mixed in culture, the entire population quickly becomes F +.

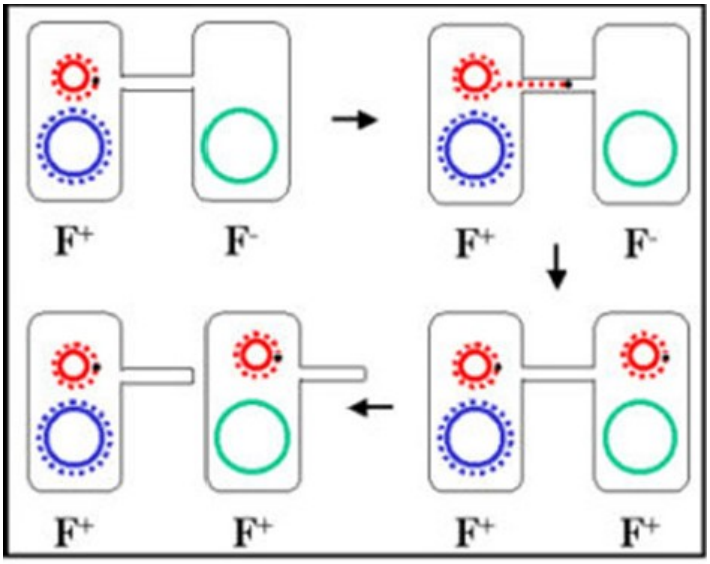


Fig.8 : Process of conjugation in bacteria

Donor : The ability of a bacterium to be a donor is a consequence of the presence in the cell of an extra piece of DNA called the F factor or fertility factor or sex factor. The F factor is a circular piece of DNA that can replicate autonomously in the cell; it is an independent replicon. Extrachromosomal pieces of DNA that can replicate autonomously are given the general name of plasmids. The F factor has genes on it that are needed for its replication and for its ability to transfer DNA to a recipient. One of the things the F factor codes for is the ability to produce a sex pilus (F pilus) on the surface of the bacterium. This pilus is important in the conjugation process. The F factor is not the only plasmid that can mediated conjugation but it is generally used as the model.

Recipient: The ability to act as a recipient is a consequence of the lack of the F factor.

Physiological states of the F factor:

Autonomous (F^+): In this state the F factor carries only those genes necessary for its replication and for DNA transfer. There are no chromosomal genes associated with the F factor in F^+ strains.
Integrated (Hfr): In this state the F factor has integrated into the bacterial chromosome (Fig.2) via a recombination event.

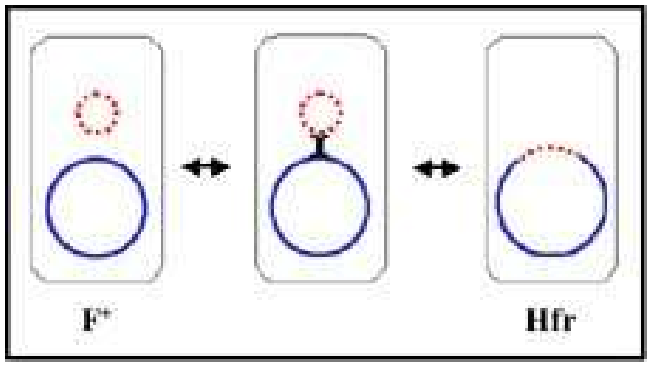


Fig.8: Integrated f factor

In crosses of the type Hfr X F^- the F^- rarely becomes Hfr and Hfr remains Hfr. In addition, there is a high frequency of transfer of donor chromosomal genes.

Autonomous with chromosomal genes (F'): In this state the F factor is autonomous but it now carries some chromosomal genes. F' factors are produced by excision of the F factor from an Hfr. Occasionally, when the F factor is excising from the Hfr chromosome, donor genes on either side of the F factor can be excised with the F factor generating an F'. F' factors are named depending on the chromosomal genes that they carry.
In crosses of the type F' X F^-, the F^- becomes F' while F' remains F'. In addition there is high frequency of transfer of those chromosomal genes on the F' and low frequency transfer of other donor chromosomal genes.

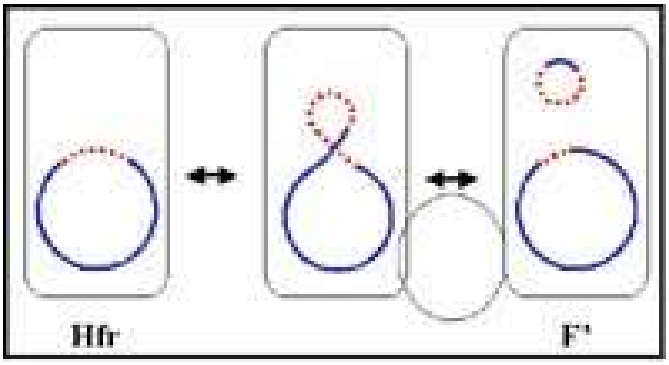


Fig 9: Transfer of genetic material to (F')

Overall mechanism of conjugation

$F^+X\ F^-$crosses

i) Pair formation

The tip of the sex pilus comes in contact with the recipient and a conjugation bridge is formed between the two cells. It is through this bridge that the DNA will pass from the donor to the recipient. Thus, the DNA is protected from environmental nucleases. The mating pairs can be separated by shear forces and conjugation can be interrupted. Consequently, the mating pairs remain associated for only a short time.

ii) DNA transfer

The plasmid DNA is nicked at a specific site called the origin of transfer and is replicated by a rolling circle mechanism. A single strand of DNA passes through the conjugation bridge and enters the recipient where the second strand is replicated. This process explains the characteristics of F^+ X F^- crosses. The recipient becomes F^+, the donor remains F^+ and there is low frequency of transfer of donor chromosomal genes. Indeed, as depicted in Figure 7 there is no transfer of donor chromosomal genes. In practice however, there is a low level of transfer of donor chromosomal genes in such crosses.

Hfr X F^-crosses

i) Pair Formation

In Hfr × F^-crosses, virtually none of the F^- parents were converted into F^+ or into Hfr. This result is in contrast with F^+ × F^-crosses, where infectious transfer of F results in a large proportion of the F^- parents being converted into F^+. During conjugation between an Hfr cell and a F^- cell a part of the chromosome is transferred with F. Random breakage interrupts the transfer before the entire chromosome is transferred. The chromosomal fragment can then recombine with the recipient chromosome. Clearly, the low level of chromosomal marker transfer observed by Lederberg and Tatum in an F^+ × F^-cross can be explained by the presence of rare Hfr cells in the population

ii) DNA transfer

The DNA is nicked at the origin of transfer and is replicated by a rolling circle mechanism. But the DNA that is transferred first is the chromosome. Depending upon where in the chromosome the F

factor has integrated and in what orientation, different chromosomal genes will betransferred at different times. However, the relative order and distances of the genes will always remain the same. Only when the entire chromosome is transferred will the F factor be transferred. Since shearing forces separate the mating pairs it is rare that the entire chromosome will be transferred. Thus, the recipient does not receive the F factor in a Hfr XF^{-}.

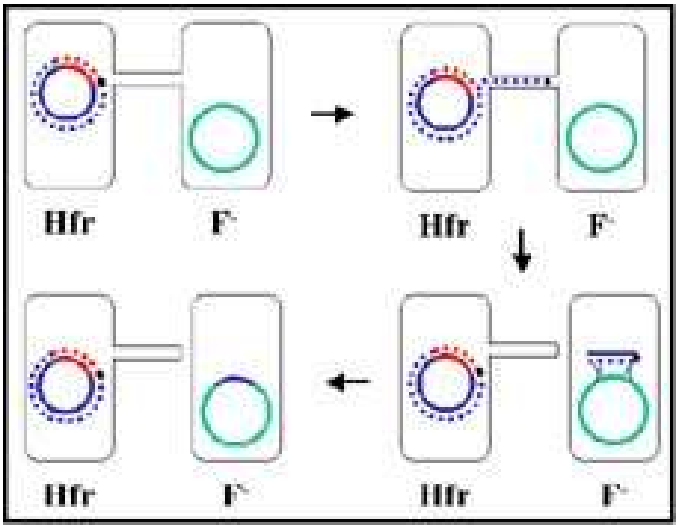


Fig 10: Cross and result of gene transfer

Significance : Among the Gram negative bacteria this is the major way that bacterial genes are transferred. Transfer can occur between different species of bacteria. Transfer of multiple antibiotic resistance by conjugation has become a major problem in the treatment of certain bacterial diseases. Since the recipient cell becomes a donor after transfer of a plasmid it is easy to see why an antibiotic resistance gene carried on a plasmid can quickly convert a sensitive population of cells to a resistant one.
Gram positive bacteria also have plasmids that carry multiple antibiotic resistance genes, in some cases these plasmids are transferred by conjugation while in others they are transferred by transduction. The mechanism of conjugation in Gram + bacteria is different than that for Gram -. In Gram + bacteria the donor makes an adhesive material which causes aggregation with the recipient and the DNA is transferred.

Bacterial Transduction

Transduction, a process of genetic recombination in bacteria in which genes from a host cell (a bacterium) are incorporated into the genome of a bacterial virus (bacteriophage) and then carried to another host cell when the bacteriophage initiates another cycle of infection. In general transduction, any of the genes of the host cell may be involved in the process; in special transduction, however, only a few specific genes are transduced. It has been exploited as a remarkable molecular biological technique for altering the genetic construction of bacteria, for locating bacterial genes, and for many other genetic experiments.
So it is the transfer of genetic information from a donor to a recipient by way of a bacteriophage. The phage coat protects the DNA in the environment so that transduction, unlike transformation, is not affected by nucleases in the environment. Not all phages can mediate transduction. In most cases gene transfer is between members of the same bacterial species. However, if a particular phage has a wide host range then transfer between species can occur. Also the ability of a phage to

mediated transduction is related to the life cycle of the phage. The discovery and initial mechanistic description of transduction were reported in 1952 by Norton Zinder and Joshua Lederberg in the journal of bacteriology.

Types of transduction

1. Generalized transduction-Generalized transduction is transduction in which potentially any bacterial gene from the donor can be transferred to the recipient.

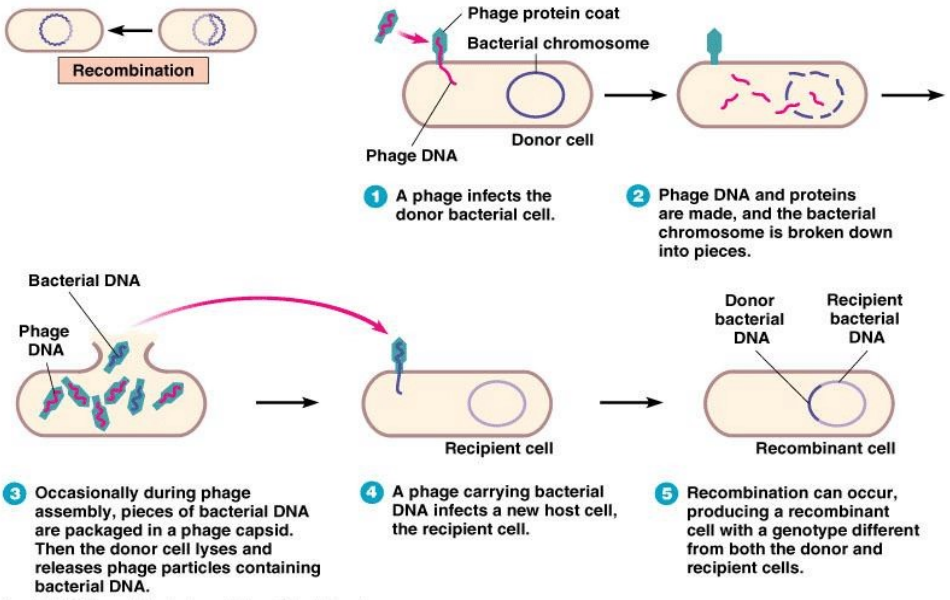


Fig.11: Mechanism of Generalized transduction

2. Specialized transduction-Specialized transduction is transduction in which only certain donor genes can be transferred to the recipient. Different phages may transfer different genes but an individual phage can only transfer certain genes. Specialized transduction is mediated by lysogenic or temperate phage and the genes that get transferred will depend on where the prophage has inserted in the chromosome. The mechanism of specialized transduction is illustrated in figure 4.phages that mediate generalized transduction generally breakdown host DNA into smaller pieces and package their DNA into the phage particle by a “head full” mechanism. Occasionally one of the pieces of host DNA is randomly packaged into a phage coat. Thus, any donor gene can be potentially transferred but only enough DNA that can fit into a phage head can be transferred. If a recipient cell is infected by a phage that contains donor DNA, donor DNA enters the recipient. In the recipient a generalized recombination event can occur which substitutes the donor DNA and recipient DNA.

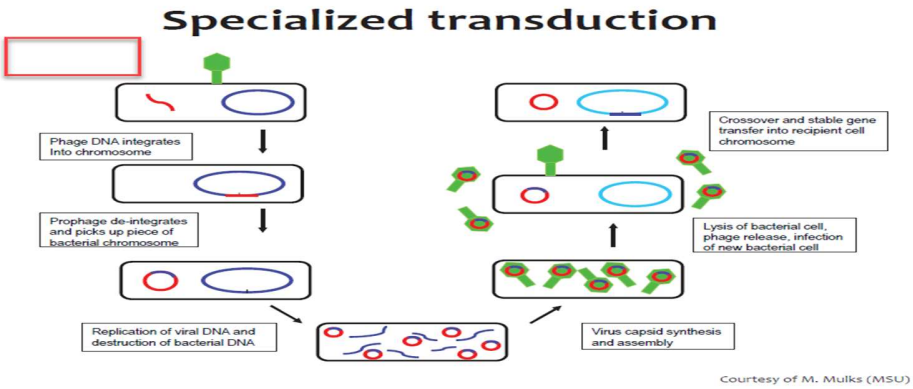


Fig. 12: The mechanism of Specialized transduction

During excision of the prophage, occasionally an error occurs where some of the host DNA is excised with the phage DNA. Only host DNA on either side of where the prophage has inserted can be transferred (i.e. specialized transduction). After replication and release of phage and infection of a recipient, lysogenization of recipient can occur resulting in the stable transfer of donor genes. The recipient will now have two copies of the gene(s) that were transferred. Legitimate recombination between the donor and recipient genes is also possible. Transduction happens through either the lytic cycle or the lysogenic cycle. If the lysogenic cycle is adopted, the phage chromosome is integrated (by covalent bonds) into the bacterial chromosome, where it can stay dormant for thousands of generations. If the lysogen is induced (by UV light for example), the phage genome is excised from the bacterial chromosome and initiates the lytic cycle, which culminates in lysis of the cell and the release of phage particles. The lytic cycle leads to the production of new phage particles which are released by lysis of the host.

Application: Correcting genetic diseases by direct modification of genetic errors

Bacterial Transformation

The conversion of one genotype into another by the introduction of exogenous DNA (that is, bits of DNA from an external source) is termed transformation. Transformation is one of three processes for horizontal gene transfer, in which exogenous genetic material passes from one bacterium to another, the other two being conjugation (transfer of genetic material between two bacterial cells in direct contact) and transduction (injection of foreign DNA by a bacteriophage virus into the host bacterium). Transformation was discovered in *Streptococcus pneumoniae* in 1928 by Frederick Griffith in 1944, Oswald T. Avery, Colin M. MacLeod, and Maclyn McCarty demonstrated that the "transforming principle" was DNA. For transformation to take place, the recipient bacteria must be in a state of competence, which might occur in nature as a time-limited response to environmental conditions such as starvation and cell density, and may also be induced in a laboratory. About 80 species of bacteria were known to be capable of transformation, almost evenly divided between Gram-positive and Gram-negative bacteria; the number might be an overestimate since several of the reports are supported by single paper.

Natural competence and transformation

Naturally competent bacteria carry sets of genes that provide the protein machinery to bring DNA

across the cell membrane (s). The transport of the exogenous DNA into the cells may require proteins that are involved in the assembly of type IV pili and type II secretion system, as well as DNA translocase complex at the cytoplasmic membrane.
Due to the differences in structure of the cell envelope between Gram-positive and Gram- negative bacteria, there are some differences in the mechanisms of DNA uptake in these cells, however most of them share common features that involve related proteins. The DNA first binds to the surface of the competent cells on a DNA receptor, and passes through the cytoplasmic membrane via DNA translocase. Only single-stranded DNA may pass through, the other strand being degraded by nucleases in the process. The translocated single-stranded DNA may then be integrated into the bacterial chromosomes by a RecA-dependent process. In Gram-negative cells, due to the presence of an extra membrane, the DNA requires the presence of a channel formed by secretins on the outer membrane. Pilin may be required for competence, but its role is uncertain. The uptake of DNA is generally non-sequence specific, although in some species the presence of specific DNA uptake sequences may facilitate efficient DNA uptake.

Natural transformation

Natural transformation is a bacterial adaptation for DNA transfer that depends on the expression of numerous bacterial genes whose products appear to be responsible for this process.The capacity for natural transformation appears to occur in a number of prokaryotes, and thus far 67 prokaryotic species (in seven different phyla) are known to undergo this process. In general, transformation is a complex, energy-requiring developmental process. In order for a bacterium to bind, takes up and recombine exogenous DNA into its chromosome, it must become competent, that is, enter a special physiological state. Competence development in *Bacillus subtilis* requires expression of about 40 genes. The DNA integrated into the host chromosome is usually (but with rare exceptions) derived from another bacterium of the same species, and is thus homologous to the resident chromosome.
In *B. subtilis* the length of the transferred DNA is greater than 1271 kb (more than 1 million bases). The length transferred is likely double stranded DNA and is often more than a third of the total chromosome length of 4215 kb. It appears that about 7-9% of the recipient cells take up an entire chromosome.
Competence for transformation is typically induced by high cell density and/or nutritional limitation, conditions associated with the stationary phase of bacterial growth. Transformation in *Haemophilus influenzae* occurs most efficiently at the end of exponential growth as bacterial growth approaches stationary phase. Transformation in Streptococcus mutants, as well as in many other streptococci, occurs at high cell density and is associated with biofilm formation. Competence in *B. subtilis* is induced toward the end of logarithmic growth, especially under conditions of amino acid limitation. After DNA was shown to be the agent that determines the polysaccharide character of *S. pneumoniae*, transformation was demonstrated for other genes, such as those for drug resistance. The transforming principle, exogenous DNA, is incorporated into the bacterial chromosome by a breakage-and-insertion process analogous to that described for Hfr × F− crosses. Note, however, that, in conjunction, DNA is transferred from one living cell to another through close contact, whereas, in transformation, isolated pieces of external DNA are taken up by a cell. Figure 1 depicts this process.

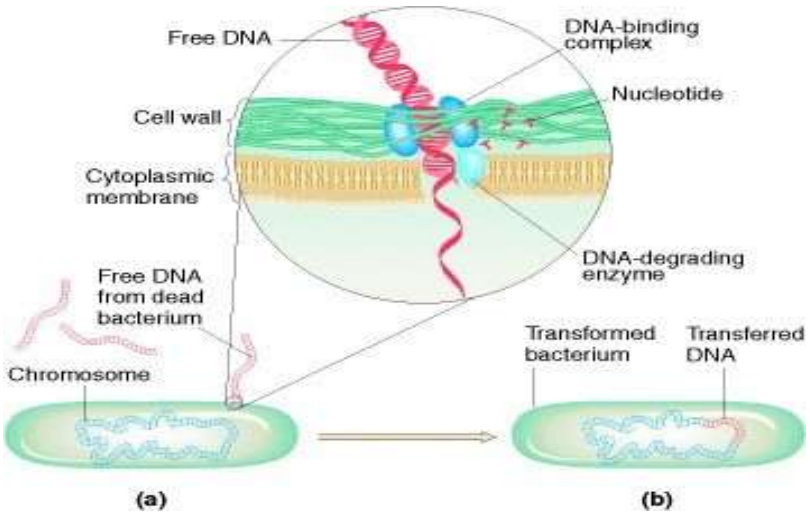


Figure 13: Bacterium undergoing transformation (a) picks up free DNA released from a dead bacterial cell. As DNA-binding complexes on the bacterial surface take up the DNA (inset), enzymes break down one strand into nucleotides; meanwhile the other strand may integrate into the bacterium's chromosome (b).

Significance: Linkage information from transformation

Transformation has been a very handy tool in several areas of bacterial research. Here we examine its usefulness in providing linkage information. When DNA (the bacterial chromosome) is extracted for transformation experiments, some breakage into smaller pieces is inevitable. If two donor genes are located close together on the chromosome, then there is a greater chance that they will be carried on the same piece of transforming DNA and hence will cause a double transformation. Conversely, if genes are widely separated on the chromosome, then they will be carried on separate transforming segments and the frequency of double transformants will equal theproductofthesingle-transformationfrequencies.Thus,itshouldbepossibletotestforcloselinkage by testing for a departure from the product rule. Unfortunately, the situation is made more complex by several factors-the most important of which is that not all cells in a population of bacteria are competent, or able to be transformed. Because single transformations are expressed as proportions, the success of the product rule depends on the absolute size of these proportions.

Transformation, as an adaptation for DNA repair

Competence is specifically induced by DNA damaging conditions. For instance, transformation is induced in *Streptococcus pneumoniae* by the DNA damaging agents mitomycin C (a DNA crosslinking agent) and fluoroquinolone (a topoisomerase inhibitor that causes double-strand breaks). In *B. subtilis*, transformation is increased by UV light, a DNA damaging agent. In *Helicobacter pylori*, ciprofloxacin, which interacts with DNA gyrase and introduces double- strand breaks, induces expression of competence genes, thus enhancing the frequency of transformation. Using *Legionella pneumophila*, Charpentier et al. tested 64 toxic molecules to determine which of these induce competence. Of these only six, all DNA damaging agents, caused strong induction. These DNA damaging agents were mitomycin C (which causes DNA inter-strand crosslinks), norfloxacin, ofloxacin and nalidixic acid (inhibitors of DNA gyrase that cause double-strand breaks, bicyclomycin (causes single- and double-strand breaks, and hydroxyurea(induces DNA base oxidation. UV light also induced competence in *L. pneumophila*. Charpentier et al. suggested that competence for transformation probably evolved as a DNA damage response

Probable Questions:

1. Describe genome organization in prokaryotes.
2. Describe the variations found in prokaryotic genome structure.
3. Describe the life cycle of MS2 bacteriophages.
4. Describe lytic cycle of a phage with examples.
5. Describe lysogenic cycle of a bacteriophage.
6. Describe significance of lysogeny.
7. Define conjugation. Describe the process in a bacteria.
8. What is transduction? What is the difference between simple and specialized transduction?
9. Define transformation in bacteria. Describe the process in brief with suitable diagram.
10. What are the significance of transformation in bacteria?

Suggested readings:

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7th Edition.
6. Gene cloning and DNA Analysis by T. A. Brown, Sixth Edition.
7. Genetics . Verma and Agarwal.

Unit-VII

Cell signalling: Modes of cell-cell signalling; steroid hormones and steroid hormone superfamily, neurotransmitters; peptide hormones and growth factors; eicosanoids, functions of cell surface receptors.

Objective: In this unit you will learn about Modes of cell-cell signalling. Steroid hormones and steroid hormone superfamily, neurotransmitters, peptide hormones and growth factors, eicosanoids and functions of cell surface receptors.

Introduction:

Eukaryotic cells and bacteria release a large number of signals and establish communication. The method of action is binding the signals with the protein receptors present on surface of large cell and triggering a series of intracellular reactions called intracellular signaling or signal transduction.

Besides, many signals (steroids and bacterial autoinducer) enter the cell, interact with signaling system and establish signal transduction. For establishing intracellular signaling, one must fully understand the operation of any cell from their origin to death.

The first signaling molecule (cyclic adenosine monophosphate or cAMP) was known during early 1960s. However, importance of intracellular signaling could be realized after the discovery of changes made by mutagenesis in signaling pathway which results in cellular transformation which is now called as cancer. One could understand their function by mutagenizing their cellular function.

It is now known that bacteria can change the eukaryotic cell signaling and invade the cells. The bacterial toxins can hijack the control of host cells. Similarly complexity of bacterial signaling is also known. Now an enormous amount of information is available on cell signaling and signal transduction pathway both in prokaryotes and eukaryotes.

The Signaling System:

Signaling system is very complex which may be compared to electronic circuits. You know that electronic system is such that can integrate, modulate and amplify inputs and generate output signals when switched on or switched off after getting suitable signals. The signaling systems include few basic type of modules. There are four main processes, but the signaling system uses the one or more processes.

The types of signaling modules used in intracellular signaling are:

(a) Receptor kinases (e.g. tyrosine kinase, serine kinase, histidine kinase),

(b) Receptor non-kinases (e.g. serpentine, cytokine, His-Asp phosphorelay),

(c) Protein kinase (intracellular enzymes e.g. cyclin families, Asp kinase),

(d) Lipid modifying intracellular enzymes (e.g. p13K, p15K,PLC),

(e) Cyclic nucleotides (e.g. cAMP, cGMP),and

(f) Metal ions (e.g.Ca^{++})

The four main processes are as follows:

(a) Protein phosphorylation by kinases,

(b) Small molecule and protein interaction, often involving phosphates,

(c) Protein-protein interaction, often mediated by common motifs (specific protein sequences) which frequently results in membrane recruitment when one component is tethered to a membrane, and

(d) Protein and DNA interaction that promotes gene expression or gene inhibition.

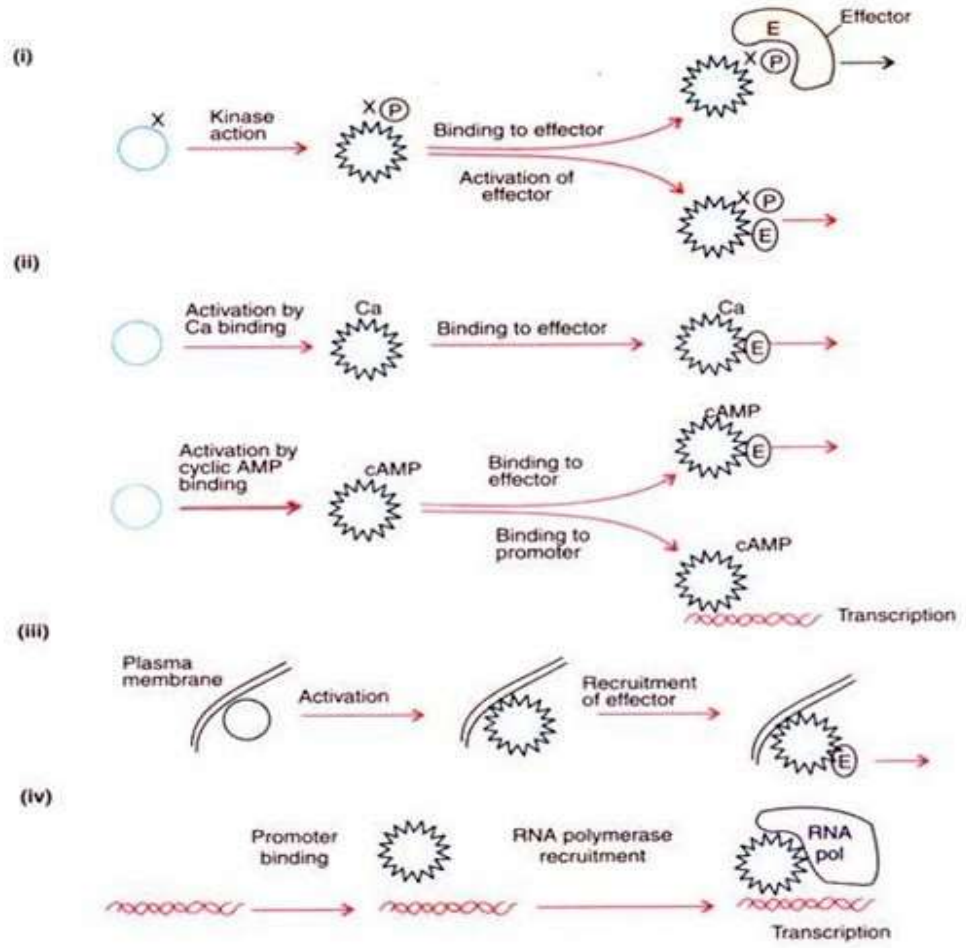


Fig. 27.8: Signalling molecules that use different types of interactions. *(i)* protein phosphorylation, where X=Tyr, Ser, Thr, His or Asp. *(ii)* interaction between small molecules and proteins, e.g. Ca^{++}, or cAMP, *(iii)* interaction between protein and protein, and *(iv)* interaction between protein and DNA which regulates transcription.

The Basic Building Blocks used in Signalling:

(a) Protein Phosphorylation: Protein phosphorylation is closely linked to cellular signaling. It exits in all signaling modules. The terminal y-phosphate is directly transferred from ARP (in some cases) to an acceptor protein by a protein kinase. The activity of the acceptor is modified example mitogen-activated protein (MAP) kinases in eukaryotes and histidyl-aspartyl phosphorelay in bacteria.

In some cases, indirect phosphorylation of protein also occurs (e.g. in G protein when binding of GTP activates their function, while GDP binding inactivates). There are secondary messengers which are used in intracellular signaling such as phosphorylated inositol's or cyclic nucleotides (cAMP, cGMP).

Kinases are regulated by any of a number of mechanisms: threonine and/or tyrosine phosphorylation, legend occupancy resulting in autophosphorylation or interaction with small molecules (e.g. cAMP or Ca^{++}).

i. Histidine Kinases:

These are found in bacteria, lower eukaryotes and plants as trans membrane protein. They are stimulated to undergo self-phosphorylation by ligand occupancy.

ii. Protein Phosphatases:

Proteins which remove phosphate groups from proteins are called protein phosphatases. Protein kinases add phosphate group to proteins and play a key role in activation of signals. Specific phosphatases e.g. dephosphorylate phosphotyrosine and phosphoserine/phosphothreonme play a key role in control of proliferation, differentiation and cell cycle Phosphoproteins take part in signaling. They moderate the phosphorylation status by regulating the balance of phosphatases and kinases.

(b) Nucleotide-Binding Proteins:

The three nucleotides (GTP, cGMP and cAMP) play a major role in the intracellular signaling.

iii. GTP-Binding Proteins:

There is a set of eukaryotic proteins (G proteins) that show GTPase activity. They bind to GTP and remove the terminal phosphate of GTP and produce GDP bound to G protein. This cycle operates similar to ATP and ADP cycles.
When GDP dissociates from the G protein and GTP binds again, the cycle is repeated (Fig. 27. 9). G proteins are of two type: the heterotrimeric G proteins (the dominating proteins), and the small G proteins or membrane of Ras super family (the intermediate member of the signaling pathways).

The heterotrimeric G proteins consist of three different subunits- α, β and y subunits The a- subunit has GTP-binding domain; hence Gβ has a role in signal transduction. The βy subunits transmit signals by non-covalent interaction with effector molecules. Activation of G proteins and Association of α-subunit from βy subunits are given in Fig. 27.9. GTPase activity results in after binding the Gα subunit with GDP and subsequent association with Gβy and down regulation

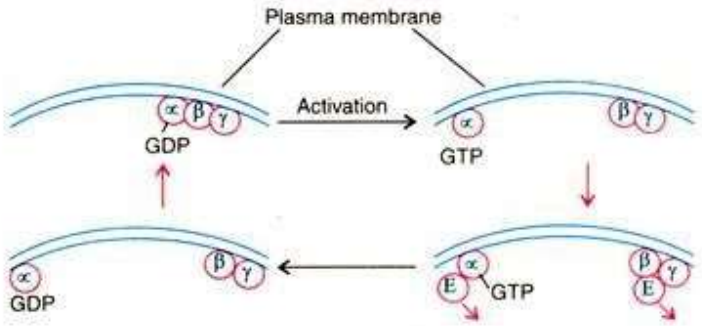


Fig. 27.9 : The function of membrane-bound heterotrimeric G proteins having α, β and γ subunits.

The small G proteins (Ras super family or p21 family) play a key role in many cellular functions

such as proliferation and differentiation (Ras family), cytoskeletal organization (Rho) and nuclear membrane transport (Ran). The activity of small G proteins is modulated after interaction with several classes of proteins (Fig.27.10).

GDP-dissociation inhibitors (GDI) inhibit the loss of bound GDP and keep the G proteins in an inactive form to attenuate signaling from the activated G proteins. GTPase activity IS stimulated by GTPase-activating proteins (GAP). The removal of the bound GDP IS helped by guanine nucleotide exchange factors (GEF) which enable the GTP to bind and activate G proteins. Some of these factors have shown to be proto-oncogenes.

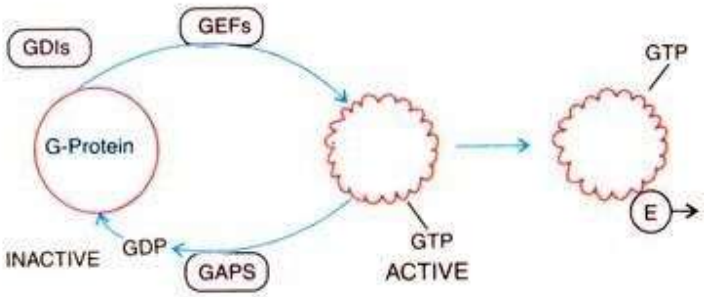


Fig. 27.10 : Functioning of small G proteins.

iv. Cyclin Nucleotide-Binding Proteins:

In 1950s, cAMP was identified as the first intracellular signaling molecules. It mediates hormone action and acts as molecules transmitting the primary signal that has been received at the cell membrane). The cAMP mediates the response to chemo-attractants. The adenylate cyclase and guanylate cyclase regulate the concentration of cAMP and cGMP, respectively.

The soluble bacterial adenylate cyclases produce cAMP which binds to c AMP receptor protein (CRP) and activate them. CRP is a transcription factor. The cAMP influences the expression of many of genes. Consequently bacteria become able to express metabolic enzymes which are required during growth. The cAMP also regulates the expression of the other genes which can cause pathogenesis. In eukaryotes heterotrimeric G proteins regulate the membrane-bound adenylate cyclases which produce cAMP. G proteins are coupled to transmembrane receptors. The cAMP-dependent protein kinases (protein kinase A) are the main effects of the cAMP signals.

While in the inactive form, protein kinase A consists of a dimer of regulatory (A) subunits and two catalytic (C) sub- units. The molecules of cAMP binds to reach R subunits and induce conformational changes. Consequently activated C subunits are released. This activated protein kinase A phosphorylates many substrates on serine or threonine (Fig. 27.11).

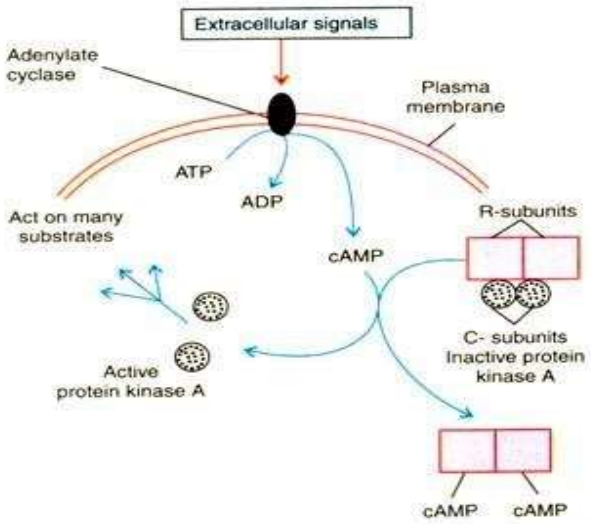


Fig. 27.11 : Production of cAMP and activation of protein kinase A.

Both the cycles work in eukaryotes by direct binding to proteins which form cation channels. Binding events result in opening of the channel. The G-protein-linked cell surface receptor generates small intracellular mediators thought cAMP pathways (Fig. 27.12).

(c) Role of Intracellular Concentration of Ca^{++} in Cell Signaling:

Calcium is found in Cytoplasm and maintained in a very low concentration(10-100 nM). But its concentration varies with cell cycle, exogenous source or release from the stores. It gets complexes in membrane bound vesicles acting as stores. A highly specific protein calmodulin (CaM) binds to Ca^{++} and transmit the signal. Ca-binding to CaM brings about changes in conformation of CaM.

Consequently CaM interacts with many effectors including CaM-modulated kinase. The most extensively studied CaM is the phosphatase calcineurin which is associated with several cellular activities such as NO synthesis, apoptosis, and induction of T lymphocytes. In eukaryotic cells Ca^{++} acts as a second messenger. Fig. 27.12 shows the two major pathways by which G-protein- linked cell surface receptors generate small intracellular mediators.

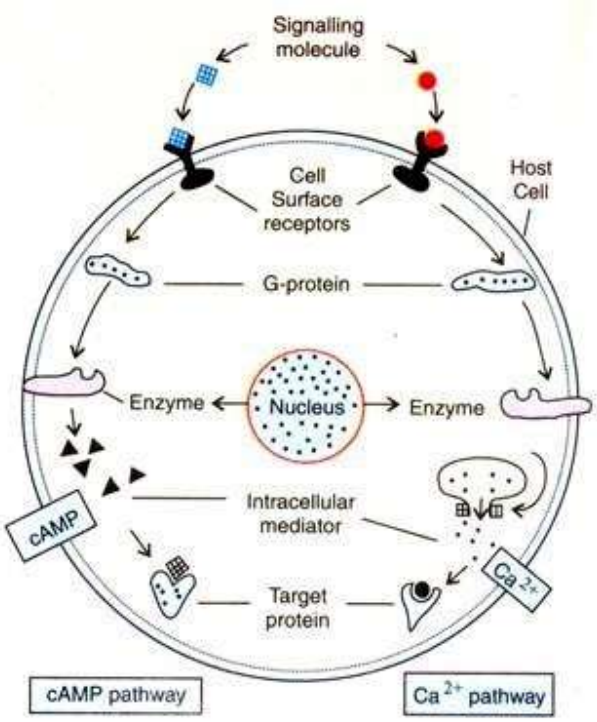


Fig. 27.12 : Generation of small intracellular mediators by G-protein-linked cell.

Role of Phosphorylated Lipids in Cell Signaling:

In eukaryotes lipids are involved in signaling process. Cellular phospholipases attack the lipid moieties of the membrane to produce different types of signaling molecules. For example, phosphatidylinositol lipids play a role in cellular stimulation. They have inositol as head, the six-membered carbon ring with a -OH group on each carbon.

On the basis of phosphorylation status of inositol head group, several phosphatidyl inositols are found in the cells. The activity of three enzymes triggers their signaling role. These are:

phosphoinositide 5′-kinase (P15p, phosphoinositide 3′-kinase (P13K), and phospholipase C (PLC). Extracellular signals regulate all these enzymes.

(d) Regulation of Transcription:

Both types of cells are able to respond to any signal by changing their gene expression. In a signaling pathway the end point acts as signal. Regulators causing changes in expression of many genes in bacteria are called ‘global regulators’. In prokaryotes post-transcriptional events regulate expression of many of the transcriptional factors for example cAMP-mediated CRP- DNA interactions.

In prokaryotes, phosphorylation or protein-protein interactions regulate the control of transcriptional factors and also select the other factors to the promotors. Besides, some other factors also get translocated from cytoplasm to the nucleus and regulate transcription.

(e) Role of Cell Membrane in Signaling:

Cell membrane acts as boundary of the cell through which extracellular signal has to enter. In bacteria histidine kinases act as receptor and directs signals across the membrane. Besides, there are many signal molecules which are associated with cell membrane because the end effect is membrane-associated.

The components can be well organized in three-dimensional way in cell membrane. The signaling components recruit the other molecules to the membrane where they interact with other factors. For example, GTP-bound Ras activates Raf kinase to recruit Raf to the membrane where the membrane-bound kinase activates it through phosphorylation.

3. Prokaryotic Signalling Mechanisms:

Intracellular signaling is very complex like electronic circuit. Genome size of different bacteria varies and those organisms work according to genes present in them.

In bacteria the generic mechanism of regulation is called signaling systems which includes:

a. The histidyl-aspartae phosphorylation systems (the main module of bacteria used to receive and process incoming signals such as chemotaxis, response to osmolarity, oxygen and phosphate, and virulence system),

b. The cAMP and CRP (involved in regulation of hundreds of genes. The cAMP is controlled at transcriptional and post-transcriptional levels. Binding of CRP- cAMP complex induces gene expression).

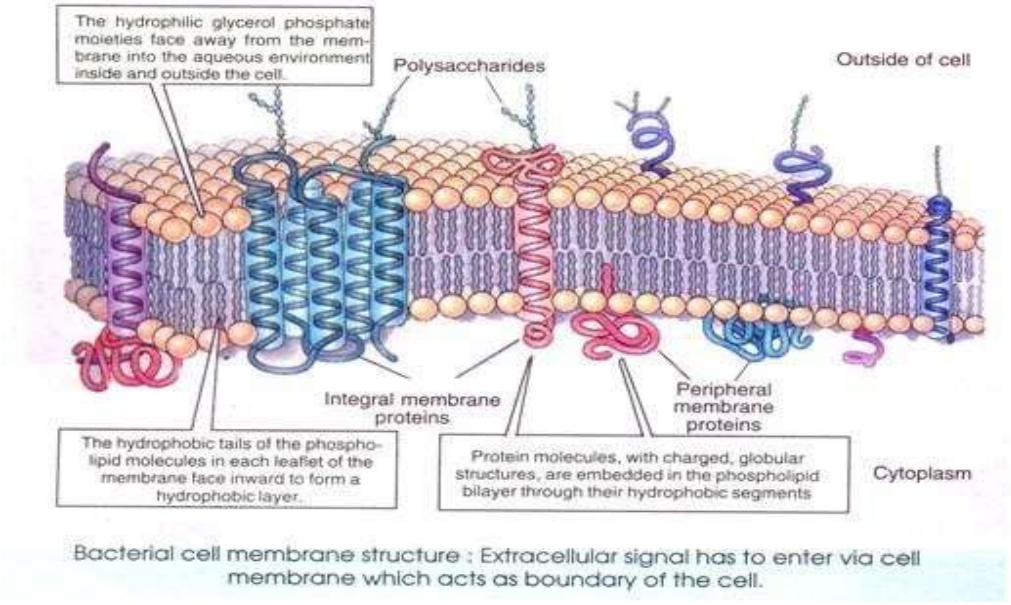
4. Eukaryotic Signalling Pathway:

Earlier it was thought that signaling process in eukaryotes was very complex to understand in molecular terms. Fragmented understanding about individual components could be known. The knowledge of signaling expanded with the development of new techniques such as genome sequencing, increasing number of reagents (isolated components, specific probes e.g. antibodies for individual components and selective inhibitors).

In spite of all these, no pathway has been fully elucidated. The best characterized pathway is the Ras activation and MAP kinases of which several details are unclarified. They are interconnected and cannot work without reference to others.

The Phospholipase C/Inositol Triphosphate Pathway:

The phospholipase C, beta or gamma is activated by membrane signaling events and cleaves PIP2 to produce diacylyglycerol (DAG) and inositol triphosphate (IP_3). These activates the release of Ca^{++} ions and results in activation of protein kinase C (PKC), which phosphorylates many additional protein substrates.



Bacterial cell membrane structure : Extracellular signal has to enter via cell membrane which acts as boundary of the cell.

The Adenylate Cyclase, cAMP and Protein Kinase A Pathway:

Adenylate cyclase is activated at the membrane by interaction with the activated heterotrimeric G protein G_5. The cAMP is generated and binds to and activates protein kinase A (PKA), which phosphorylates many substrates.

Integrin's, the Rho Family and Organization of Cytoskeletal:

The integrins are the signalling molecules that interact with the extracellular matrix on the outside of the cell and various proteins-linked to actin on the cells interior. The proteins involved include α-actin, lalin, tensin, vinculin and pavilin.

A local adhesion is formed upon activation that includes focal adhesion kinase (FAK). The Src kinase is recruited and several proteins in the complex are activated by phosphorylation by Src and FAC. These signals lead to the Ras/Raf, Rho signaling pathway and to cytoskeletal rearrangement. In eukaryotes, the central role of signaling pathway of a cell is to define its phenotype and function. The increasing novel knowledge about the components of signaling pathways and the types of genes which they interact are already being applied in new strategy to combat the cancer. For example, genetically engineered viruses are attempted to grow in such cells that lack functional p53 and kill these cells.

There are about 2000-5000 signal transduction proteins in mammalian cells. Bacteria have capacity to utilize eukaryotic signaling pathway during the process of infection. These findings make a line between the signaling pathways involved in infection and the other responsible for the pathology in diseases such as cancer and inflammation.

Eicosanoids (Gr. ecosn = 20):

These are derived from arachidonic acid, a C-20 fatty acid with 4 double bonds, e.g., prostaglandins, thrombaxanes and leukotriene's. These are called local hormones because they are short lived and have autocrine and paracrine effect.

Cytokine receptors:

Cytokine receptors are receptors that bind cytokines. In recent years, the cytokine receptors have come to demand the attention of more investigators than cytokines themselves, partly because of their remarkable characteristics, and partly because a deficiency of cytokine receptors has now been directly linked to certain debilitating immunodeficiency states. In this regard, and also because the redundancy and pleiotropy of cytokines are a consequence of their homologous receptors, many authorities are now of the opinion that a classification of cytokine receptors would be more clinically and experimentally useful.

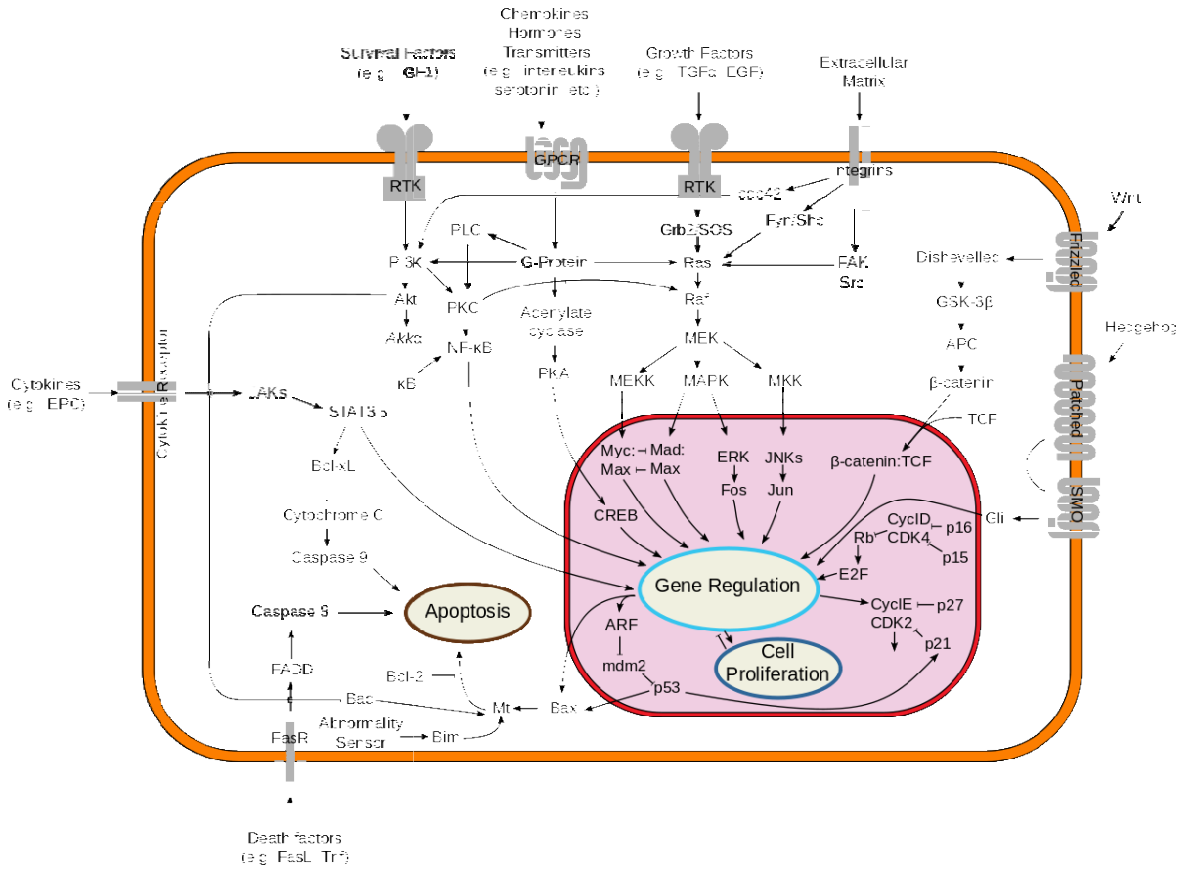


Fig : Signal transduction. (Cytokine receptor at center left.)

Classification of Cytokine Receptors

A classification of cytokine receptors based on their three- dimensional structure has been attempted. (Such a classification, though seemingly cumbersome, provides several unique perspectives for attractive pharmacotherapeutic targets.)

Type I cytokine receptors whose members have certain conserved motifs in their extracellular amino-acid domain. The IL-2 receptor belongs to this chain, whose γ chain (commonto several other cytokines) deficiency is directly responsible for the X-linked form of Severe Combined Immunodeficiency (X-SCID).

Type II cytokine receptors, whose members are receptors mainly for interferons.

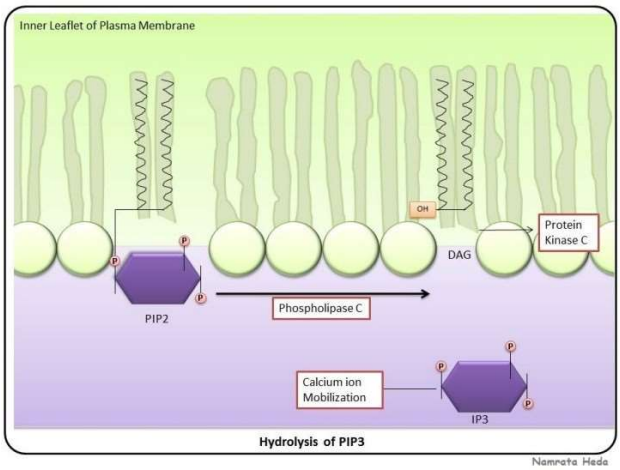
Immunoglobulin (Ig) superfamily, which are ubiquitously present throughout several cells and tissues of the vertebrate body

Tumor necrosis factor receptor family, whose members share a cysteine-rich common extracellular binding domain, and includes several other non-cytokine ligands like receptors, CD40, CD27 and CD30, besides the ligands on which the family is named (TNF).

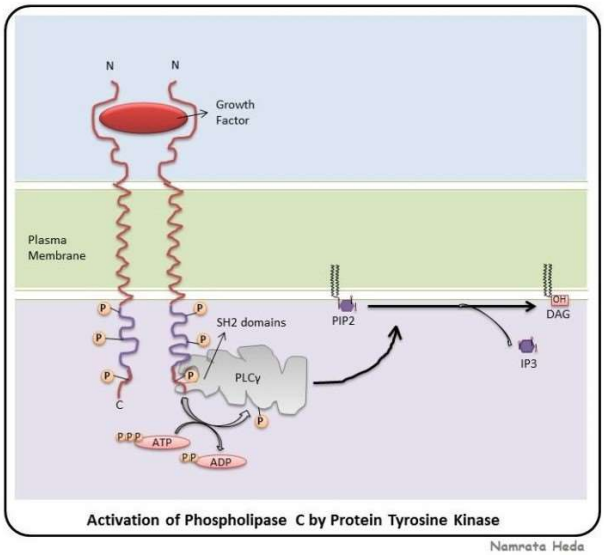
Chemokine receptors, two of which acting as binding proteins for HIV (CXCR4 and CCR5). They are G protein coupled receptors.

Phospholipids and Ca ion mediated signaling :

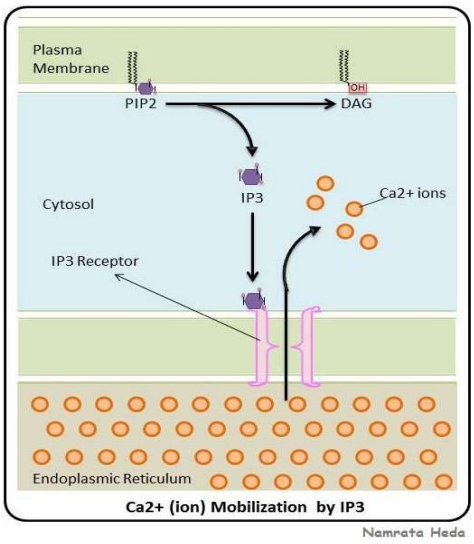
Phosphatidylinositol 4,5-bisphosphate abbreviated as PIP2 is a phospholipid present in the inner leaflet of the bilayer of the plasma membrane. The second messengers are derived from this small component (phospholipid) and the pathway is based on these messengers.



The hydrolysis of PIP2 takes place by the enzyme phospholipase C as can be seen in the adjacent figure. It is interesting to note that the enzyme phospholipase C is ultimately activated by G- protein coupled receptors (GPCRs) or protein tyrosine kinases. This is so because one form of phospholipase C (PLC-β) is stimulated by G proteins while another form of phospholipase C (PLC-γ) contains SH2 domains (as can be seen in the figure shown below) and hence it associates with activated receptor protein tyrosine kinases. This interaction helps PLC-γ to localize to plasma membrane and also leads to its phosphorylation. This tyrosine phosphorylation increases PLC-γ activity, which in turn stimulates hydrolysis ofPIP2.



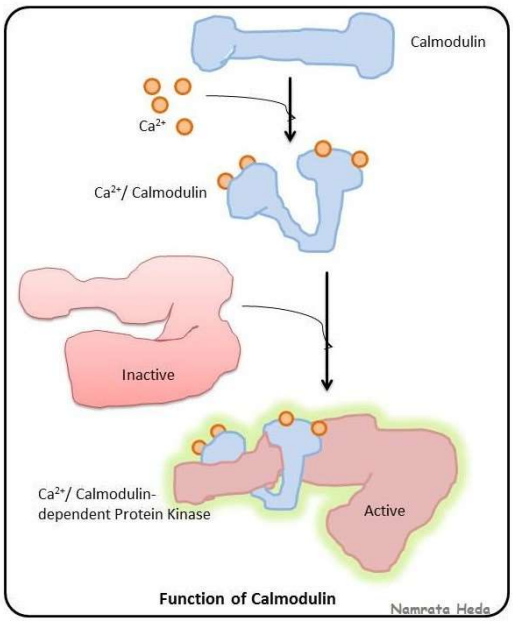
The hydrolysis of PIP2 produces two distinct second messengers as diacylglycerol and inositol 1,4,5-triphosphate which is abbreviated as IP3. Both these messengers stimulate different downstream signaling pathways thereby triggering two distinct cascades of intracellular signaling. Diacylglycerol stimulates protein kinase C mobilization while IP3 stimulates Ca2+(ions) mobilization. The diacylglycerol as second messenger activates serine/threonine kinases which belongs to the protein kinase C family which play an important role in cell growth anddifferentiation.IP3, another second messenger is released into the cytosol and it acts to release the Ca2+(ions) from intracellular stores. The level of the Ca2+(ions) inside the cell is very low and is maintained by pumping through Ca2+(ion) pumps across the plasma membrane.



The Ca2+(ions) are pumped into the ER and hence ER is considered to be the store of intracellular Ca2+(ions). Here, IP3 binds to the receptors in the ER membrane as can be seen in the adjacent diagram. These receptors are ligand-gated ion channels and hence, there is efflux of Ca2+(ions) into the cytosol.

This increase of Ca2+(ions) in the cytosol has an effect on variety of proteins like protein kinases. For example, there are some members of protein kinase C (PKC) family that requires Ca2+(ions) as well as diacylglycerol for their functioning. Hence, these PKC family members are regulated by both IP3 and diacylglycerol.

Calmodulin is another very important protein to mention while we are studying about Ca2+(ions). The word 'calmodulin' means - cal(cium) + modul(ate) + in(g). Thus, calmodulin is 'calcium modulating' protein that mediates most of the activities of Ca2+(ions). Calmodulin is dumbbell shaped protein which has four Ca2+(ions) binding sites (figure is shown below). When the Ca2+(ions) concentration in the cell increases, calmodulin is activated. This active Ca2+/calmodulin complex then binds to a variety of target proteins, like Ca2+ion/calmodulin - dependent protein kinases thereby rendering them active. The examples of Ca2+ion/calmodulin dependent-protein kinases are: myosin light-chain kinase and members.



When there is a change in plasma membrane's potential i.e.; when there is membrane depolarization, the voltage-gated Ca2+ ion channels are opened in the plasma membrane. Because of the opening, there is influx of Ca2+(ions) from the extracellular fluid into the cytosol of the cell. This increase in the levels of Ca2+(ions) further triggers the opening of the another receptor called the ryanodine receptor in the plasma membrane which further releases the Ca2+(ions) from the intracellular stores. This increase in the Ca2+(ions) results in triggering the release of neurotransmitter. Hence, we can say that Ca2+ ion plays an important role in converting electric signals to chemical signals. In muscle cells, the ryanodine receptors on the sarcoplasmic reticulum. These receptors maybe opened directly when there is membrane depolarization.

Probable Questions:

1. Describe the role of protein phosphorylation in signal transduction.
2. Describe role of Intracellular Concentration of Ca^{++} in Cell Signaling.
3. Describe the role of Phosphorylated Lipids in Cell Signaling.
4. Classify cytokine receptors in details.
5. Write down the role of calmodulin in signal transduction.

Suggested readings:

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7th Edition.
6. Gene cloning and DNA Analysis by T. A. Brown, Sixth Edition.
7. Genetics . Verma and Agarwal.

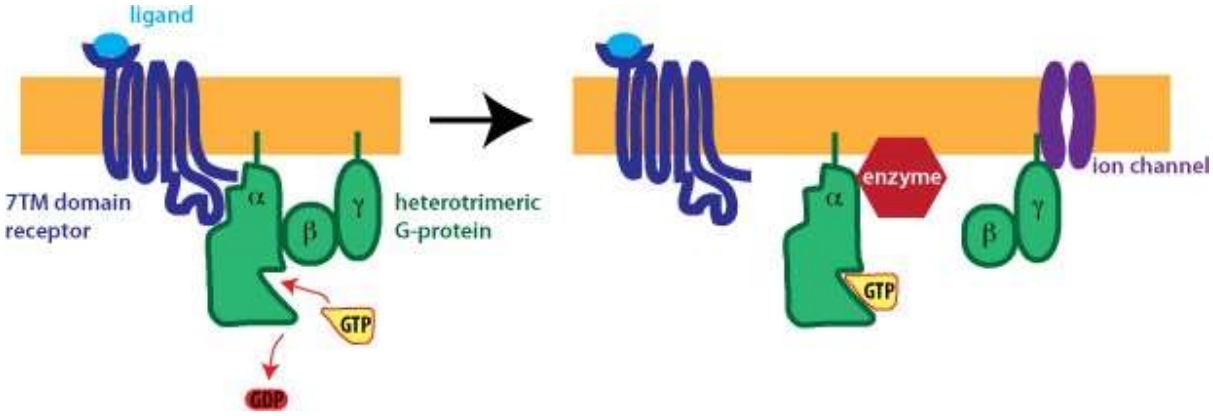
Unit-VIII

G-protein coupled receptors, tyrosine kinases, cytokine receptors; pathways of intercellular signal transduction, camp, C GMP pathways; Phospholipids and Ca ion, Ras, Raf and MAP kinase pathway, JAK/STAT pathway

Objective: In this unit you will know about G-protein coupled receptors, tyrosine kinases, cytokine receptors; pathways of intercellular signal transduction, camp, C GMP pathways; Phospholipids and Ca ion, Ras, Raf and MAP kinase pathway, JAK/STAT pathway.

G-protein coupled receptors:

The largest family of cell surface receptors are the G-protein coupled receptors (GPCRs). There are hundreds of different GPCR proteins, and nearly a third of all drugs target this type of receptor. A diverse set of ligands bind to this type of receptor, including peptide hormones, neurotransmitters, and odor molecules. These receptors all have a similar structure with seven transmembrane domains. On the basis of their seven transmembrane domain structure, many GPCRs have been identified in the human genome. Proteins that were identified by sequence homology, but whose ligands are not known, are termed orphan receptors.



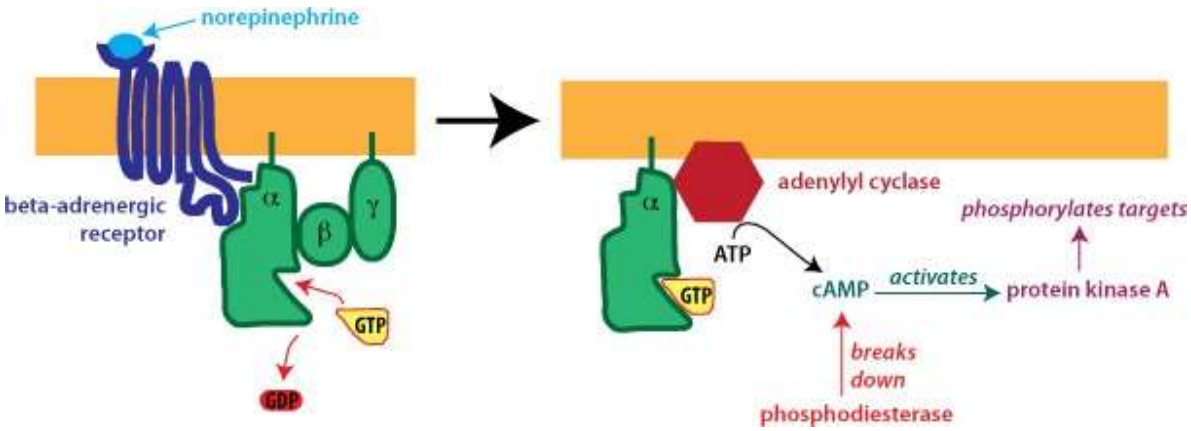
GPCRs associate with heterotrimeric G-proteins (green), that is, G-proteins composed of three different subunits: alpha, beta, and gamma. The subunits are tethered at the membrane surface by covalently attached lipid molecules.

When a ligand binds, the receptor activates the attached G-protein by causing the exchange of GTP (yellow) for GDP (red). The activated G-protein then dissociates into an alpha (G-alpha) and a beta-gamma complex. G-alpha bound to GTP is active, and can diffuse along the membrane surface to activate (and sometimes inhibit) target proteins, often enzymes that generate second messengers. Likewise, the beta-gamma complex is also able to diffuse along the inner membrane surface and affect protein activity. Inactivation occurs because G-alpha has intrinsic GTPase activity. After GTP hydrolysis, G-alpha bound to GDP will reassociate with a beta-gamma complex to form an inactive G-protein that can again associate with a receptor.

The GTPase activity of the G-alpha can be made faster by other proteins--sometimes the target protein, sometimes a separate regulatory protein. Cholera toxin causes a chemical modification that *prevents* GTP hydrolysis and leads to unregulated signaling.

Different G-alpha proteins activate different second messenger pathways. There are several different classes of heterotrimeric G-proteins that are defined by their different G-alpha subunits. One type of G-alpha activates the enzyme adenylyl cyclase, which catalyzes the formation of the second messenger cyclic AMP (cAMP). Because an activated adenylyl cyclase can generate many molecules of cAMP, this is a means to amplify the signal. cAMP can have several effects, but a major effect is to bind to and activate protein kinase A (PKA; also known as cAMP- dependent kinase). PKA then phosphorylates target proteins in the cell. cAMP is rapidly broken down by phosphodiesterases, limiting the length of the signal.

A specific example of a receptor that couples to this type of G-protein is the beta-1 adrenergic receptor found in the heart. Beta 1 receptors are the principal type of adrenergic receptor found in the heart. The ligand for this receptor is norepinephrine, the neurotransmitter that is released by sympathetic postganglionic neurons. (As well, the hormone epinephrine, released from the adrenal medulla, is also a ligand for these receptors.) Stimulation of beta-1 receptors causes increased cAMP and PKA activation. PKA phosphorylates various target proteins in cardiac cells to cause an increase in both the heart rate and the strength of cardiac muscle contraction. Beta-1 receptors are the targets of drugs (beta blockers) that are used to treat heart failure and hypertension.



Another example involving GPCR signaling that stimulates adenylyl cyclase is the regulation of secretion in the small intestine. This regulation is disrupted by cholera toxin. The effect of cholera toxin is to lead to persistent activation of adenylyl cyclase because it destroys the GTPase activity of G-alpha. There is over-production of cAMP, continuous activation of PKA, and continuous phosphorylation of CFTR, causing excessive fluid secretion.

A different type of G-alpha activates the enzyme phospholipase C. This type of G-alpha couples to various GPCRs found on smooth muscle, such as the oxytocin receptor shown in the example below

Phospholipase C is an enzyme that cuts PIP2, a membrane phospholipid, to generate two second messengers, IP_3 and diacylglycerol (DAG). IP_3 is water soluble, diffusing through the cytosol to bind to and open a ligand-gated Ca^{++} channel in the endoplasmic reticulum (or sarcoplasmic reticulum in muscle cells). Thus, stimulation of a receptor linked to this G-alpha is a way to

increase Ca^{++} inside the cytosol. Ca^{++} in the cytosol exerts its effects by binding to Ca^{++}-binding proteins such as calmodulin. In the uterus, the increase in intracellular Ca^{++} that results from oxytocin signaling causes the smooth muscle to contract. DAG is lipid soluble and stays in the membrane. It activates protein kinase C (PKC), which, like PKA phosphorylates particular target proteins.

Desensitization

In the continuing presence of ligand, many GPCRs show desensitization. The mechanism is shown in the figure. A protein known as a G-protein Receptor Kinase (GRK) phosphorylates the receptor on particular residues. This increases its affinity for a protein called beta-arrestin (red), that binds to the receptor. This reduces signaling by *preventing* the association with the G-protein.

There are several potential outcomes once beta-arrestin binds to the receptor. One possibility is that beta-arrestin targets the receptor for endocytosis, leading to receptor downregulation (a decreased number of receptors on the cell surface). Another possibility is the activation of beta-arrestin-dependent signaling pathways that are independent of G-protein signaling. Beta-arrestin can act as a scaffold that binds and brings together other intracellular signaling proteins. The physiological significance of beta-arrestin-dependent signaling is still being worked out.

Steroid Hormone superfamily mediated signal transduction:

As already noted, all signaling molecules act by binding to receptors expressed by their target cells. In many cases, these receptors are expressed on the target cell surface, but some receptors are intracellular proteins located in the cytosol or the nucleus. These intracellular receptors respond to small hydrophobicsignalling molecules that are able to diffuse across the plasma membrane. The steroid hormones are the classic examples of this group of signaling molecules, which also includes thyroid hormone, vitamin D_3, and retinoic acid.

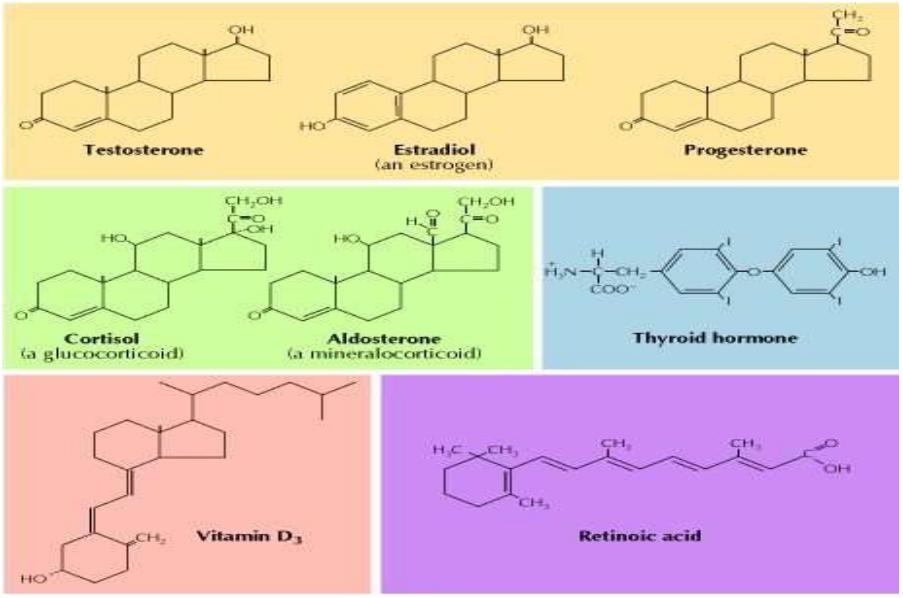


Figure : Structure of steroid hormones, thyroid hormone, vitamin D $_3$, and retinoic acid

The steroid hormones (including testosterone, estrogen, progesterone, the corticosteroids, and ecdysone) are all synthesized from cholesterol. Testosterone, estrogen, and progesterone are the sex steroids, which are produced by the gonads. The corticosteroids are produced by the adrenal gland. They include the glucocorticoids, which act on a variety of cells to stimulate production of glucose, and the mineralocorticoids, which act on the kidney to regulate salt and water balance. Ecdysone is an insect hormone that plays a key role in development by triggering the metamorphosis of larvae to adults.

Although thyroid hormone, vitamin D_3, and retinoic acid are both structurally and functionally distinct from the steroids, they share a common mechanism of action in their target cells. Thyroid hormone is synthesized from tyrosine in the thyroid gland; it plays important roles in development and regulation of metabolism. Vitamin D_3 regulates Ca^{2+} metabolism and bone growth. Retinoic acid and related compounds (retinoids) synthesized from vitamin A play important roles in vertebrate development.

Because of their hydrophobic character, the steroid hormones, thyroid hormone, vitamin D_3, and retinoic acid are able to enter cells by diffusing across the plasma membrane . Once inside the cell, they bind to intracellular receptors that are expressed by the hormonally responsive target cells. These receptors, which are members of a family of proteins known as the steroid receptor superfamily, are transcription factors that contain related domains for ligand binding, DNA binding, and transcriptional activation. Ligand binding regulates their function as activators or repressors of their target genes, so the steroid hormones and related molecules directly regulate gene expression.

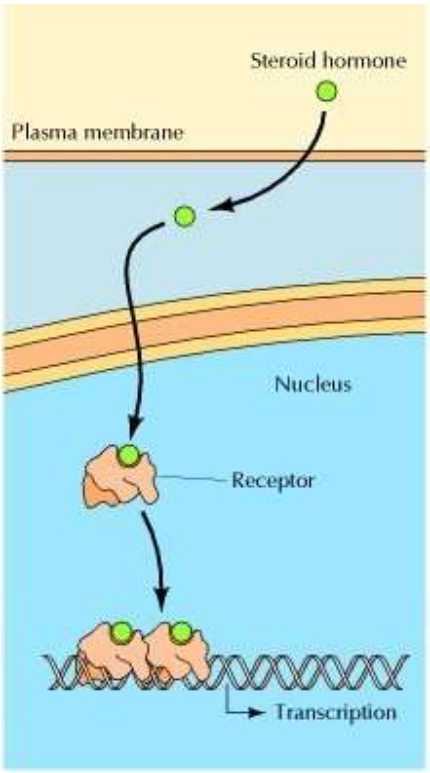


Figure: Action of steroid hormones. The steroid hormones diffuse across the plasma membrane and bind to nuclear receptors, which directly stimulate transcription of their target genes. The steroid hormone receptors bind DNA as dimers.

Ligand binding has distinct effects on different receptors. Some members of the steroid receptor superfamily, such as the estrogen and glucocorticoid receptors, are unable to bind to DNA in the absence of hormone. The binding of hormone induces a conformational change in the receptor, allowing it to bind to regulatory DNA sequences and activate transcription of target genes. In other cases, the receptor binds DNA in either the presence or absence of hormone, but hormone binding alters the activity of the receptor as a transcriptional regulatory molecule. For example, thyroid hormone receptor acts as a repressor in the absence of hormone, but hormone binding converts it to an activator that stimulates transcription of thyroid hormone-inducible genes

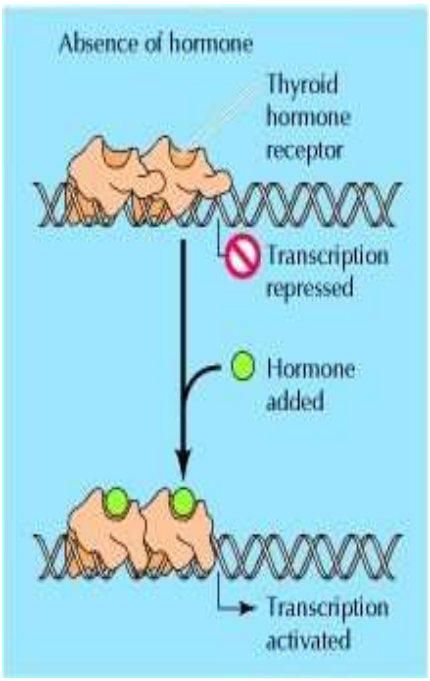


Figure: Gene regulation by the thyroid hormone receptor. Thyroid hormone receptor binds DNA in either the presence or absence of hormone. However, hormone binding changes the function of the receptor from a repressor to an activator of target gene transcription

Neurotransmitter Receptors in Cell Signaling Transduction

Chemical transmission is the major means by which nerves communicate with one another in the nervous system. Many different types of neurotransmitters play an important role in the process of chemical transdmittion. The neurotransmitters achieve cell signaling transduction through neurotransmitter receptors on the postsynaptic membrane. The neurotransmitter receptor perform large specificity and potency. Many receptors have been isolated and purified biochemically, and many have also been cloned and sequenced. Neurotransmitter receptors can also be grouped according to the type of primary effector to which they couple. This classification leads to four major categories of receptors.

Four groups of Neurotransmitter Receptor in cell signaling transduction

Group I: Ligand-gated ion channels as neurotransmitter receptors

These ligand-gated ion channels include nAChR, GluN1, GluN2A-D, GluN3A,B, GluA1-4, GluK1-3, 4-5, GABAA, GlyR, IP3-R1, IP3-R2, IP3-R3, 5HT3, P2X1-7 and Nicotinic cholinergic (muscle [αβγδε] and neuronal [α or αβ] subtypes).

Receptors in this category include those that are activated by synaptically released neurotransmitter and occur on the cell surface (mostly, the intracellular ligand-gated receptor for IP3 is present in the smooth endoplasmic reticulum). Upon the binding of an agonist to these ligand-gated ion channels, the receptors undergo a conformational change that facilitates opening of the intrinsic ion channel (some ligand gated ion channel receptors (e.g., NMDA and GABAA) are also found at extrasynaptic locations).

The permeability to specific ions is a characteristic of the receptor; for example, both the neuronal nicotinic cholinergic receptors (nAChR) and N-methyl D-aspartate (NMDA) receptors are selectively permeable to Na+ and Ca2+ ions, whereas GABAA and glycine

receptors are primarily permeable to Cl- ions. As a result of the changes in ion conductance, the membrane potential may become either depolarized, as occurs for nAChRs or NMDA receptors, or hyperpolarized, as observed for GABAA or glycine receptors.

Group II: Receptors with intrinsic guanylyl cyclase activity as neurotransmitter receptors

The representative receptor with intrinsic guanylyl cyclase activity is GC-B. Receptors in the this group possess intrinsic guanylyl cyclase activity and generate cyclic GMP (cGMP) upon activation of a receptor. These receptors consist of an extracellular binding domain, a single transmembrane-spanning domain (TMD), a protein kinase–like domain and a guanylyl cyclase catalytic domain. Ligand binding results in a conformational change in the receptor and activation of the guanylyl cyclase catalytic region. Receptors with intrinsic guanylyl cyclase activity are often very highly phosphorylated in the absence of agonist and rapidly undergo dephosphorylation upon activation.

Group III: Receptors with intrinsic or associated tyrosine kinase activity as neurotransmitter receptors:

Receptors with intrinsic or associated tyrosine kinase activity include TrkB, EGFR, FGFR1-FGFR4, IGFR-1, Trk A, ErbB2, ErbB3, ErbB4, Trk C, PDGFR α and β, gp130 + CNTFRα and LIFRβ, 2 x gp130 + IL6Rα and gp130 + LIFRβ. Receptors in the third group possess intrinsic receptor tyrosine kinase (RTK) activity themselves or are closely associated with cytoplasmic tyrosine kinases (RATK). Structurally, RTKs possess an extracellular ligand binding domain, a single TMD and an intracellular catalytic kinase domain.

Three distinct events underlie signal transduction at RTKs: (1) Initially, upon ligand binding to an RTK, the receptor undergoes a dimerization that results in the juxtaposition of the two cytoplasmic domains. (2) Contact between these domains is thought to result in a stimulation of catalytic activity, (3) which in turn results in an intermolecular autophosphorylation of tyrosine residues both within and outside of the kinase domain. Once auto phosphorylated, RTKs can recruit a number of cytoplasmic proteins and initiate a series of reactions involving protein–protein interactions.

RATKs, such as those for the neurotrophic cytokines (leukemia inhibitory factor, interleukin-6 or ciliary neurotrophic factor) do not possess intrinsic tyrosine kinase activity themselves, but upon activation, they undergo dimerization and are then able to recruit cytoplasmic tyrosine kinases (such as Janus kinase). The latter then phosphorylate the RATK on tyrosine residues (in addition to being tyrosine phosphorylated themselves) and facilitate protein–protein interactions, as observed for RTKs.

Group IV: G-protein-coupled receptors as neurotransmitter receptors

G-protein-coupled receptors within neurotransmitter receptors include Acetylcholine receptors, Adenosine receptors, ATP receptors, Dopamine receptors…… This group of receptors involves G proteins. Numerically, more diverse types of receptors have been demonstrated to operate via an intervening G protein than by any other mechanism. These G protein–coupled receptors(GPCRs) have a characteristic seven TMD structure. G-protein-coupled neurotransmitter receptors can be further divided into four functional categories: (1) Some GPCRs, such as GABAB, α2-adrenergic, D2-dopaminergic or M2 muscarinic (mAChR), regulate the changes in K+ conductance independently of second-messenger production. (2) A second group of GPCRs is linked to the modulation of adenylyl cyclase activity. This regulation may be either positive, as in the case of activation of the β2-adrenergic receptor, or negative, as occurs following activation of the α2-adrenergic receptor. Changes in the concentrations of cAMP regulate the activity of protein kinase A (PKA). (3) A third group of GPCRs is linked to the activation of phosphoinositide-specific phospholipase C (PLC) with the attendant breakdown of PIP2 and formation of IP3 and DAG. These receptors are linked to changes in Ca2+ homeostasis and protein phosphorylation via the action of protein kinase C (PKC). Other effector enzymes that may be regulated by IP3-linked GPCRs include phospholipases A2 and D. (3) A fourth, and unique, mechanism for the activation of a GPCR is that utilized by the visual pigment rhodopsin, which structurally is a prototypical GPCR. However, in this case it is light, rather than a chemical stimulus, that triggers the activation of rhodopsin. Photoactivated rhodopsin activates transducin, a G-protein, which is coupled to cGMP phosphodiesterase with a concomitant increased rate in the hydrolysis of cGMP to GMP.

Ras / Raf and MAP Kinase Pathway :

The gene family ras encodes small GTPases that are involved in cellular signal transduction. Ras the super-family of proteins regulates diverse cell behaviors such as cell growth, differentiation and survival. Since Ras communicates signals from outside the cell to the nucleus, mutations in ras genes can permanently activate it and cause inappropriate transmission inside the cell even in the absence of extracellular signals. Because these signals result in cell growth and division, disregulated Ras signaling can ultimately lead to oncogenesis and cancer.

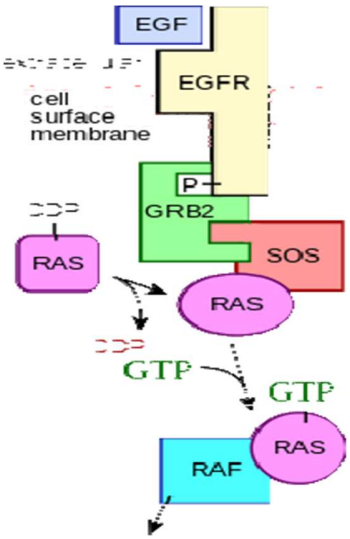
Ras proteins function as binary molecular switches that control intracellular signaling networks. Ras-regulated signal pathways control processes such as actin- cytoskeletal integrity, proliferation, differentiation, cell adhesion, apoptosis, and cell migration. Ras and Ras-related proteins are often deregulated in cancers, leading to increased invasion and metastasis, and decreased apoptosis. Activated Ras activates the protein kinase activity of RAF kinase. RAF kinase phosphorylates and activates MEK. MEK phosphorylates and activates a mitogen-activated protein kinase (MAPK).

Mitogen-activated protein (MAP) kinases are serine/threonine-specific protein kinases that respond to extracellular stimuli (mitogens, osmotic stress, heat shock and pro-inflammatory cytokines) and regulate various cellular activities, such as gene expression, mitosis,

differentiation, proliferation, and cell survival/apoptosis. MAPK pathways are activated within the protein kinase cascades called “MAPK cascade”. Each one consists of three enzymes, MAP kinase, MAP kinase kinase (MKK, MEKK, or MAP2K) and MAP kinase kinase kinase (MKKK or MAP3K) that are activated in series. A MAP3K that is activated by extracellular stimuli, which phosphorylates a MAP2K on its serine and threonine residues and this MAP2K activates a MAP kinase through phosphorylation on its serine and tyrosine residues.

The phosphorylation of tyrosine precedes to the phosphorylation of threonine, although phosphorylation of either residue can occur in the absence of the other. Because both tyrosine and threonine phosphorylations are required to activate the MAP kinases, phosphatases that remove phosphate from either sites will inactivate them. This MAP kinase signaling cascade has been evolutionary well-conserved from yeast to mammals. Cascades convey information to effectors, coordinates incoming information from other signaling pathways, amplify signals, and allow for a variety of response patterns.

Down-regulation of MAP kinase pathways may occur through dephosphorylation by serine/threonine phosphatases, tyrosine phosphatases, or dual-specificity phosphatases and through feedback inhibitory mechanisms that involve the phosphorylation of upstream kinases. Drugs that selectively down-regulate MAP kinase cascades could prove to be valuable as therapeutic agents in the control of malignant disease.



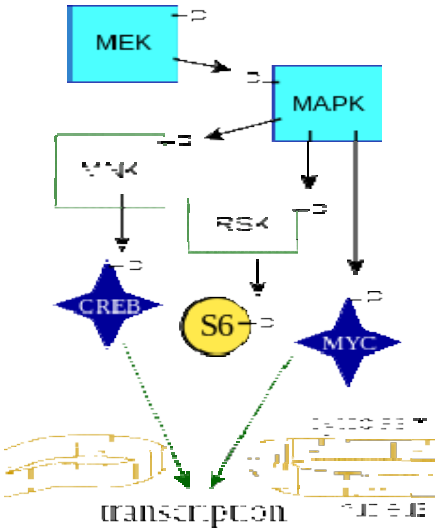


Figure: Key components of the MAPK/ERK pathway. "P" represents phosphate, which communicates the signal. Top, epidermal growth factor (EGF) binds to the EGF receptor(EGFR) in the cell membrane, starting the cascade of signals. Further downstream, phosphate signal activates MAPK (also known as ERK). Bottom, signal enters the cell nucleus and causes transcription of DNA, which is then expressed as protein.

JAK-STAT signaling pathway

The JAK-STAT signaling pathway is a chain of interactions between proteins in a cell, and is involved in processes such as immunity, cell division, cell death and tumour formation. The pathway communicates information from chemical signals outside of a cell to the cell nucleus, resulting in the activation of genes through a process called transcription. There are three key parts of JAK-STAT signaling: Janus kinases (JAKs), Signal Transducer and Activator of Transcription proteins (STATs), and receptors (which bind the chemical signals).[1] Disrupted JAK-STAT signaling may lead to a variety of diseases, such as skin conditions, cancers, and disorders affecting the immune system.

Here are 4 JAK proteins: JAK1, JAK2, JAK3 and TYK2. JAKs contains a FERM domain

(approximately 400 residues), an SH2-related domain (approximately 100 residues), a kinase domain (approximately 250 residues) and a pseudokinase domain (approximately 300 residues). The kinase domain is vital for JAK activity, since it allows JAKs to phosphorylate (add phosphate molecules to) proteins.

There are7STATproteins:STAT1,STAT2,STAT3,STAT4,STAT5A,STAT5BandSTAT6.

STAT proteins contain many different domains, each with a different function, of which the most conserved region is the SH2 domain. The SH2 domain is formed of 2 α-helices and a β-sheet and is formed approximately from residues 575-680.STATs also have transcriptional activation

domains (TAD), which are less conserved and are located at the C-terminus. In addition, STATs also contain: tyrosine activation, amino-terminal, linker, coiled-coil and DNA-binding domains.

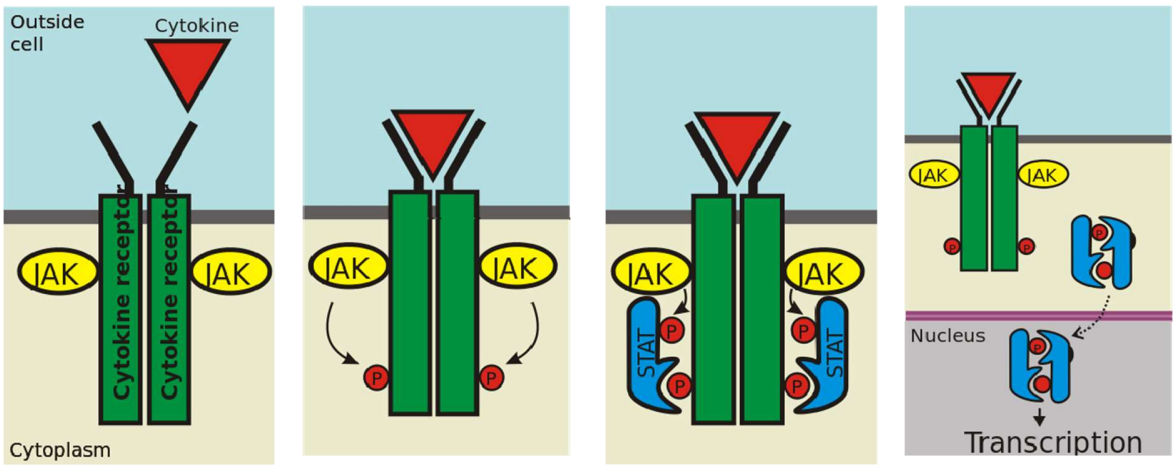


Fig: Key steps of the JAK-STAT pathway. JAK-STAT signalling is made of three major proteins: cell-surface receptors, Janus kinases (JAKs), and signal transducer and activator of transcription proteins (STATs). Once a ligand (red triangle) binds to the receptor, JAKs add phosphates (red circles) to the receptor. Two STAT proteins then bind to the phosphates, and then the STATs are phosphorylated by JAKs to form a dimer. The dimer enters the nucleus, binds to DNA, and causes transcription of target genes.

Probable Questions:

1. Describe the basic components of G-protein coupled receptor.
2. What is desensitization?
3. How signal is transmitted by steroid hormones.
4. Describe the role of neurotransmitter receptors in signal transduction.
5. How signal is transmitted thorough Ligand-gated ion channels ?
6. Describe Ras / Raf and MAP Kinase Pathway with suitable diagrams.
7. Describe JAK-STAT signaling pathway with suitable diagram.

Suggested readings:

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7th Edition.
6. Gene cloning and DNA Analysis by T. A. Brown, Sixth Edition.
7. Genetics . Verma and Agarwal.

Disclaimer:

The study materials of this book have been collected from various books, e-books, journals and other e-sources.

Post-Graduate Degree Programme (CBCS)

in

ZOOLOGY

SEMESTER-III

ELECTIVE THEORY II
CELL AND DEVELOPMENTAL BIOLOGY

ZET-302

SELF LEARNING MATERIAL

DIRECTORATE OFOPEN AND DISTANCE LEARNING
UNIVERSITY OF KALYANI

Kalyani, Nadia
West Bengal, India

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Director's Message

Satisfying the varied needs of distance learners, overcoming the obstacle of distance and reaching the unreached students are the threefold functions catered by Open and Distance Learning (ODL) systems. The onus lies on writers, editors, production professionals and other personnel involved in the process to overcome the challenges inherent to curriculum design and production of relevant Self Learning Materials (SLMs). At the University of Kalyani a dedicated team under the able guidance of the Hon'ble Vice-Chancellor has invested its best efforts, professionally and in keeping with the demands of Post Graduate CBCS Programmes in Distance Mode to devise a self-sufficient curriculum for each course offered by the Directorate of Open and Distance Learning (DODL), University of Kalyani.

Development of printed SLMs for students admitted to the DODL within a limited time to cater to the academic requirements of the Course as per standards set by Distance Education Bureau of the University Grants Commission, New Delhi, India under Open and Distance Mode UGC Regulations, 2017 had been our endeavour. We are happy to have achieved our goal.

Utmost care and precision have been ensured in the development of the SLMs, making them useful to the learners, besides avoiding errors as far as practicable. Further suggestions from the stakeholders in this would be welcome.

During the production-process of the SLMs, the team continuously received positive stimulations and feedback from Professor (Dr.) Sankar Kumar Ghosh, Hon'ble Vice- Chancellor, University of Kalyani, who kindly accorded directions, encouragements and suggestions, offered constructive criticism to develop it within proper requirements. We gracefully, acknowledge his inspiration and guidance.

Sincere gratitude is due to the respective chairpersons as well as each and every member of PGBOS (DODL), University of Kalyani. Heartfelt thanks is also due to the Course Writers-faculty members at the DODL, subject-experts serving at University Post Graduate departments and also to the authors and academicians whose academic contributions have enriched the SLMs. We humbly acknowledge their valuable academic contributions. I would especially like to convey gratitude to all other University dignitaries and personnel involved either at the conceptual or operational level of the DODL of University of Kalyani.

Their persistent and co-ordinated efforts have resulted in the compilation of comprehensive, learner-friendly, flexible texts that meet the curriculum requirements of the Post Graduate Programme through Distance Mode.

All the Self Learning Materials are self writing and collected from e-book, journals and websites.

Prof Manas Mohan Adhikary
Director
Directorate of Open and Distance Learning
University of Kalyani

ELECTIVE THEORY II

CELL AND DEVELOPMENTAL BIOLOGY (ZET 302)

Module	Unit	Content	Credit	Class	Time (h)	Page No.
ZET - 301 (Cell and Developmental Biology)	I	Morphogenesis: Meaning of morphogenesis, morphogenetic processes, cell shape, Cell death, morphogenetic movements, cell sorting, morphogenetic field, regionalization.	2	1	1	
	II	Teratogenesis: Genetic teratology, Environmental teratology, Developmental mechanism, Contribution of teratology to Developmental Biology.		1	1	
	III	Ageing: Cellular basis of aging, Causes of aging, Free Radical Theory of Aging, Ageing of connective tissue		1	1	
	IV	Differentiation: i) Processes, determination, induction, competence, mechanism of differentiation		1	1	
	V	Reversibility of differentiated state, criteria for dedifferentiation, metaplasia and transdifferentiation, modulation		1	1	
	VI	Neural crest cell migration based differentiation		1	1	
	VII	Cartilage: a. Structure, differentiation		1	1	
	VIII	Experimental induction of cartilage and proteoglycan synthesis		1	1	

Unit- I

Morphogenesis: Meaning of morphogenesis, morphogenetic processes, cell shape, Cell death, morphogenetic movements, cell sorting, morphogenetic field, regionalization

Objective: In this unit, you will learn about Morphogenesis: Meaning of morphogenesis, morphogenetic processes, cell shape, Cell death, morphogenetic movements, cell sorting, morphogenetic field, regionalization.

Developmental Ramifications

Morphogenesis

Morphogenesis is the biological process that causes an organism to develop its shape. It is one of three fundamental aspects of developmental biology along with the control of cell growth and cellular differentiation, unified in evolutionary developmental biology (evo-devo).
The process controls the organized spatial distribution of cells during the embryonic development of an organism. Morphogenesis can take place also in a mature organism, in cell culture or inside tumor cell masses. Morphogenesis also describes the development of unicellular life forms that do not have an embryonic stage in their life cycle, or describes the evolution of a body structure within a taxonomic group.

Morphogenetic responses may be induced in organisms by hormones, by environmental chemicals ranging from substances produced by other organisms to toxic chemicals or radionuclides released as pollutants, and other plants, or by mechanical stresses induced by spatial patterning of the cells.
Several types of molecules are important in morphogenesis. Morphogens are soluble molecules that can diffuse and carry signals that control cell differentiation via concentration gradients. Morphogens typically act through binding to specific protein receptors. An important class of molecules involved in morphogenesis is transcription factor proteins that determine the fate of cells by interacting with DNA. These can be coded for by master regulatory genes, and either activate or deactivate the transcription of other genes; in turn, these secondary gene products can regulate the expression of still other genes in a regulatory cascade of gene regulatory networks. At the end of this cascade are classes of molecules that control cellular behaviors such as cell migration, or, more generally, their properties, such as cell adhesion or cell contractility. For example, during gastrulation, clumps of stem cells switch off their cell-to-cell adhesion, become migratory, and take up new positions within an embryo where they again activate specific cell adhesion proteins and form new tissues and organs. Developmental signaling pathways implicated in morphogenesis include Wnt, Hedgehog, and ephrins

Cellular basis of morphogenesis:
At a tissue level, ignoring the means of control, morphogenesis arises because of cellular proliferation and motility. Morphogenesis also involves changes in the cellular structure or how cells interact in tissues. These changes can result in tissue elongation, thinning, folding, invasion or separation of one tissue into distinct layers. The latter case is often referred ascell sorting. Cell "sorting out" consists of cells moving so as to sort into clusters that maximize contact between cells of the same type. The ability of cells to do this has been proposed to arise from differential cell adhesion by Malcolm Steinberg through his Differential Adhesion Hypothesis. Tissue separation can also occur via more dramatic cellular

differentiation events during which epithelial cells become mesenchymal. Mesenchymal cells typically leave the epithelial tissue as a consequence of changes in cell adhesive and contractile properties. Following epithelial-mesenchymal transition, cells can migrate away from an epithelium and then associate with other similar cells in a new location.

Cell-cell adhesion

During embryonic development, cells are restricted to different layers due to differential affinities. One of the ways this can occur is when cells share the same cell-to-cell adhesion molecules. For instance, homotypic cell adhesion can maintain boundaries between groups of cells that have different adhesion molecules. Furthermore, cells can sort based upon differences in adhesion between the cells, so even two populations of cells with different levels of the same adhesion molecule can sort out. In cell culture cells that have the strongest adhesion move to the center of mixed aggregates of cells. Moreover, cell-cell adhesion is often modulated by cell contractility, which can exert forces on the cell-cell contacts so that two cell populations with equal levels of the same adhesion molecule can sort out. The molecules responsible for adhesion are called cell adhesion molecules (CAMs). Several types of cell adhesion molecules are known and one major class of these molecules are cadherins. There are dozens of different cadherins that are expressed on different cell types. Cadherins bind to other cadherins in a like-to-like manner: E-cadherin (found on many epithelial cells) binds preferentially to other E-cadherin molecules. Mesenchymal cells usually express other cadherin types such as N-cadherin.

Extracellular matrix

The extracellular matrix (ECM) is involved in keeping tissues separated, providing structural support or providing a structure for cells to migrate on. Collagen, laminin, and fibronectin are major ECM molecules that are secreted and assembled into sheets, fibers, and gels. Multisubunit transmembrane receptors called integrins are used to bind to the ECM. Integrins bind extra cellularly to fibronectin, laminin, or other ECM components, and intracellularly to microfilament-binding proteins α-actinin and talin to link the cytoskeleton with the outside. Integrins also serve as receptors to trigger signal transductioncascades when binding to the ECM. A well-studied example of morphogenesis that involves ECM is mammary gland ductal branching.

Cell contractility

Tissues can change their shape and separate into distinct layers via cell contractility. Just as in muscle cells, myosin can contract different parts of the cytoplasm to change its shape or structure. Myosin-driven contractility in embryonic tissue morphogenesis is seen during the separation of germ layers in the model organisms *Caenorhabditis elegans, Drosophilaand zebrafish*. There are often periodic pulses of contraction in embryonic morphogenesis. A model called the cell state splitter involves alternating cell contraction and expansion, initiated by a bistable organelle at the apical end of each cell. The organelle consists of microtubules and microfilaments in mechanical opposition. It responds to local mechanical perturbations caused by morphogenetic movements. These then trigger traveling embryonic differentiation waves of contraction or expansion over presumptive tissues that determine cell type and is followed by cell differentiation. The cell state splitter was first proposed to explain neural plate morphogenesis during gastrulation of the axolotl and the model was later generalized to all of morphogenesis.

Morphogenetic movement

In addition to the mechanism by which the complicated bodies of animals are formed via cleavage of the fertilized egg and repeated cell specification, discussed earlier, another important mechanism in animal development is morphogenetic movement. Morphogenetic movement is caused by large-scale

and dynamic movement of embryoniccells. It rearranges the distribution of embryonic cells, thereby allo wing the interaction between germ layers that previously existed separately. The first morphogenetic movement triggered is gastrulation, which forms the future digestive tract. Gastrulation is one of the most important morphogenetic movements in the formation of the basic tubularstructure of animals. Morphogenetic movement consists of several basic cellular deforma tions and movements, including the invagination movement, in which contraction of one side of the embryo causes bending and encourages epithelial cells to extend inward into the embryo, the extension movement caused by the rearrangement of epithelial cells, and the ingression movement of epithelial cells migrating into the embryo (Figure 19-10).

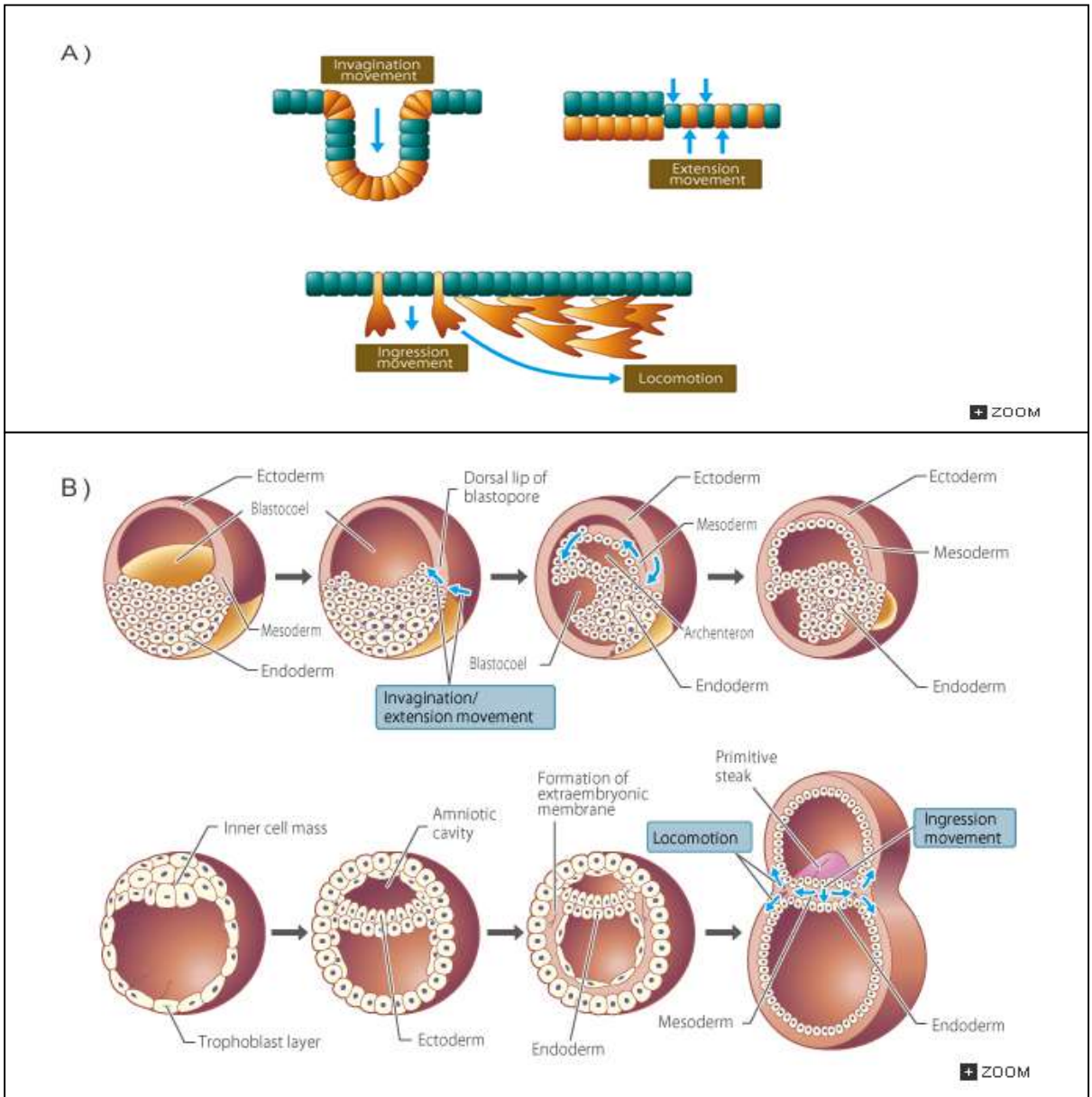


Figure 19-10 Morphogenetic movement: (A) Morphogenetic movement in the early embryo takes place mainly through the movement and deformation of the epithelial cells,which constitute the embryo, and includes: a) invagination movement of epithelium, b) extensio n movement caused byrearrangement of epithelialcells, andc) locomotive movement (migration) of e pithelialtissues. (B) Examples of morphogeneticmovement in amphibians and mammalian embryos. The cells move in the directions of the red arrows shown in the diagram. a) Gastrulation ofthe frog embryo: the mesoderm and endoderm move intothe interior ofthe embryo thr ough invagination andextension movements and form an inner lining ofthe ectoderm. b) Gastrulation ofthe mammalianembryo: cells separate fromthecentral part ofthe ectoderm and move intothe interior

Morphogenetic field:
In the developmental biology of the early twentieth century, a morphogenetic fieldis a group of cells able to respond to discrete, localized biochemical signals leading to the development of specific morphological structures or organs. The spatialand temporal extents of the embryonic field are dynamic, and within the field is a collection of interacting cells out of which a particular organ is formed. As a group, the cells within a given morphogenetic field are constrained: thus, cells in a limb fieldwill become a limb tissue, those in a cardiac field will become heart tissue. However, specific cellular programming of individual cells in a field is flexible: an individual cell in a cardiac field can be redirected via cell-to-cell signaling to replace specific damaged or missing cells. Imaginal discs in insect larvae are examples of morphogenetic fields.

Cell sorting
Cell sorting is a method used to separate cells isolated from an organism's tissues according to their type. Cells are mostly commonly separated relying on differences in cell size, shape (morphology), and surface protein expression. The resulting homogenous populations of cells have important applications in research and as therapeutics.

Methods
Currently there are several methods for cell sorting. Some are primitive and do not require special equipment whereas others rely on sophisticated electronic appliances. Three major types of cell sorting are fluorescent activated cell sorting, magnetic cell selection and single cell sorting.

Single cell sorting
Single cell sorting provides a method for sorting a heterogeneous mixture of cells based upon intracellular and extracellular properties. There are several methods for sorting single cells:

- The IsoRaft array provides a rapid, cost-effective method for isolating cells, analyzing cells over time, and generating clonal populations with the unique ability to monitor all intra- and extracellular properties. This system is ideal for both adherent and non-adherent cell types.

- The DEPArray lab-on-a-chip technology platform is designed to individually identify, manipulate and sort specific cells within a heterogeneous population based on intra- and extracellular properties, not including morphology. The DEPArray cell-sorting and isolation technology, followed by NGS analysis, can reveal comprehensive genomic information from any FFPE sample, regardless of sample cellularity and size of the specimen. Moreover, the methodology informs a new model for conducting clinical biopsies of tumors, as well as for performing translational cancer research and the way new cancer drugs are developed and biomarkers discovered.

Fluorescent activated cell sorting
Fluorescent Activated Cell Sorting, or FACS, utilizes Flow cytometry to provide a fast, objective and quantitative measurement of intra- and extracellular properties, not including morphology, for sorting a heterogeneous mixture of cells.

Magnetic cell sorting
Magnetic cell sorting provides a method for enriching a heterogeneous mixture of cells based upon extracellular properties, typically cell-surface proteins (antigens). There are several types of magnetic cell sorting:
- Magnetic-activated cell sorting (MACS) is a column based separation technique where labeled cells are passed through a magnetic column.

- SEP system provides a column-free cell separation technique in which a tube of labeled cells is placed inside a magnetic field. Positively selected cells are retained in the tube while negatively selected cells are in the liquid suspension.

Buoyancy activated cell sorting
Buoyancy activated cell sorting (BACS), developed by Akadeum Life Sciences, is a separation technique in which microbubbles bind to cells through antibodies binding to the surface of cells. The targeted cells are then removed from a biological sample through flotation.

Cell death
Cell death is the event of a biological cell ceasing to carry out its functions. This may be the result of the natural process of old cells dying and being replaced by new ones, or may result from such factors as disease, localized injury, or the death of the organism of which the cells are part. Kinds of cell death include the following:

Programmed cell death (or PCD) is cell death mediated by an intracellular program. PCD is carried out in a regulated process, which usually confers advantage during an organism's life-cycle. For example, the differentiation of fingers and toes in a developing human embryo occurs because cells between the fingers apoptose; the result is that the digits are separate. PCD serves fundamental functions during both plant and metazoa (multicellular animals) tissue development.

Apoptosis or Type I cell-death, and autophagy or Type II cell-death are both forms of programmed cell death, while necrosis is a non-physiological process that occurs as a result of infection or injury. Necrosis is cell death caused by external factors such as trauma or infection, and occurs in several different forms. Recently a form of programmed necrosis, called necroptosis, has been recognized as an alternate form of programmed cell death. It is hypothesized that necroptosis can serve as a cell-death backup to apoptosis when the apoptosis signaling is blocked by endogenous or exogenous factors such as viruses or mutations.

Mitotic catastrophe is a mode of cell death that is due to premature or inappropriate entry of cells into mitosis. It is the most common mode of cell death in cancer cells exposed to ionizing radiation and many other anti-cancer treatments. Autophagy is cytoplasmic, characterized by the formation of large vacuoles that eat away organelles in a specific sequence prior to the destruction of the nucleus.

Apoptosis is the process of programmed cell death (PCD) that may occur in multicellular organisms. Biochemical events lead to characteristic cell changes (morphology) and death. These changes include blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation. It is now thought that – in a developmental context – cells are induced to positively commit suicide whilst in a homeostatic context; the absence of certain survival factors may provide the impetus for suicide. There appears to be some variation in the morphology and indeed the biochemistry of these suicide pathways; some treading the path of "apoptosis", others following a more generalized pathway to deletion, but both usually being genetically and synthetically motivated. There is some evidence that certain symptoms of "apoptosis" such as endonuclease activation can be spuriously induced without engaging a genetic cascade, however, presumably true apoptosis and programmed cell death must be genetically mediated. It is also becoming clear that mitosis and apoptosis are toggled or linked in some way and that the balance achieved depends on signals received from appropriate growth or survival factors.

Macroautophagy, often referred to as autophagy, is a catabolic process that results in the autophagosomic-lysosomaldegradation of bulk cytoplasmic contents, abnormal protein aggregates, and excess or damaged organelles. Autophagy is generally activated by conditions of nutrient deprivation but has also

been associated with physiological as well as pathological processes such as development, differentiation, neurodegenerative diseases, stress, infection and cancer. Other pathways of programmed cell death have been discovered. Called "non-apoptotic programmed cell-death" (or "caspase-independent programmed cell-death" or "necroptosis"), these alternative routes to death are as efficient as apoptosis and can function as either backup mechanisms or the main type of PCD.

Other forms of programmed cell death include anoikis, almost identical to apoptosis except in its induction; cornification, a form of cell death exclusive to the eyes; excitotoxicity; ferroptosis, an iron-dependent form of cell death and Wallerian degeneration. Plant cells undergo particular processes of PCD similar to autophagic cell death. However, some common features of PCD are highly conserved in both plants and metazoa.

Activation-induced cell death (AICD) is a programmed cell death caused by the interaction of Fas receptor (Fas, CD95) and Fas ligand (FasL, CD95 ligand). It occurs as a result of repeated stimulation of specific T-cell receptors (TCR) and it helps to maintain the periphery immune tolerance. Therefore, an alteration of the process may lead to autoimmune diseases. In the other words AICD is the negative regulator of activated T-lymphocytes. Ischemic cell death, or oncosis, is a form of accidental, or passive cell death that is often considered a lethal injury. The process is characterized by mitochondrial swelling, cytoplasm vacuolization, and swelling of the nucleus and cytoplasm.

Immunogenic cell death or immunogenic apoptosis is a form of cell death caused by some cytostatic agents such asanthracyclines, oxaliplatin and bortezomib, or radiotherapy and photodynamic therapy (PDT). Pyroptosis is a highly inflammatory form of programmed cell death that occurs most frequently upon infection with intracellular pathogens and is likely to form part of the antimicrobial response in myeloid cells.

The term "cell necrobiology" has been used to describe the life processes associated with morphological, biochemical, and molecular changes which predispose, precede, and accompany cell death, as well as the consequences and tissue response to cell death. The word is derived from the Greek νεκρό meaning "death", βìο meaning "life", and λόγος meaning "the study of". The term was initially coined to broadly define investigations of the changes that accompany cell death, detected and measured by multiparameter flow- and laser scanning- cytometry. It has been used to describe the real-time changes during cell death, detected by flow cytometry

Programmed cell death:

Programmed cell death protein 1, also known as PD-1 andCD279 (cluster of differentiation 279), is a protein found on the surface of cells that has a role in regulating the immune system's response to the cells of the human body by down-regulating the immune system and promoting self tolerance by suppressing T cell inflammatory activity. This prevents autoimmune diseases, but it can also prevent the immune system from killing cancer cells.
PD-1 is an immune checkpoint and guards against autoimmunity through two mechanisms. First, it promotes apoptosis (programmed cell death) of antigen-specific T-cells in lymph nodes. Second, it reduces apoptosis in regulatory T cells (anti-inflammatory, suppressive T cells). PD-1 inhibitors, a new class of drugs that block PD-1, activate the immune system to attack tumors and are used to treat certain types of cancer. The PD-1 protein in humans is encoded by the PDCD1 gene. PD-1 is a cell surface receptor that belongs to the immunoglobulin superfamily and is expressed on T cells and pro-B cells. PD-1 binds two ligands, PD-L1 and PD-L2.

Regionalization :

In the field of developmental biology, regionalization is the process by which different areas are identified in the development of the early embryo. The process by which the cells become specified differs between organisms.
In terms of developmental commitment, a cell can either be specified or it can be determined. Specification is the first stage in differentiation. A cell that is specified can have its commitment reversed while the determined state is irreversible. There are two main types of specification: autonomous and conditional. A cell specified autonomously will develop into a specific fate based upon cytoplasmic determinants with no regard to the environment the cell is in. A cell specified conditionally will develop into a specific fate based upon other surrounding cells or morphogen gradients. Another type of specification is syncytial specification, characteristic of most insect classes.
Specification in sea urchins uses both autonomous and conditional mechanisms to determine the anterior/posterior axis. The anterior/posterior axis lies along the animal/vegetal axis set up during cleavage. The micromeres induce the nearby tissue to become endoderm while the animal cells are specified to become ectoderm. The animal cells are not determined because the micromeres can induce the animal cells to also take on mesodermal and endodermal fates. It was observed that β-catenin was present in the nuclei at the vegetal pole of the blastula. Through a series of experiments, one study confirmed the role of β-catenin in the cell-autonomous specification of vegetal cell fates and the micromeres inducing ability. Treatments of LiCl sufficient to vegetalize the embryo resulted in increases in nuclearly localized b-catenin. Reduction of expression of β-catenin in the nucleus correlated with loss of vegetal cell fates. Transplants of micromeres lacking nuclear accumulation of β-catenin were unable to induce a second axis.

For the molecular mechanism of β-catenin and the micromeres, it was observed that Notch was present uniformly on the apical surface of the early blastula but was lost in the secondary mesenchyme cells (SMCs) during late blastula and enriched in the presumptive endodermal cells in late blastula. Notch is both necessary and sufficient for determination of the SMCs. The micromeres express the ligand for Notch, Delta, on their surface to induce the formation of SMCs.

The high nuclear levels of b-catenin results from the high accumulation of the disheveled protein at the vegetal pole of the egg. disheveled inactivates GSK-3 and prevents the phosphorylation of β-catenin. This allows β-catenin to escape degradation and enter the nucleus. The only important role of β-catenin is to activate the transcription of the gene Pmar1. This gene represses a repressor to allow micromere genes to be expressed.

The aboral/oral axis (analogous to the dorsal/ventral axes in other animals) is specified by a nodal homolog. This nodal was localized on the future oral side of the embryo. Experiments confirmed that nodal is both necessary and sufficient to promote development of the oral fate. Nodal also has a role in left/right axis formation.

Cell Shape :
The variety of cell shapes seen in prokaryotes and eukaryotes reflects the functions that each cell has, confirming the **structure-function relationship** seen throughout biology. Each cell type has evolved a shape that is best related to its function. For example, the neuron in **Figure** below has long, thin extensions (**axons** and **dendrites**) that reach out to other nerve cells. The extensions help the neuron pass chemical and electrical messages quickly through the body. The shape of the red blood cells (**erythrocytes**) enable these cells to easily move through **capillaries**. The spikes on the pollen grain help it stick to a pollinating insect or animal so that it can be transferred to and pollinate another flower. The long whip-like **flagella** (tails) of the algae *Chlamydomonas* help it swim in water. Different cells within a single organism can come in a variety of sizes and shapes. They may not be very big, but their shapes can

be very different from each other. However, these cells all have common abilities, such as obtaining and using food energy, responding to the external environment, and reproducing. In part, a cell's shape determines its function.

Cell Size:

If cells are the main structural and functional unit of an organism, why are they so small? And why are there no organisms with huge cells? The answers to these questions lie in a cell's need for fast, easy food. The need to be able to pass nutrients and gases into and out of the cell sets a limit on how big cells can be. The larger a cell gets, the more difficult it is for nutrients and gases to move in and out of the cell.

As a cell grows, its volume increases more quickly than its surface area. If a cell was to get very large, the small surface area would not allow enough nutrients to enter the cell quickly enough for the cell's needs. This idea is explained in **Figure** below. However, large cells have a way of dealing with some size challenges. Big cells, such as some white blood cells, often grow more nuclei so that they can supply enough proteins and RNA for the cell's requirements. Large, metabolically active cells often have lots of cell protrusions, resulting in many folds throughout the membrane. These folds increase the surface area available for transport of materials into or out of the cell. Such cell types are found lining your small intestine, where they absorb nutrients from your food through protrusions called microvilli**.**

Scale of Measurements

- 1 centimeter (cm) = 10 millimeters (mm) = 10^{-2} meters (m)
- 1 mm = 1000 micrometers (μm) = 10^{-3} m
- 1 μm = 1000 nanometers (nm) = 10^{-6} m
- 1 nm = 10^{-3} μm

Imagine cells as little cube blocks. If a small cube cell like the one in Figure above is one unit (u) in length, then the total surface area of this cell is calculated by the equation:

- height × width × number of sides × number of boxes
- $1u \times 1u \times 6 \times 1 = 6u^2$

The volume of the cell is calculated by the equation:

- height × width × length × number of boxes
- $1u \times 1u \times 1u \times 1 = 1u^3$

The surface-area to volume ratio is calculated by the equation:

- area ÷ volume
- $6 \div 1 = 6$

A larger cell that is 3 units in length would have a total surface area of

- $3u \times 3u \times 6 \times 1 = 54u^2$

and a volume of:

- $3u \times 3u \times 3u \times 1 = 27u^3$

The surface-area to volume ratio of the large cell is:

- $54 \div 27 = 2$

Now, replace the three unit cell with enough one unit cells to equal the volume of the single three unit cell. This can be done with 27 one unit cells. Find the total surface area of the 27 cells:

- $1u \times 1u \times 6 \times 27 = 162u^2$

The total volume of the block of 27 cells is:

- $1 \times 1 \times 1 \times 27 = 27u^3$

The surface-area to volume ratio of the 27 cells is:

- $162 \div 27 = 6$

An increased surface area to volume ratio means increased exposure to the environment. This means that nutrients and gases can move in and out of a small cell more easily than in and out of a larger cell.

The cells you have learned about so far are much smaller than the period at the end of this sentence, so they are normally measured on a very small scale. The smallest **prokaryotic cell** currently known has a diameter of only 400 nm. **Eukaryotic cells** normally range between 1– 100μm in diameter. The mouse

cells in **Figure** above are about 10 µm in diameter. One exception, however, is **eggs**. Eggs contain the largest known single cell, and the ostrich egg is the largest of them all. The ostrich egg in **Figure** above is over 10,000 times larger than the mouse cell.

Probable questions:

1. What is developmental ramification?
2. What do you mean by morphogenesis?
3. Define programmed cell death.

Suggested readings:

GILBERT, S. F., & BARRESI, M. J. F. (2016). *Developmental biology*.

Unit-II

Teratogenesis: Genetic teratology, Environmental teratology, Developmental mechanism, Contribution of teratology to Developmental Biology

Objective: In this unit you will learn about Teratogenesis: Genetic teratology, Environmental teratology, Developmental mechanism, Contribution of teratology to Developmental Biology.

Teratogenesis

Introduction:

Teratology is the study of abnormal development in embryos and the causes of congenital malformations or birth defects. These anatomical or structural abnormalities are present at birth although they may not be diagnosed until later in life. They may be visible on the surface of the body or internal to the viscera. Congenital malformations account for approximately 20% of deaths in the perinatal period. Approximately 3% of newborn infants will have major malformations and another 3% will have malformations detected later in life.

There are a variety of causes of congenital malformations including: 1) genetic factors (chromosomal abnormalities as well as single gene defects); 2) environmental factors (drugs, toxins, infectious etiologies, mechanical forces); and 3) multifactorial etiologies including a combination of environmental and genetic factors. The graph below (Fig. 23-1) divides these etiologies by percentages.

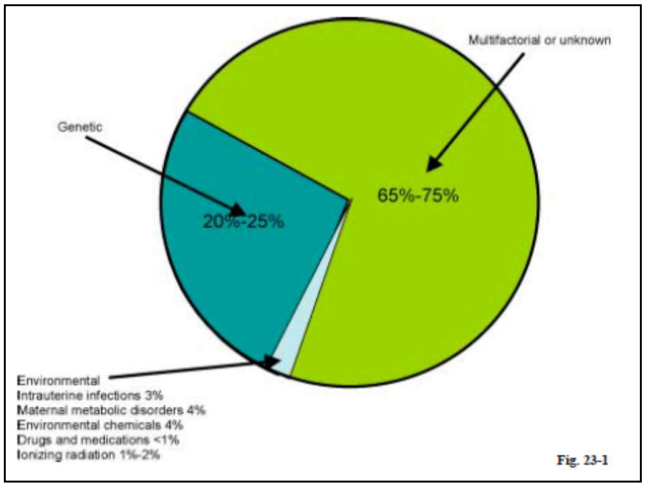


Figure: Factors responsible for teratogenesis.

Malformations may be single or multiple and have major or minor clinical significance. Single minor malformations are observed in approximately 14% of newborns. These malformations are usually of no clinical consequence and may include features such a simian crease or ear tags. Specific minor malformations suggest the possibility of an associated major malformation. For instance, the finding of a single umbilical artery should suggest the possibility of associated congenital heart problems. The greater the number of minor malformations, the greater the likelihood of an associated major malformation. The

more severe and the greater the number of major malformations, the greater the likelihood of a spontaneous miscarriage or shortened life span.

Genetic etiologies of malformations

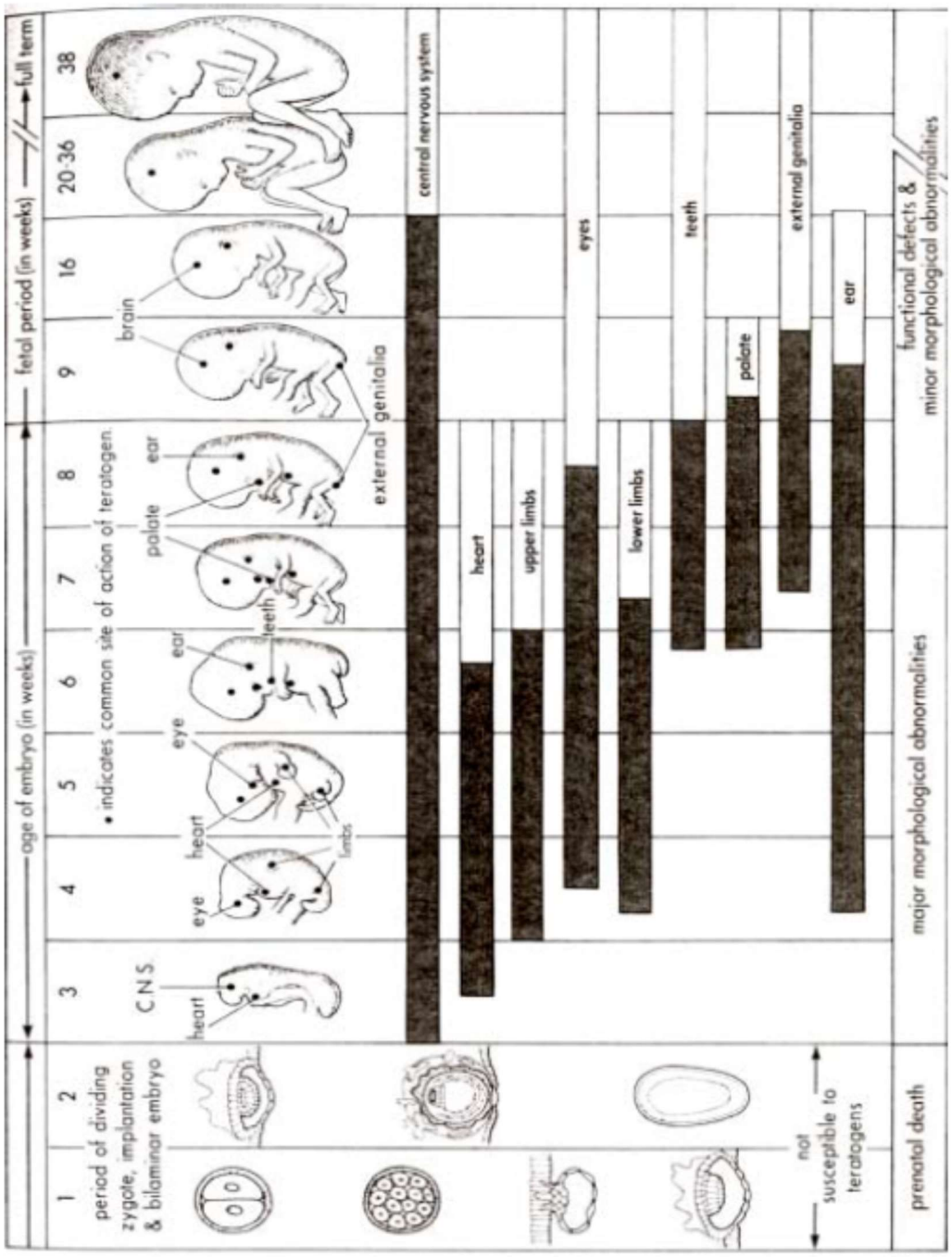
Genetic factors are the most common causes of congenital malformations and account for approximately one fourth of all congenital malformations. Chromosomal abnormalities including numerical and structural abnormalities are a common cause of congenital malformations. Specific genetic syndromes are associated with the most common of these chromosomal defects. Trisomy 21 is referred to as Down syndrome and has associated characteristic facial features, congenital heart disease, growth retardation, and mental retardation. Monosomy of the X-chromosome is referred to as Turner syndrome and is associated with webbing of the neck, lymphedema of the hands and feet, and later in life short stature and infertility. Trisomy 13 is associated with midline defects including cleft lip and cleft palate, central nervous system malformations, microphthalmia, and congenital heart disease. Infants with this disorder rarely live beyond the first year of life. Trisomy 18 is associated with intrauterine growth restriction, clenched hands, rocker bottom feet, and congenital heart disease. Similar to trisomy 13, infants with the syndrome also rarely live beyond the first year of life. Other chromosomal abnormalities including interstitial deletions, interstitial duplications, and unbalanced translocations are often associated with congenital anomalies. The most common deletions have named clinical syndromes with which they are associated.

In addition to gross chromosomal abnormalities, there are multiple single gene defects that can result in congenital malformations. Many of these genes include developmentally important transcription factors and genes important in intermediary metabolism. Teratogenic agents cause approximately 7% of congenital malformations. A teratogenic agent is a chemical, infectious agent, physical condition, or deficiency that, on fetal exposure, can alter fetal morphology or subsequent function. Teratogenicity depends upon the ability of the agent to cross the placenta. Certain medications such as heparin cannot cross the placenta due to its high molecular weight and are therefore not teratogenic. The embryo is most susceptible to teratogenic agents during periods of rapid differentiation. The stage of development of the embryo determines susceptibility to teratogens. The most critical period in the development of an embryo or in the growth of a particular organ is during the time of most rapid cell division. The critical period for each organ is pictured below. For instance, the critical period for brain growth and development is from three to 16 weeks. However the brain's differentiation continues to extend into infancy. Teratogens can produce mental retardation during both embryonic and fetal periods.

Specific types of major malformations and the times of development usually associated with exposure to the teratogenic agent are outlined in the table below. Each organ of an embryo has a critical period during which its development may be disrupted. The type of congenital malformation produced by an exposure depends upon which organ is most susceptible at the time of the teratogenic exposure. For instance, high levels of radiation produce abnormalities of the central nervous system and eyes specifically at eight to 16 weeks after fertilization. Embryological timetables such as the one above are helpful in studying the etiology of human malformations. However, it is wrong to assume that malformations always result from a single event occurring during a single critical sensitive period or that one can determine the exact day on which a malformation was produced.

A teratogen is any agent that can induce or increase the incidence of a congenital malformation. Recognition of human teratogens offers the opportunity to prevent exposure at critical periods of development and prevent certain types of congenital malformations. In general, drugs, food additives, and pesticides are tested to determine their teratogenicity to minimize exposure of pregnant women to teratogenic agents. To prove that a specific agent is teratogenic means to prove that the frequency of

congenital malformations in women exposed to the agent is prospectively greater than the background frequency in the general population. These data are oftentimes not available for humans and thus cannot be determined in an unbiased fashion. Therefore, testing is often done in animal models and often times administered at higher than the usual therapeutic doses. There are clearly species differences between teratogenic effects, limiting this testing in animals. Based upon either anecdotal information on exposures in humans or on the basis of testing in animals, drugs are classified as to their teratogenic potential. It should be emphasized that less than 2% of congenital malformations are caused by drugs or chemicals. There are small numbers of drugs that have been positively implicated as teratogenic agents that should be avoided either during or prior to conception. However, because of the unknown, subtle effects of many



agents, women preparing to conceive or already pregnant refrain from taking any medications that are not absolutely necessary. Women are especially urged to avoid using all medications during the first 8 weeks after conception unless there is a strong medical reason. Effects of teratogens during this period of developmental often times results in an "all or none effect." That is, the effect of the teratogen, if it is to have any effect, will be so profound as to cause a spontaneous abortion.

Nicotine does not produce congenital malformations but nicotine does have a effect on fetal growth. Maternal smoking is a well-established cause of intrauterine growth restriction. Heavy cigarette smokers were also more likely to have a premature delivery. Nicotine constricts uterine blood vessels and causes decreased uterine blood flow thereby decreasing the supply of oxygen and nutrients available to the embryo. This compromises cell growth and may have an adverse effect on mental development.

Alcohol is a common drug abused by women of childbearing age. Infants born to alcoholic mothers demonstrate prenatal and postnatal growth deficiency, mental retardation, and other malformations. There are subtle but classical facial features associated with fetal alcohol syndrome including short palpebral fissures, maxillary hypoplasia, a smooth philtrum, and congenital heart disease. Binge drinking also likely has a harmful effect on embryonic brain developments at all times of gestation.

Tetracycline, the type of antibiotic, can cross the placental membrane and is deposited in the embryo in bones and teeth. Tetracycline exposure can result in yellow staining of the primary or deciduous teeth and diminished growth of the long bones. Tetracycline exposure after birth has similar effects. Anticonvulsant agents such as phenytoin produce the fetal hydantoin syndrome consisting of intrauterine growth retardation, microcephaly, mental retardation, distal phalangeal hypoplasia, and specific facial features.

Anti-neoplastic or chemotherapeutic agents are highly teratogenic as these agents inhibit rapidly dividing cells. These medications should be avoided whenever possible but are occasionally used in the third trimester when they are urgently needed to treat the mother.

Retinoic acid or vitamin A derivatives are extremely teratogenic in humans. Even at very low doses, oral medications such as isotretinoin, used in the treatment of acne, are potent teratogens. The critical period of exposure appears to be from the second to the fifth week of gestation. The most common malformations include craniofacial dysmorphisms, cleft palate, thymic aplasia, and neural tube defects.

The tranquilizer **thalidomide** is one of the most famous and notorious teratogens. This hypnotic

agent was used widely in Europe in 1959, after which an estimated 7000 infants were born with the thalidomide syndrome or meromelia. The characteristic features of this syndrome include limb abnormalities that span from absence of the limbs to rudimentary limbs to abnormally shortened limbs. Additionally, thalidomide also causes malformations of other organs including absence of the internal and external ears, hemangiomas, congenital heart disease, and congenital urinary tract malformations. The critical period of exposure appears to be 24 to 36 days after fertilization.

Infectious agents can also cause a variety of birth defects and mental retardation when they cross the placenta and enter the fetal blood stream. Congenital rubella or German measles consists of the triad of cataracts, cardiac malformation, and deafness. The earlier in the pregnancy that the embyro is exposed to maternal rubella, the greater the likelihood that it will be affected. Most infants exposed during the first four to five weeks after fertilization will have stigmata of this exposure. Exposure to rubella during the second and third trimester results in a much lower frequency of malformation, but continues to pose a risk of mental retardation and hearing loss.

Congenital cytomegalovirus infection is the most common viral infection of the fetus. Infection of the early embryo during the first trimester most commonly results in spontaneous termination. Exposure later

in the pregnancy results in intrauterine growth retardation, micromelia, chorioretinitis, blindness, microcephaly, cerebral calcifications, mental retardation, and hepatosplenomegaly.

Ionizing radiation can injure the developing embryo due to cell death or chromosome injury. The severity of damage to the embryo depends on the dose absorbed and the stage of development at which the exposure occurs. Study of survivors of the Japanese atomic bombing demonstrated that exposure at 10 to 18 weeks of pregnancy is a period of greatest sensitivity for the developing brain. There is no proof that human congenital malformations have been caused by diagnostic levels of radiation. However, attempts are made to minimize scattered radiation from diagnostic procedures such as x-rays that are not near the uterus. The standard dose of radiation associated with a diagnostic x-ray produces a minuscule risk to the fetus. However, all women of childbearing age are asked if they are pregnant before any exposure to radiation.

Maternal medical conditions can also produce teratogenic risks. Infants of diabetic mothers have an increased incidence of congenital heart disease, renal, gastrointestinal, and central nervous system malformations such as neural tube defects. Tight glycemic control during the third to sixth week post-conception is critical. Infants of mothers with phenylketonuria who are not well controlled and have high levels of phenylalanine have a significant risk of mental retardation, low birth weight, and congenital heart disease.

Mechanical forces can also act as teratogens. Malformations of the uterus may restrict fetal movements and be associated with congenital dislocation of the hip and clubfoot. Oligohydramnios can have similar results and mechanically induce abnormalities of the fetal limbs. These abnormalities would be classified as deformations or abnormal forms, shapes, or positions of body parts caused by physical constraints. Amniotic bands are fibrous rings and cause intrauterine amputations or malformations of the limbs as well. These abnormalities would be classified as disruptions or defects from interference with a normally developing organ system usually occurring later in gestation.

Most common congenital malformations have familial distributions consistent with multifactorial inheritance. Multifactorial inheritance may be presented by a model in which liability to a disorder is a continuous variable that is dependent on a combination of environmental and genetic factors. Development of the malformation is dependent upon passing a threshold that is the sum of a combination of many of these factors. Traits that demonstrate this mode of inheritance include cleft lip, cleft palate, neural tube defects, pyloric stenosis, and congenital dislocation of the hip.

Environmental Teratology: It is estimated that approximately 10–15% of congenital structural anomalies are the result of the adverse effect of environmental factors on prenatal development. This means that approximately 1 in 250 newborn infants have structural defects caused by an environmental exposure and, presumably, a larger number of children have growth retardation or functional abnormalities resulting from nongenetic causes, in other words, from the effects of teratogens. A teratogen is defined as any environmental factor that can produce a permanent abnormality in structure or function, restriction of growth, or death of the embryo or fetus. A dose-response relationship should be demonstrated in animals or humans so that the greater the exposure during pregnancy, the more severe the phenotypic effects on the fetus. Factors comprise medications, drugs, chemicals, and maternal conditions or diseases, including infections.

Teratogenic exposures during prenatal development cause disruptions regardless of the developmental stage or site of action. Most structural defects caused by teratogenic exposures occur during the embryonic period, which is when critical developmental events are taking place and the foundations of organ systems are being established. Different organ systems have different periods of susceptibility to exogenous agents.

Radiation: Ionizing radiation can injure the developing embryo due to cell death or chromosome injury. There is no proof that human congenital malformations have been caused by diagnostic levels of radiation. The most critical exposure period is 8–15 week after fertilization. Before implantation, the mammalian embryo is insensitive to the teratogenic and growth-retarding effects of radiation and sensitive to the lethal effects. The risks of 1-rad (0.10Gy) or 5-rad (0.05Gy) acute exposure are far below the spontaneous risks of the developing embryo because 15% of human embryos abort, 2.7 – 3.0% of human embryos have major malformations, 4% have intrauterine growth retardation, and 8–10% have early- or late-stage onset genetic disease. Permanent growth retardation is more severe after midgestation radiation.

Because of its extended periods of organogenesis and histogenesis, the central nervous system (CNS) retains the greatest sensitivity of all organ systems to the detrimental effects of radiation through the later fetal stages. In utero radiation produces microcephaly and mental retardation. Later in life there is increased incidence of hematopoietic malignancies and leukemia.

Infectious Agents: The lethal or developmental effects of infectious agents are the result of mitotic inhibition, direct cytotoxic effects, or a vascular disruptive event on the embryo or fetus. However, a repair process may result in scarring or calcification, which causes further damage by interfering with histogenesis. Infections that do not result in congenital malformations but do cause fetal or neonatal death include enteroviruses (coxsackie virus, poliovirus and echovirus) and hepatitis, variola, vaccina, and mumps viruses. Non-radioactive in-situ hybridization of formalin-fixed, paraffin-embedded placental and fetal tissue, using virus-specific DNA or RNA probes, is helpful for diagnosing fetal virus infections such as cytomegalovirus, parvovirus B-19, and varicellazoster virus that cause fetal hydrops, placentitis, and abortion.

Varicella: Varicella (or chickenpox) is a highly infectious disease, usually occurring in childhood. By adulthood, more than 95 percent of Americans have had chickenpox. Eighty-five to ninety-five percent of pregnant women are immune to chickenpox, which means that there is no need to be concerned about this during pregnancy, even if the woman is exposed to someone with chickenpox. Nearly seven women out of 10,000 will develop chickenpox during pregnancy, however, because they are not immune.

The disease is caused by the varicellazoster virus (VZV), which is a form of the herpes virus. Transmission occurs from person-to-person by direct contact or through the air. Chicken pox is contagious from 1 to 2 days before the appearance of the rash until the blisters have dried and become scabs. Once a person is exposed to the virus, chickenpox may take up to 14 to 18 days to develop. When a woman has a varicella infection during the first 20 wk of pregnancy, there is a 2% chance that the baby will have a group of defects called the congenital varicella syndrome, which includes scars, defects of muscle and bone, malformed and paralyzed limbs, small head size, blindness, seizures, and mental retardation. This syndrome is rarely seen if the infection occurs after 20 weeks of pregnancy.

Another time that there is a concern about a varicella infection is in the newborn period, if the mother develops the rash during the period from 5 days before to 2 days after delivery. Between 25% and 50% of newborns will be infected in this case, and they develop a rash between 5 and 10 days after birth. Up to 30% of infected babies will die if not treated. If the mother develops a rash between 6 and 21 days before delivery, the baby faces some risk of mild infection.

Mumps virus: Mumps virus during pregnancy does not cause malformations, but endocardial fibroelastosis has been noted in infants with a positive mumps antigen skin test; this relationship has not been consistent.

Influenza virus: There is no compelling evidence to incriminate influenza virus infection during pregnancy as a cause of malformations.

Parvovirus: Human parvovirus B-19 is able to cross the placenta and results in fetal infection, which may occur whether the mother is symptomatic or asymptomatic. It is associated with a higher than average fetal loss and may lead to spontaneous abortion in the first trimester, hydrops fetalis in the second trimester, and stillbirth at term. Generalized myocarditis, myositis of skeletal muscles, and abnormalities of the eyes are reported. Human parvovirus B-19 has an affinity for the erythropoietic tissue of the host and is therefor associated with fetal anemia leading to cardiac failure.

Mechanism of teratogenesis:

The Mechanism of Teratogenesis fall into broad categories based on the etiology of congenital malformations:

(a) Errors in genetic programming based on deviations in the genotypes of the embroy or the low probability for error of the normal genotype ; and

(b) Environmental agants or factors that interact with an embryo during the period of development (drugs, chemicals, rediation, hyperthermia, infections, abnormal maternal metabolic states, or mechanical factors).

The etiology of human malformations includes both genetic and environmental factors.

Most developmental defects have complex etiology following from interactions of gene environment lifestyle factors.

• Recognizing a teratogen is a very different problem than understanding its mechanism of action.

• Mechanistic information is essential to understanding how drugs and chemicals perturb development.

• Identifies important molecular initiating events for which rapid and cost efficient screens can be developed.

• Understanding mechanisms is needed for appropriate intervention and preventive public health strategies.

What defines a teratogenic mechanism ?

*The means by which a lesion is produced and propagated through a series of measurable events in development.

* Starts with exposure (eg, maternal) and ends with an adverse developmental outcome (eg, malformation).

* Implies detailed molecular knowledge of the initial point of chemical-biological interaction (initiating event).

* Considers downstream pathogenesis that can be linked to dynamic changes in cell fate and behavior.

Large number of teratogens and adverse developmental outcomes makes it difficult to pinpoint unifying mechanisms. Principal teratogenic mechanisms based on associations of major birth defects with medications used by women of reproductive age:

1. Folate antagonism

2. Neural crest cell disruption

3. Endocrine disruption

4. Oxidative stress

5. Vascular disruption

6. Specific receptor- or enzyme-mediated teratogenesis.

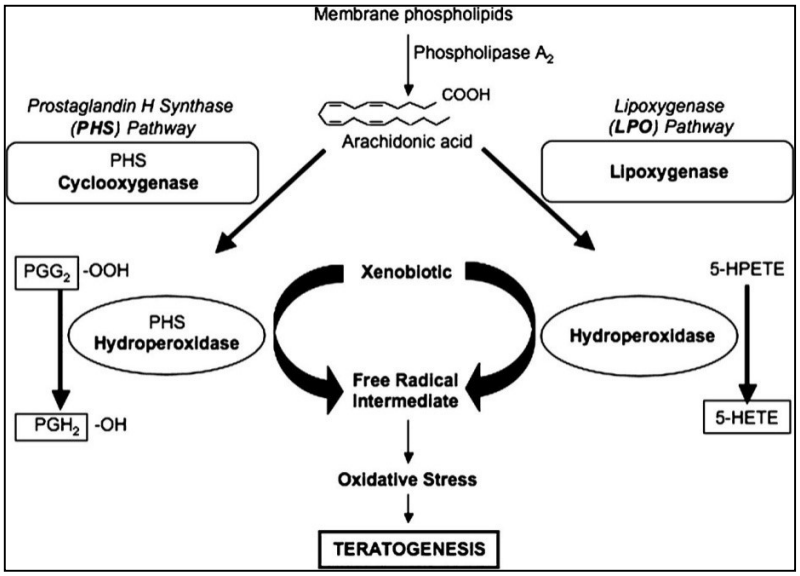


Figure: Mechanism of teratogen.

Various mechanisms are involved in teratogenic effects:

i. Interference with Nucleic Acids:

Various teratogenic agents interfere with nucleic acid replication, transcription, or RNA translation. These include alkylating agents, antimetabolites, intercalating agents and amino acid antagonists.

ii. Inhibition of Enzymes:

Inhibitors of enzymes, e.g. 5-flourouracil, may induce malformation through interference with differentiation or growth by inhibiting thymidylate synthatase. Other examples include 6-aminonicotinamide, which inhibits glucose-6-phosphate dehydrogenase, and folate antagonists which inhibit dihydrofolate reductase.

iii. Deficiency of Energy Supply and Osmolarity:

Certain teratogens can affect the energy supply for the metabolism by restricting the availability of substrates either directly (e.g., dietary deficiencies) or through the presence of analogs for antagonists of vitamins, essential amino acids, and others.

In addition, hypoxia and agents i.e., CO and CO_2, can be teratogenic by depriving the metabolic process of the required O_2 and probably also by the production of osmolar imbalances. These can induce edema, which, in turn, cause mechanical distortion and tissue ischemia. Physical agents that can cause malformations include radiation, hypothermia, hyperthermia and mechanical trauma.

It shall not be out of place to mention that the mode of action of many teratogens is yet uncertain. Furthermore, a potential teratogen may or may not exert teratogenic effects depending on such factors as

bio-activating mechanism, stability and detoxifying capability of the embryonic tissues. Appropriate experimental testing for the teratogenicity of toxicants is, therefore, essential.

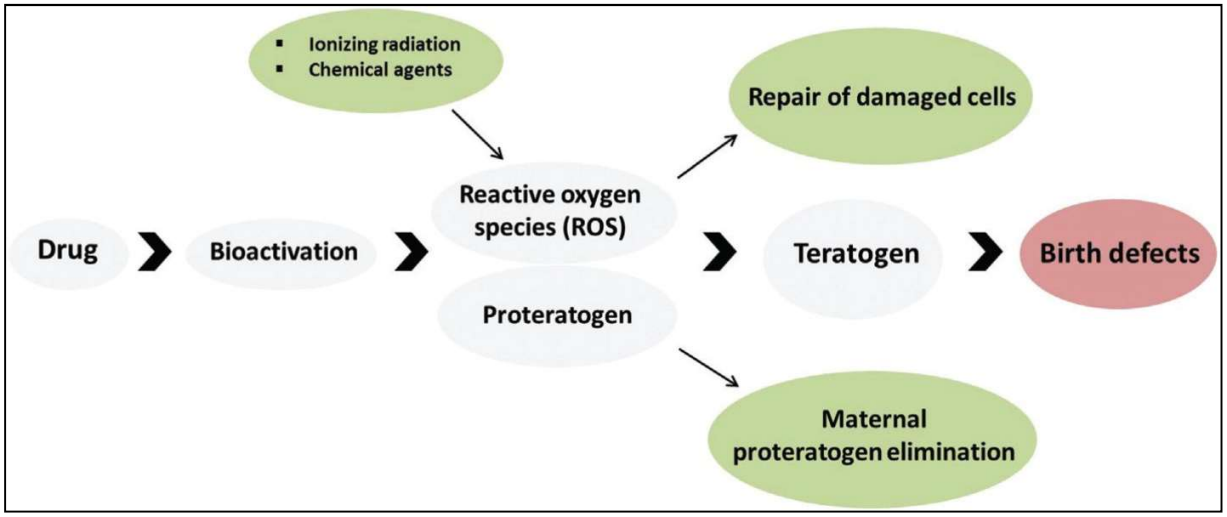


Figure: Mechanism of action of teratogen.

Contribution of teratology in developmental biology:

Teratology is the branch of medical science which studies the causes and underlying mechanisms of congenital birth defects. A teratogen can consist of any chemical, infectious or environmental agent capable of disrupting normal embryo-fetal development following exposure during pregnancy. Understanding the biological processes by which a teratogen causes congenital malformations requires the integration of fundamental scientific knowledge from a diverse range of disciplines including toxicology, developmental and molecular biology. Research within the field of teratology, can be utilised to inform the pharmaceutical industry and government agencies of preventative measures and treatment strategies for pregnant females and their offspring. This scientific knowledge can be further translated into a clinical setting within the specialties of prenatal medicine, obstetrics, neonatology and paediatrics. Alcohol, one of the oldest intoxicants in the world, has long been documented as a human teratogen and is a primary focus within this research stream.

While embryologists could look at embryos to describe the evolution of life and how different animals form their organs, physicians became interested in embryos for more practical reasons. About 2% of human infants are born with a readily observable anatomical abnormality. These abnormalities may include missing limbs, missing or extra digits, cleft palate, eyes that lack certain parts, hearts that lack valves, and so forth. Physicians need know the causes of these birth defects in order to counsel parents as to the risk of having another malformed infant. In addition, the different birth defects can tell us how the human body is normally formed. In the absence of experimental data on human embryos, we often must rely on nature's “experiments” to learn how the human body becomes organized. Some birth defects are produced by mutant genes or chromosomes, and some are produced by environmental factors that impede development.

Abnormalities caused by genetic events (gene mutations, chromosomal aneuploidies and translocations) are called malformations. Malformations often appear as syndromes (from the Greek, “running together”), where several abnormalities are seen concurrently. For instance, a human malformation called piebaldism, shown in Figure 1.15A, is due to a dominant mutation in a gene (KIT) on the long arm of chromosome 4. The syndrome includes anemia, sterility, unpigmented regions of the skin and hair, deafness, and the absence of the nerves that cause peristalsis in the gut. The common feature underlying

these conditions is that the KIT gene encodes a protein that is expressed in the neural crest cells and in the precursors of blood cells and germ cells. The Kit protein enables these cells to proliferate. Without this protein, the neural crest cells—which generate the pigment cells, certain ear cells, and the gut neurons—do not multiply as much as they should (resulting in underpigmentation, deafness, and gut malformations), nor do the precursors of the blood cells (resulting in anemia) or the germ cells (resulting in sterility).

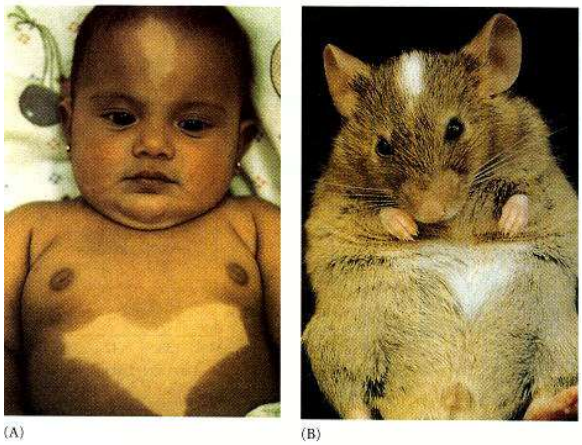


Figure 1.15: Developmental anomalies caused by genetic mutation. (A) Piebaldism in a human infant. This genetically produced condition results in sterility, anemia and underpigmented regions of the skin and hair, along with defective development of gut neurons and the ear. Piebaldism is caused by a mutation in the KIT gene. The Kit protein is essential for the proliferation and migration of neural crest cells, germ cell precursors, and blood cell precursors. (B) A piebald mouse with a mutation of the Kit gene. Mice provide important models for studying human developmental diseases.

Probable questions:

1. Define teratogenesis.
2. State the mechanism of action of a teratogen.
3. Describe the contribution of teratology to Developmental Biology.

Suggested readings:

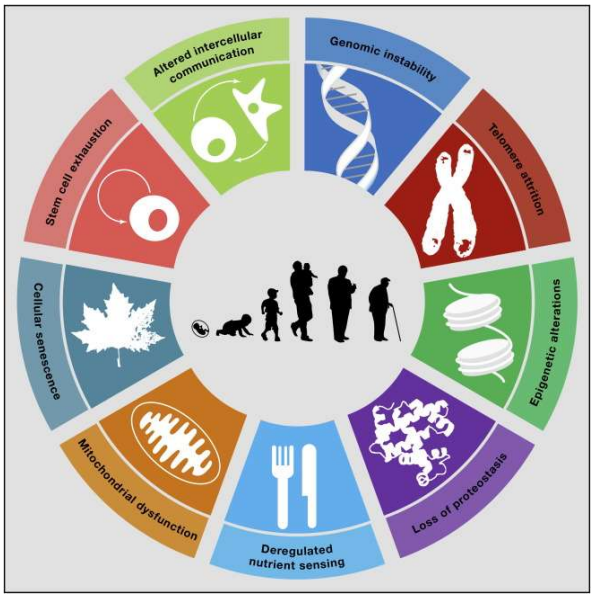
GILBERT, S. F., & BARRESI, M. J. F. (2016). *Developmental biology*.

Unit-III

Ageing: Cellular basis of aging, Causes of aging, Free Radical Theory of Aging, Ageing of connective tissue

Objective: In this unit you will learn about Ageing: Cellular basis of aging, Causes of aging, Free Radical Theory of Aging, Ageing of connective tissue

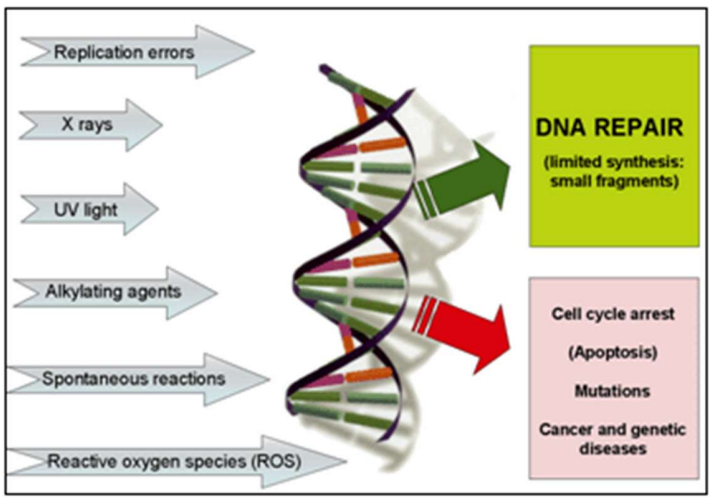
Cellular basis of aging



Normal cells have only a finite life span before they die. The process known as aging may occur as a result of continued damage to the cell or as a result of expression of predetermined information within the genetic structure of the cell. Both processes lead to progressive cellular dysfunction which is evidenced by the organs of the body as aging.The rate of aging is controlled, at least to some extent, by genetic pathways and biochemical processes conserved in evolution. There are mainly ninetentative hallmarks that represent common denominators of aging in different organismswhich includes the following:

Genomic Instability

One common denominator of aging is the accumulation of genetic damage throughout life. The stability of DNA is continuously challenged by exogenous physical, chemical, and biological agents, as well as by endogenous threats, including DNA replication errors, spontaneous hydrolytic reactions, and reactive oxygen species (ROS). The genetic lesions arising from extrinsic or intrinsic damages are highly diverse and include point mutations, translocations, chromosomal gains and losses, telomere shortening, and gene disruption caused by



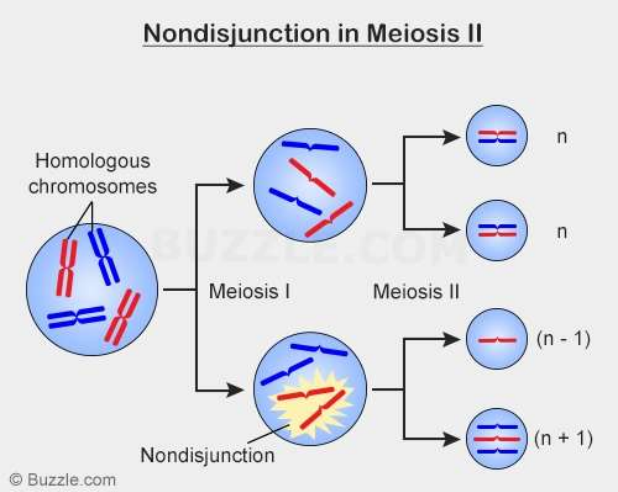
the integration of viruses or transposons. To minimize these lesions,organisms have evolved a complex network of DNA repair mechanisms that are collectively capable of dealing with most of the damages inflicted to nuclear DNA.

1. Nuclear DNA:

Somatic mutations accumulate within cells from aged humans and model organisms.Other forms of DNA damage are as follows:

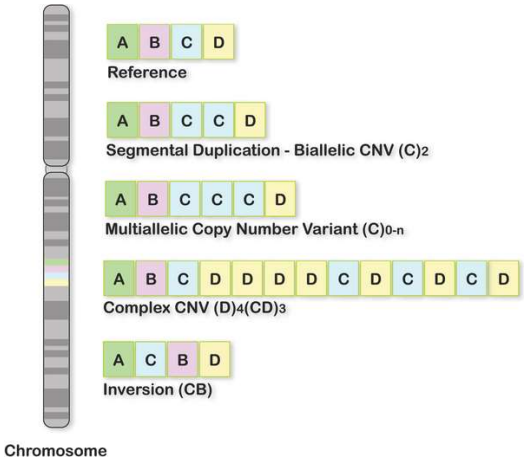
Chromosomal Aneuploidies:

Aneuploidy is the presence of an abnormal numbers of chromosomes in a cell, for example a human cell having 45 or 47 chromosomes instead of the usual 46. During meiosis, when germ cells divide to create sperm and egg (gametes), each half should have the same number of chromosomes. But sometimes, the whole pair of chromosomes will end up in one gamete, and the other gamete will not get that chromosome at all.This *Nondisjunction* usually occurs as the result of a weakened mitotic checkpoint, as these checkpoints tend to arrest or delay cell division until all components of the cell are ready to enter the next phase.

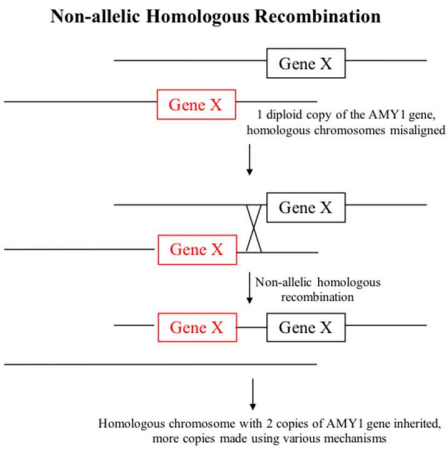


Copy Number Variations:

Copy number variation **(CNV)** is a phenomenon in which sections of the genome are repeated and the number of repeats in the genome varies between individuals in the human population.Copy number variation is a type of structural variation: specifically, it is a type of duplication or deletion event that affects a considerable number of base pairs.One of the most well-known examples of a short copy number variation is the tri-nucleotide repeat of the CAG base pairs in the Huntingtin gene, the gene that is responsible for the neurological disorder, Huntington's disease.

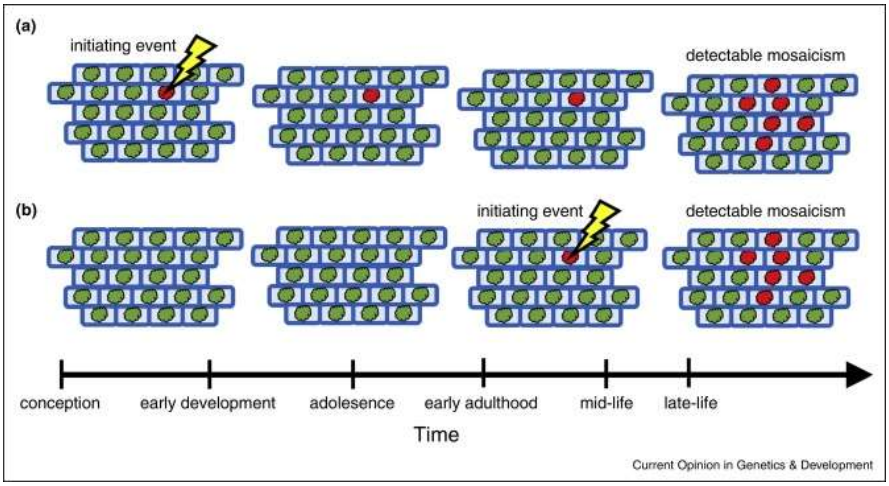


One of the best-recognized theories that leads to copy number variations as well as deletions and inversions is non-allelic homologous recombinations. During meiotic recombination, homologous chromosomes pair up and form two ended double-stranded breaks leading to Holliday junctions. However, in the aberrant mechanism, during the formation of Holliday junctions, the double-stranded breaks are



misaligned and the crossover lands in non-allelic positions on the same chromosome.

Clonal Mosaicism:



Human genetic mosaicism is the presence of two or more cellular populations with distinct genotypes in an individual who developed from a single fertilized ovum.While initially observed across a spectrum of rare genetic disorders, detailed assessment of data from genome-wide association studies now reveal that detectable clonal mosaicism involving large structural alterations (> 2 Mb) can also be seen in populations of apparently healthy individuals. The characterization of detectable genetic mosaicism reveals that there could be important dynamic changes in the human genome associated with the aging process, which could be associated with risk for common disorders, such as cancer, cardiovascular disease, diabetes, and neurological disorders.Genetic mosaicism arises from a post-zygotic mutational event in a cell or group of cells.The timing of a somatic event can be later and due to either an increase in somatic alterations as a result of aging (e.g., breakdown in key DNA repair or stability pathways) alone or in combination with decreased genomic stability due to telomere attrition.

All of these forms of DNA alterations may affect essential genes and transcriptional pathways, resulting in dysfunctional cells that, if not eliminated by apoptosis or senescence, may jeopardize tissue and organismal homeostasis. This is especially relevant when DNA damage impacts the functional competence of stem cells, thus compromising their role in tissue renewal. It is also being found that deficiencies in DNA repair mechanisms cause accelerated aging in mice and underlie several human progeroid syndromes, such as Werner syndrome, Bloom syndrome, xeroderma pigmentosum, trichothio dystrophy, Cockayne syndrome, and Seckel syndrome. Moreover, transgenic mice overexpressing BubR1, a mitotic checkpoint component that ensures accurate segregation of chromosomes, exhibit an increased protection against aneuploidy and cancer, as well as extended healthy lifespan. The latter findings provide experimental evidence that artificial reinforcement of nuclear DNA repair mechanisms may delay aging.

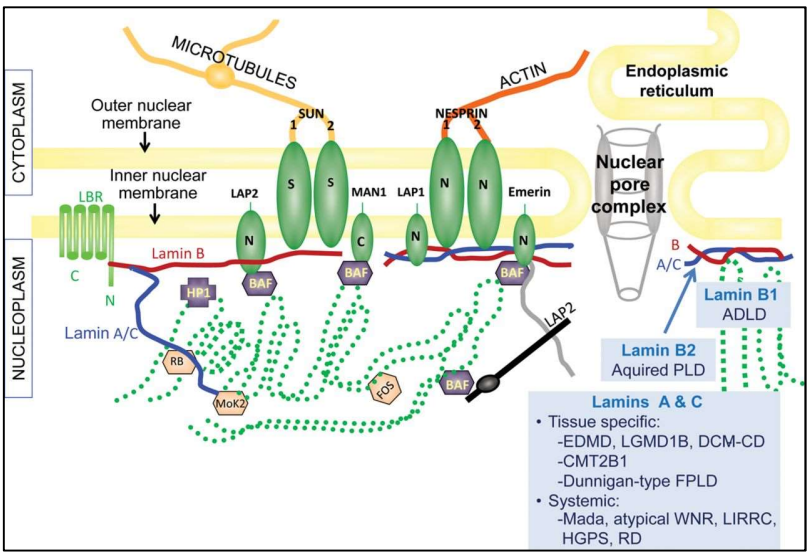
Mitochondrial DNA :

Mutations and deletions in aged mtDNA may also contribute to aging (Park and Larsson, 2011). mtDNA has been considered a major target for aging-associated somatic mutations due to the oxidative microenvironment of the mitochondria, the lack of protective histones in the mtDNA, and the limited efficiency of the mtDNA repair mechanisms compared to those of nuclear DNA. The causal implication of mtDNA mutations in aging has been controversial because of the multiplicity of mitochondrial genomes, which allows for the coexistence of mutant and wild-type genomes within the same cell, a phenomenon that is referred to as ‘‘heteroplasmy.’’ However, single-cell analyses have revealed that, despite the low overall level of mtDNA mutations, the mutational load of individual aging cells becomes significant and may attain a state of homoplasmy in which one mutant genome prevails (Khrapko et al., 1999). Interestingly, contrary to previous expectations, most mtDNA mutations in adult or aged cells appear to be caused by replication errors early in life, rather than by oxidative damage. These mutations

may undergo polyclonal expansion and cause respiratory chain dysfunction in different tissues. Studies of accelerated aging in HIV-infected patients treated with antiretroviral drugs, which interfere with mtDNA replication, have supported the concept of clonal expansion of mtDNA mutations originated early in life.

Further causative evidence comes from studies on mice that are deficient in mitochondrial DNA polymerase g. These mutant mice exhibit aspects of premature aging and reduced lifespan in association with the accumulation of random point mutations and deletions in mtDNA.

Nuclear Architecture:

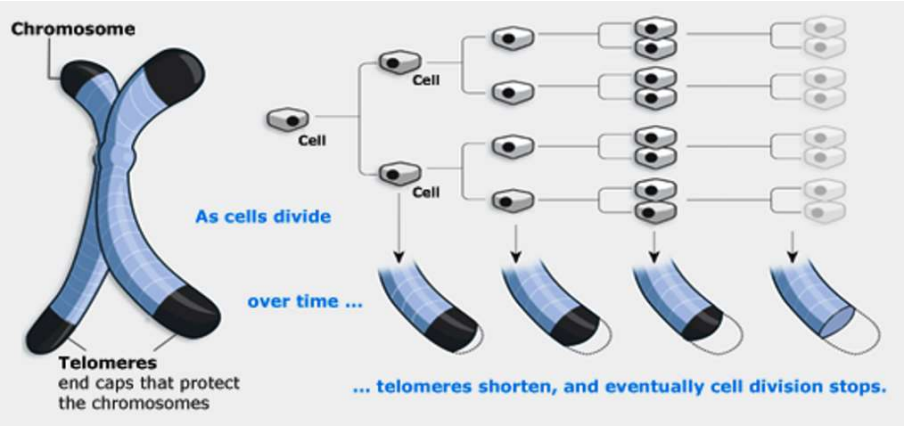


Defects in the nuclear lamina can also cause genome instability. Nuclear lamins constitute the major components of the nuclear lamina and participate in genome maintenance by providing a scaffold for tethering chromatin and protein complexes that regulate genomic stability. Researchers have found that mutations in genes encoding protein components of this structure, or factors affecting their maturation and dynamics, causes accelerated aging syndromes such as the Hutchinson-Gilford and the Ne´stor-Guillermo progeria syndromes (HGPS and NGPS, respectively). Alterations of the nuclear lamina and production of an aberrant prelaminA isoform called progerin have also been detected during normal human aging. Telomere dysfunction also promotes progerin production in normal human fibroblasts upon prolonged in vitro culture, suggesting intimate links between telomere maintenance and progerin expression during normal aging.

In addition to these age-associated changes in A-type lamins, lamin B1 levels decline during cell senescence, pointing to its utility as a biomarker of this process (Freund et al., 2012; Shimi et al., 2011). Animal and cellular models have facilitated the identification of the stress pathways elicited by aberrations in the nuclear lamina characteristic of HGPS. These pathways include the activation of p53, deregulation of the somatotrophic axis (Marin˜ o et al., 2010), and attrition of adult stem cells. The causal relevance of nuclear lamina abnormalities in premature aging has been supported by the observation that decreasing prelamin A or progerin levels delays the onset of progeroid features and extends lifespan in mouse models of HGPS. This can be achieved by systemic injection of antisense oligonucleotides, farnesyltransferase inhibitors, or a combination of statins and aminobisphosphonates. Restoration of the somatotrophic axis through hormonal treatments or inhibition of NF-kB signaling also extends lifespan in these progeroid mice.

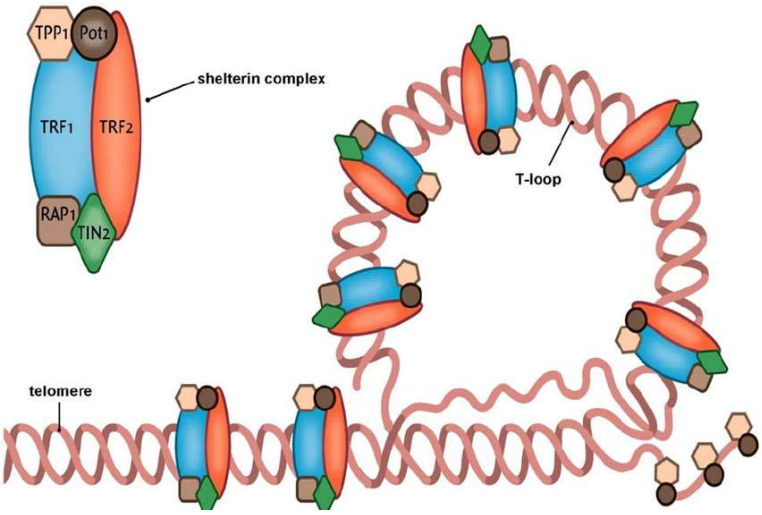
Telomere Attrition

Accumulation of DNA damage with age appears to affect the genome near to randomly, but there are some chromosomal regions, such as telomeres, that are particularly susceptible to age-related deterioration. Replicative DNA polymerases lack the capacity to replicate completely the terminal ends of linear DNA molecules, a function that is proprietary of a specialized DNA polymerase known as telomerase.



However, most mammalian somatic cells do not express telomerase, and this leads to the progressive and cumulative loss of telomere-protective sequences from chromosome ends. Telomere exhaustion explains the limited proliferative capacity of some types of in-vitro-cultured cells, the so-called replicative senescence, or **Hayflick limit** (the number of times a normal human cell population will divide before cell division stops). Importantly, telomere shortening is also observed during normal aging both in human and in mice.

Telomeres are bound by a characteristic multiprotein complex known as **shelterin** (Palm and de Lange, 2008). A main function of this complex is to prevent the access of DNA repair proteins to the telomeres. Otherwise, telomeres would be ‘‘repaired’’ as DNA breaks leading to chromosome fusions. Due to their restricted DNA repair, DNA damage at telomeres is notably persistent and highly efficient in inducing senescence and/or apoptosis.



Telomerase deficiency in humans is associated with premature development of diseases, such as pulmonary fibrosis, dyskeratosiscongenita, and aplastic anemia, which involve the loss of the regenerative capacity of different tissues. Telomere uncapping and rampant chromosome fusions can also result from deficiencies in shelterin components (Palm and de Lange, 2008). Shelterin mutations have been found in some cases of aplastic anemia and dyskeratosiscongenita. Various loss-of-function models for shelterin components are characterized by rapid decline of the regenerative capacity of tissues and accelerated aging, a phenomenon that occurs even in the presence of telomeres with a normal length.

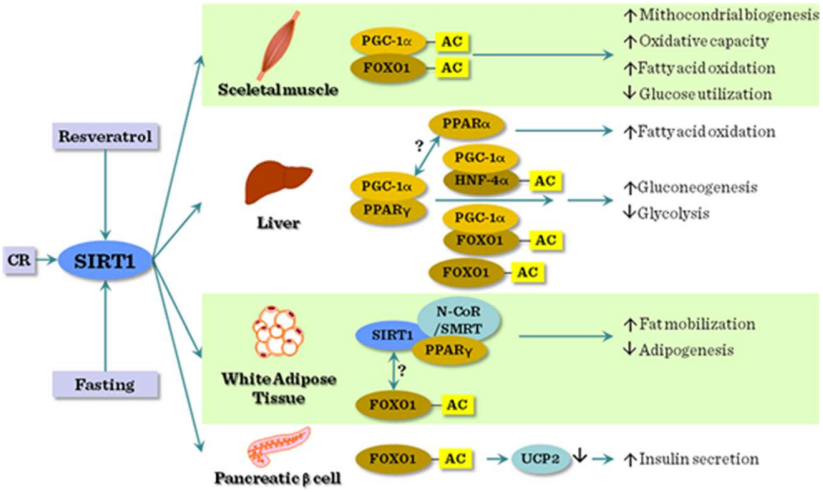
Genetically modified animal models have established causal links between telomere loss, cellular senescence, and organismal aging. Thus, mice with shortened or lengthened telomeres exhibit decreased or increased lifespans, respectively. Recent evidence also indicates that aging can be reverted by telomerase activation.

Epigenetic Alterations

A variety of epigenetic alterations affects all cells and tissues throughout life (Figure 2B). Epigenetic changes involve alterations in DNA methylation patterns, posttranslational modification of histones, and chromatin remodeling. Increased histone H4K16 acetylation, H4K20 trimethylation, or H3K4 trimethylation, as well as decreased H3K9 methylation or H3K27 trimethylation, constitute age-associated epigenetic marks. The multiple enzymatic systems assuring the generation and maintenance of epigenetic patterns include DNA methyltransferases, histone acetylases, deacetylases, methylases, and demethylases, as well as protein complexes implicated in chromatin remodeling.

Histone Modifications:

Histone methylation meets the criteria for a hallmark of aging in invertebrates. Deletion of components of histone methylation complexes (for H3K4 and for H3K27) extends longevity in nematodes and flies, respectively (Greer et al., 2010; Siebold et al., 2010). Moreover, inhibition of histone demethylases (for H3K27) in worms may extend lifespan by targeting components of key longevity routes such as the insulin/IGF-1 signaling pathway (Jin et al., 2011). It is not yet clear whether manipulations of histone-modifying enzymes can influence aging through purely epigenetic mechanisms, by impinging on DNA repair and genome stability, or through transcriptional alterations affecting metabolic or signaling pathways outside of the nucleus.



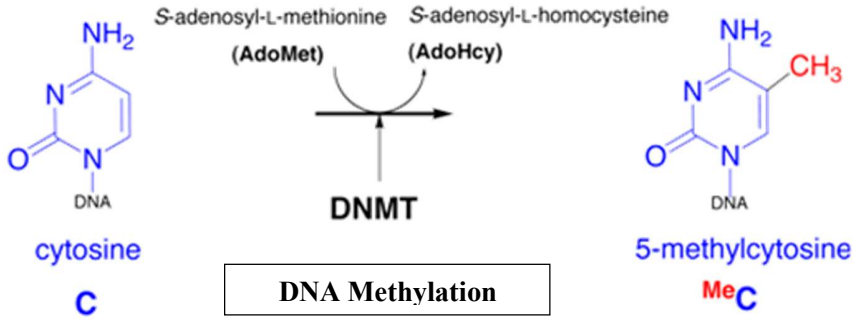
Members of the sirtuin family of NAD-dependent protein deacetylases and ADP ribosyltransferases have been studied extensively as potential anti-aging factors. Interest in this family of proteins in relation to aging stems from a series of studies in yeast, flies, and worms, which reported that the single sirtuin gene of these organisms, named Sir2, had a remarkable longevity activity (Guarente, 2011). Overexpression of Sir2 was first shown to extend replicative lifespan in Saccharomyces cerevisiae (1198 Cell 153, June 6, 2013 ª2013 Elsevier Inc.), and subsequent reports indicated that enhanced expression of the worm (sir-2.1) and fly (dSir2) orthologs could extend lifespan in both invertebrate model systems.

Regarding mammals, several of the seven mammalian sirtuin paralogs can ameliorate various aspects of aging in mice. In particular, transgenic overexpression of mammalian SIRT1, which is the closest homolog to invertebrate Sir2, improves aspects of health during aging but does not increase longevity. More compelling evidence for a sirtuin-mediated prolongevity role in mammals has been obtained for SIRT6, which regulates genomic stability, NF-kB signaling, and glucose homeostasis through histone H3K9 deacetylation. Mutant mice that are deficient in **SIRT6** exhibit accelerated aging (Mostoslavsky et al., 2006), whereas male transgenic mice overexpressing SIRT6 have a longer lifespan. Interestingly, the mitochondria-located sirtuin SIRT3 has been reported to mediate some of the beneficial effects of **dietary restriction (DR)** in longevity, though its effects are not due to histone modifications but, rather, due to the deacetylation of mitochondrial proteins.Very recently, overexpression of **SIRT3** has been reported to improve the regenerative capacity of aged hematopoietic stem cells (Brown et al., 2013). Therefore, in mammals, at least three members of the sirtuin family—**SIRT1, SIRT3 and SIRT6**—contribute to healthy aging.

DNA Methylation:

The relationship between DNA methylation and aging is complex. Early studies described an age-associated global hypomethylation, but subsequent analyses revealed that several loci, including those corresponding to various tumor suppressor genes and **Polycomb target genes(Polycomb-group proteins** are a family of proteins first discovered in fruit flies that can remodel chromatin such that epigenetic silencing of genes takes place. Polycomb-group proteins are well known for silencing **Hox gene**s through modulation of chromatin structure during embryonic development in fruit flies (*Drosophila melanogaster*)**)**, actually become hypermethylated with age (Maegawa et al., 2010).

Cells from patients and mice with progeroid syndromes exhibit DNA methylation patterns and histone modifications that largely recapitulate those found in normal aging. All of these epigenetic defects or epimutations accumulated throughout life may specifically affect the behavior and functionality of stem cells causing **Stem Cell Exhaustion** (described later in the text).

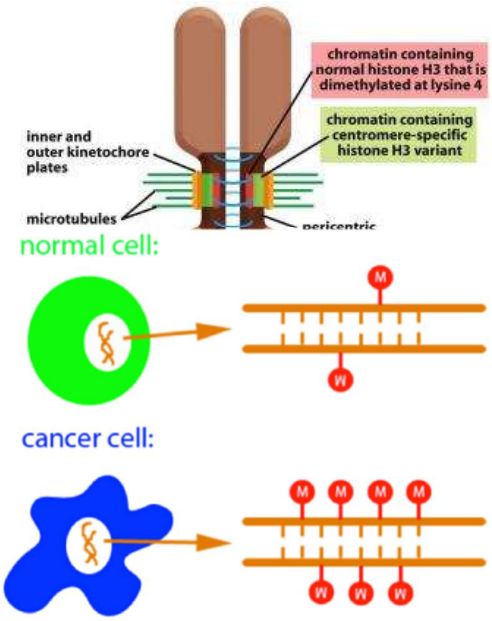


DNA Methylation

Nevertheless, there is no direct experimental demonstration thus far that organismal lifespan can be extended by altering patterns of DNA methylation.

Chromatin Remodelling:

DNA- and histone-modifying enzymes act in concert with key chromosomal proteins, such as the heterochromatin protein 1a (HP1a), and chromatin remodeling factors, such as Polycomb group proteins or the **NuRD complex**, whose levels are diminished in both normally and pathologically aged cells. Along with the above discussed epigenetic modifications in histones and DNA methylation, alterations in these epigenetic factors determine changes in chromatin architecture, such as global heterochromatin loss and redistribution, which constitute characteristic features of aging.



The causal relevance of these chromatin alterations in aging is supported by the finding that flies with loss-of-function mutations in HP1a have a shortened lifespan, whereas overexpression of this heterochromatin protein extends longevity in flies and delays the muscular deterioration characteristic of old age (Larson et al., 2012). Supporting the functional relevance of epigenetically mediated chromatin alterations in aging, there is a notable connection between heterochromatin formation at repeated DNA domains and chromosomal stability. In particular, heterochromatin assembly at pericentric regions requires trimethylation of histones H3K9 and H4K20, as well as HP1a binding, and is important for chromosomal stability (Schotta et al., 2004).

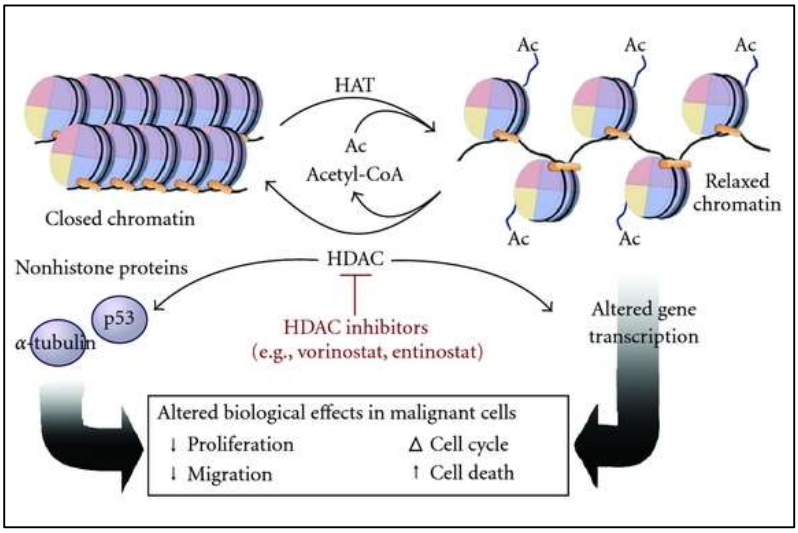
Mammalian telomeric repeats are also enriched for these chromatin modifications, indicating that chromosome ends are assembled into heterochromatin domains. Subtelomeric regions also show features of constitutive heterochromatin, including H3K9 and H4K20 trimethylation, HP1a binding, and DNA hypermethylation. Thus, epigenetic alterations can directly impinge on the regulation of telomere length, one of the hallmarks of aging.

Transcriptional Alterations:

Aging is associated with an increase in transcriptional noise and an aberrant production and maturation of many mRNAs. Microarray-based comparisons of young and old tissues from several species have identified age-related transcriptional changes in genes encoding key components of inflammatory, mitochondrial, and lysosomal degradation pathways (de Magalha˜ es et al., 2009). These aging-associated transcriptional signatures also affect noncoding RNAs, including a class of miRNAs (gero-miRs) that is associated with the aging process and influences lifespan by targeting components of longevity networks or by regulating stem cell behaviour. **Gain- and loss-off function** studies have confirmed the capacity of several miRNAs to modulate longevity in Drosophila melanogaster and *C. elegans*.

Reversion of Epigenetic Changes:

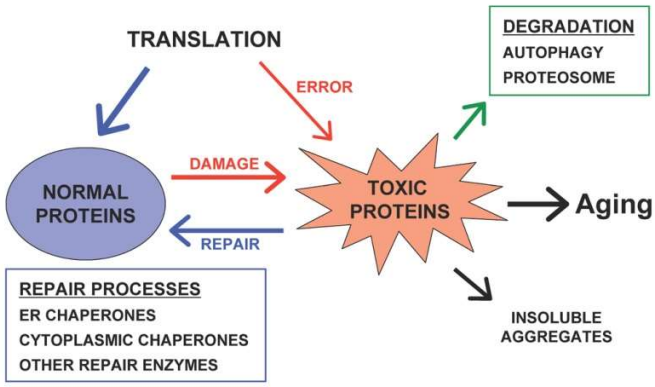
Unlike DNA mutations, epigenetic alterations are—at least theoretically—reversible, hence offering opportunities for the design of novel anti-aging treatments. Restoration of physiological H4 acetylation through administration of histone deacetylase inhibitors avoids the manifestation of age-associated memory impairment, indicating that reversion of epigenetic changes may have neuroprotective effects.



Inhibitors of histone acetyltransferases also improves the premature aging phenotypes of progeroid mice and extend their lifespan (Krishnan et al., 2011). Moreover, the recent discovery of transgenerational epigenetic inheritance of longevity in *C. elegans* suggests that manipulation of specific chromatin modifications in parents can induce an epigenetic memory of longevity in their descendants (Greer et al., 2011). Conceptually similar to histone acetyltransferase inhibitors, histone deacetylase activators may conceivably promote longevity. Resveratrol has been extensively studied in relation to aging, and among its multiple mechanisms of action are the upregulation of SIRT1 activity, as well as other effects associated with energetic deficits.

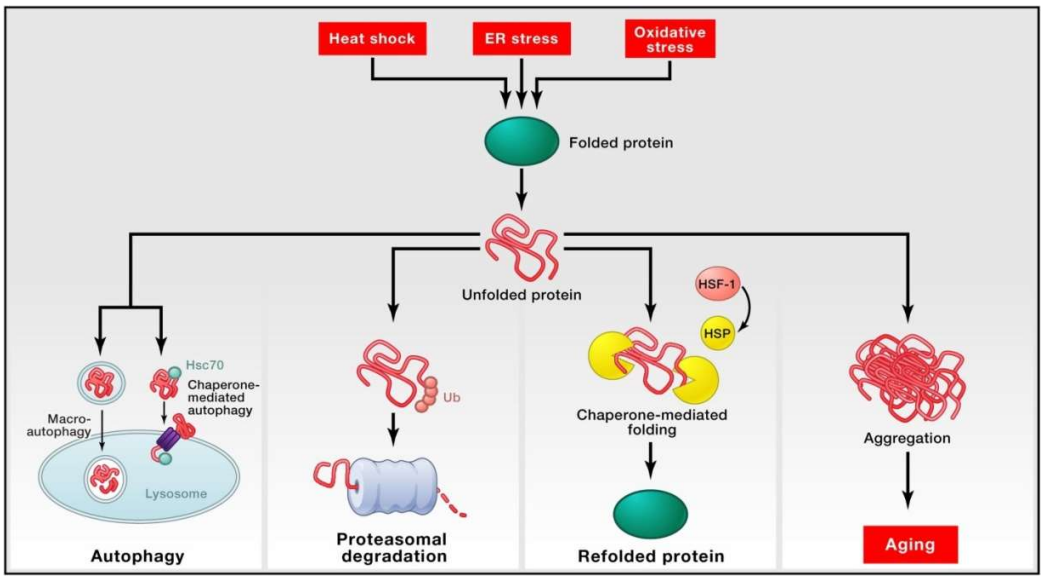
Loss of Proteostasis :

Aging and some aging-related diseases are linked to impaired protein homeostasis or proteostasis (Figure above). All cells take advantage of an array of quality control mechanisms to preserve the stability and functionality of their proteomes. Proteostasis involves mechanisms for the stabilization of correctly folded proteins—most prominently, the heatshock family of proteins—and mechanisms for the degradation of proteins by the proteasome or the lysosome.



Moreover, there are regulators of age-related **proteotoxicity**, such as MOAG-4, that act through an alternative pathway distinct from molecular chaperones and proteases. All of these systems function in a coordinated fashion to restore the structure of misfolded polypeptides or to remove and degrade them completely, thus preventing the accumulation of damaged components and assuring the continuous renewal of intracellular proteins.

Accordingly, many studies have demonstrated that proteostasis is altered with aging (Koga et al., 2011). In particular, transgenic worms and flies overexpressing chaperones are long-lived (Morrow et al., 2004; Walker and Lithgow, 2003). Also, mutant mice deficient in a cochaperone of the heat-shock family exhibit accelerated aging phenotypes, whereas long-lived mouse strains show a marked upregulation of some heat-shock proteins. Moreover, activation of the master regulator of the heat-shock response, the transcription factor HSF-1, increases longevity and thermotolerance in nematodes.

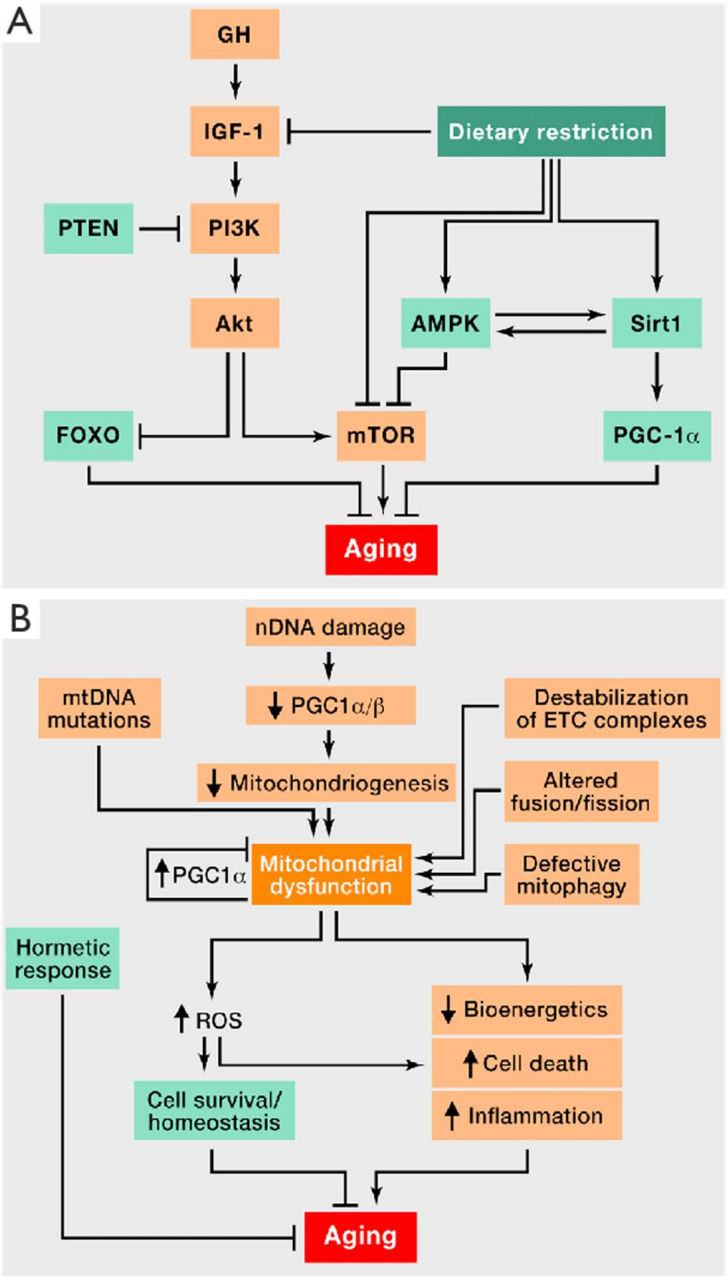


Deregulated Nutrient Sensing :

The somatotrophic axis in mammals comprises the growth hormone (GH), which is produced by the anterior pituitary, and its secondary mediator, insulin-like growth factor 1 (IGF-1), produced in response to GH by many cell types, most notably hepatocytes.

The intracellular signaling pathway of **IGF-1(Insulin like Growth Factor-1)** is the same as that elicited by **insulin**, which informs cells of the presence of glucose. For this reason, **IGF-1** and **insulin signaling** are known as the **insulin and IGF-1 signaling (IIS) pathway**. Remarkably, the IIS pathway is the most conserved aging-controlling pathway in evolution, and among its multiple targets are the FOXO family of transcription factors and the mTOR complexes, which are also involved in aging and conserved through evolution (Barzilai et al., 2012; Fontana et al., 2010;Kenyon, 2010).

Genetic polymorphisms or mutations that reduce the functions of GH, IGF-1 receptor, insulin receptor, or downstream intracellular effectors such as **AKT**, **mTOR**, and **FOXO** have been linked to longevity, both in humans and in model organisms, illustrating the major impact of bioenergetic pathways on longevity.



Mitochondrial Dysfunction

As cells and organisms age, the efficacy of the respiratory chain tends to diminish, thus increasing electron leakage and reducing ATP generation (Green et al., 2011).

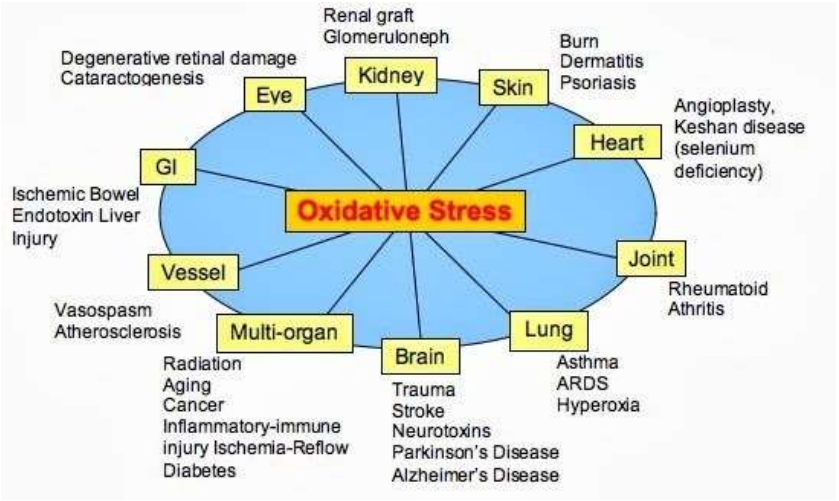
Reactive Oxygen Species:

The **mitochondrial free radical theory of aging** proposes that the progressive mitochondrial dysfunction that occurs with aging results in increased production of ROS, which in turn causes further mitochondrial deterioration and global cellular damage (Harman, 1965).

Of particular impact has been the unexpected observation that increased ROS may prolong lifespan in yeast and *C. elegans* (Doonan et al., 2008; Mesquita et al., 2010; Van Raamsdonk and Hekimi, 2009). Similarly unexpected, genetic manipulations in mice that increase mitochondrial ROS and oxidative damage do not accelerate aging (Van Remmen et al., 2003; Zhang et al., 2009), mice with increased antioxidant defenses do not present an extended lifespan (Pe´rez et al., 2009), and, finally, genetic

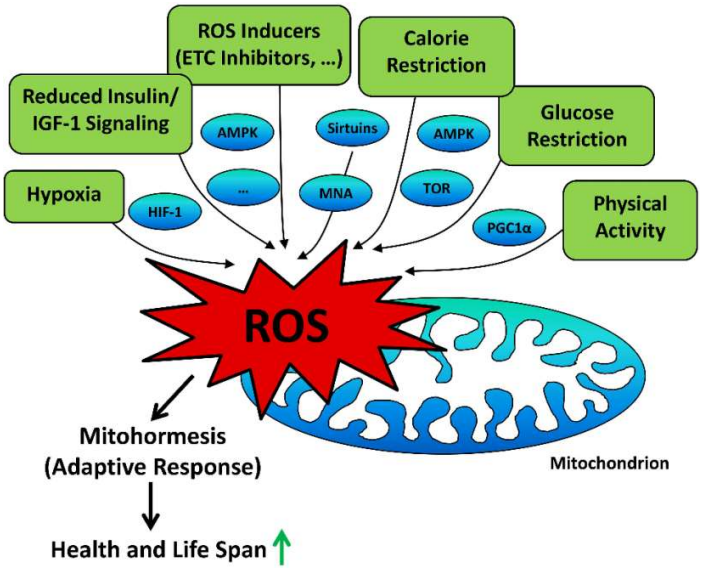
manipulations that impair mitochondrial function but do not increase ROS accelerate aging. These and other data have paved the way to a reconsideration of the role of ROS in aging (Ristow and Schmeisser, 2011). Indeed, parallel and separate to the work on the damaging effects of ROS, the field of intracellular signaling has accumulated solid evidence for the role of ROS in triggering proliferation and survival in response to physiological signals and stress conditions (Sena and Chandel, 2012).

Beyond a certain threshold, ROS levels betray their original homeostatic purpose and eventually detoreorates, rather than decreasing the age-associated damage (Hekimi et al., 2011). This new conceptual framework may accommodate apparently conflicting evidence regarding the positive, negative, or neutral effects of ROS on aging.

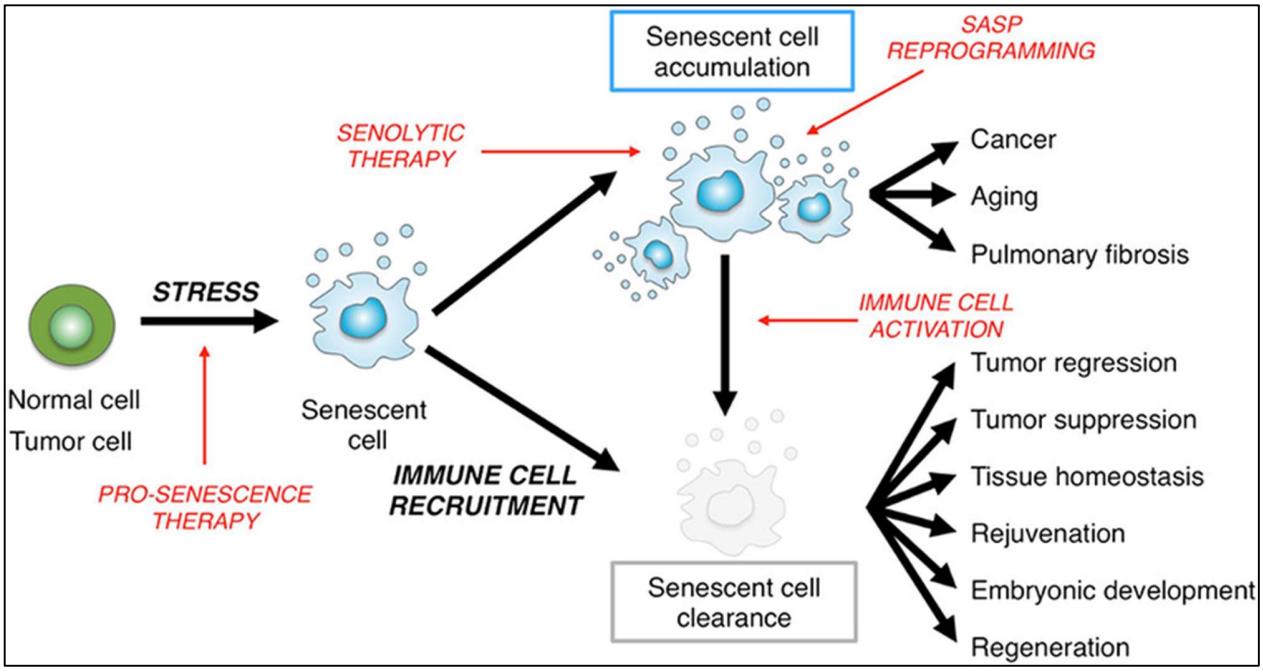


Mitohormesis:

Mitochondrial dysfunctions during aging are also connected with **hormesis**. According to this concept, mild toxic treatments trigger beneficial compensatory responses. Thus, although severe mitochondrial dysfunction is pathogenic, mild respiratory deficiencies may increase lifespan, perhaps due to a hormetic response. Hormetic reactions may trigger a mitochondrial defensive response both in the same tissue in which mitochondria are defective and even in distant tissues, as shown in C. elegans (Durieux et al., 2011). There is compelling evidence that compounds such as metformin and resveratrol are mild mitochondrial poisons that induce a lowenergy state characterized by increased AMP levels and activation of AMPK (Hawley et al., 2010). Importantly, metformin can increase mouse lifespan when administered from early life (Anisimov et al., 2011).



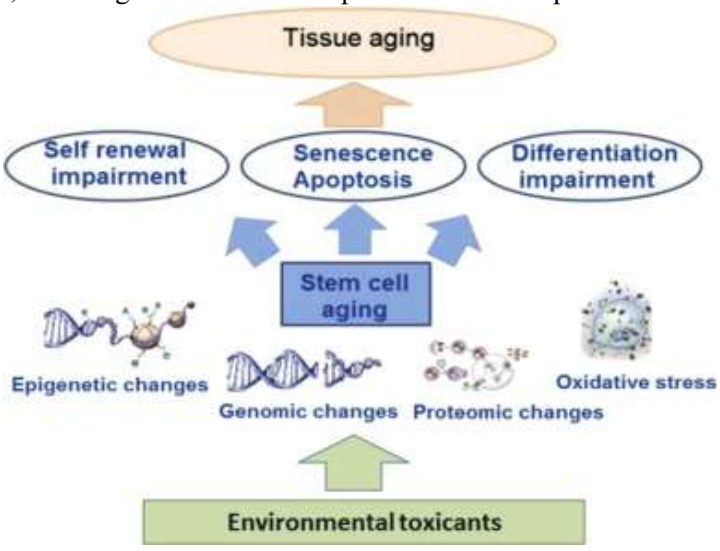
Cellular Senescence



Cellular senescence is one phenomenon by which normal cells cease to divide. This phenomenon was originally described by Hayflick in human fibroblasts serially passaged in culture. Today, we know that the senescence observed by Hayflick is caused by **telomere shortening** (Bodnar et al., 1998), but there are other aging-associated stimuli that trigger senescence independently of this telomeric process. Most notably, nontelomeric DNA damage, which progressively occur with aging, are also capable of inducing senescence.

Stem Cell Exhaustion

The decline in the regenerative potential of tissues is one of the most obvious characteristics of aging. For example, **hematopoiesis** declines with age, resulting in a diminished production of adaptive immune cells—a process termed **immunosenescence**—and in an increased incidence of anemia and myeloid malignancies (Shaw et al., 2010).



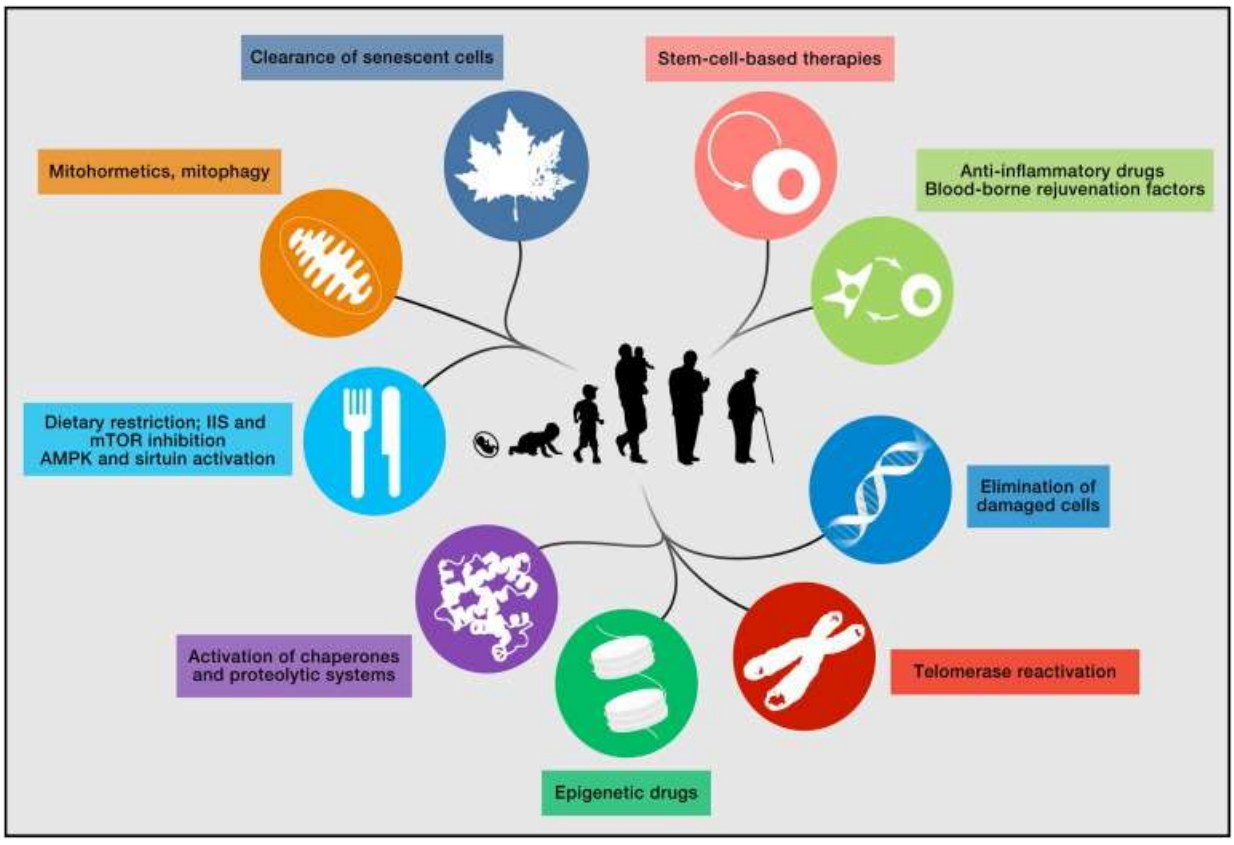
Studies on aged mice have revealed an overall decrease in cell-cycle activity of hematopoietic stem cells (HSCs), with old HSCs undergoing fewer cell divisions than young HSCs. This correlates with the accumulation of **DNA damage** and with the overexpression of **cell-cycle inhibitory proteins** such as **p16INK4a**. Although deficient proliferation of stem and progenitor cells is obviously detrimental for the long-term maintenance of the organism, an excessive proliferation of stem and progenitor cells can also be

deleterious by accelerating the exhaustion of stem cell niches. Also, recent studies have shown that **DR(dietary restriction)** has been reported to increase intestinal and muscle stem functions (Cerletti et al., 2012; Yilmaz et al., 2012). An important debate regarding the decline in stem cell function is the relative role of cell-intrinsic pathways compared to cellextrinsic ones.

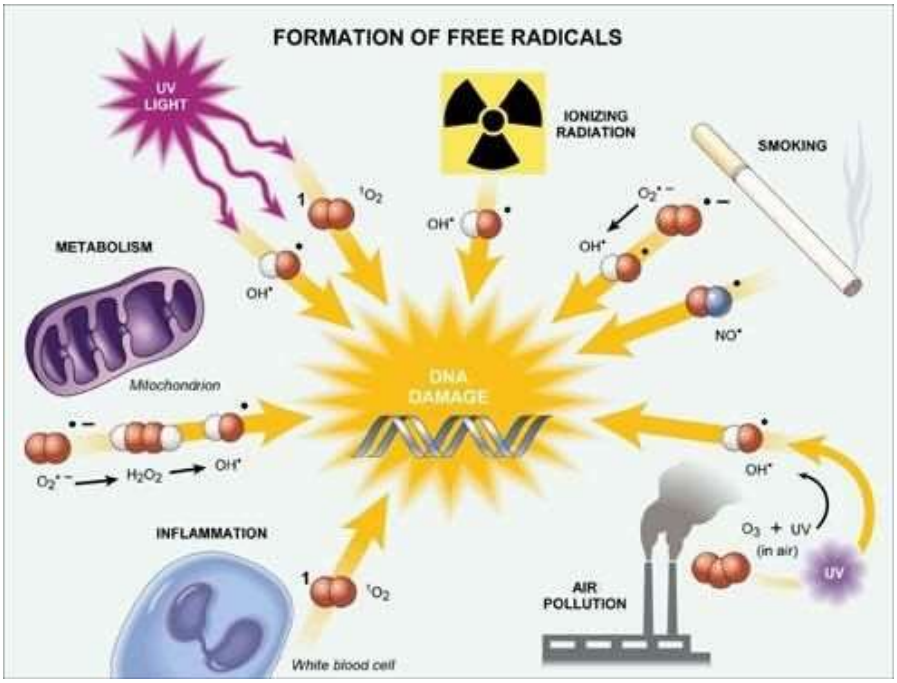
Altered Intercellular Communication

Aging involves changes at the level of intercellular communication, be it endocrine, neuroendocrine, or neuronal. Thus, neurohormonalsignaling (e.g., renin-angiotensin, adrenergic, insulin-IGF1 signaling) tends to be deregulated in aging as inflammatory reactions increase, immunosurveillance against pathogens and premalignant cells declines, and the composition of the peri- and extracellular environment changes.

Inflammation is prominent aging-associated alteration in intercellular communication. It may result from multiple causes, such as the accumulation of proinflammatory tissue damage, the failure of an ever more dysfunctional immune system to effectively clear pathogens and dysfunctional host cells, the propensity of senescent cells to secrete proinflammatory cytokines.



Free Radical Theory of Aging (FRTA)



The **free radical theory of aging (FRTA)**was conceived by Denham Harman in the 1950s, when prevailing scientific opinion held that free radicals were too unstable to exist in biological systems.The free radical theory of aging states that organisms age because cells accumulate free radical damage over time. A free radical is any atom or molecule that has a single unpaired electron in an outer shell. While a few free radicals such as melanin are not chemically reactive, most biologically relevant free radicals are highly reactive.For most biological structures, free radical damage is closely associated with oxidative **damage**.The free radical theory proposes that ageing is the cumulative result of oxidative damage to the cells and tissues of the body that arises primarily as a result of aerobic metabolism.

Several lines of evidence have been used to support this hypothesis including the claims that:

(1) Variation in species life span is correlated with metabolic rate and protective antioxidant activity;

(2) Enhanced expression of antioxidative enzymes in experimental animals can produce a significant increase in longevity;

(3) Cellular levels of free radical damage increases with age; and

(4) Reduced calorie intake leads to a decline in the production of reactive oxygen species and an increase in life span.

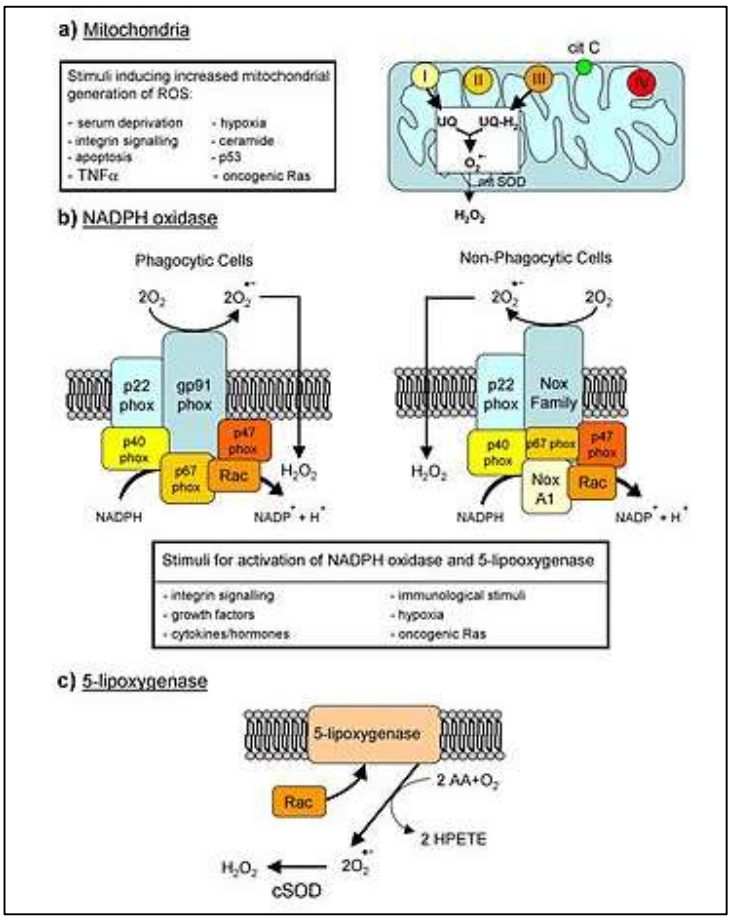
The free radical theory may also be used to explain many of the structural features that develop with ageing including the lipid peroxidation of membranes, formation of age pigments, cross-linkage of proteins, DNA damage and decline of mitochondrial function. Free radicals only occur in trace quantities in biological tissues, their cellular levels and actions cannot be measured in vivo, and definitive proof that oxidised molecules are the primary cause of ageing is lacking. Moreover, ageing is also likely to be a multifactorial process and not reducible to any one single cause.

The free radical theory is only concerned with free radicals such as superoxide (O_2^-), but it has since been expanded to encompass oxidative damage from other Reactive Oxygen Species**(ROS)** such as hydrogen peroxide **(H_2O_2)**, or peroxynitrite **($OONO^-$)**.

he free radical theory was expanded to include not only aging, but also age-related diseases. Free radical damage within cells has been linked to a range of disorders including cancer, arthritis, atherosclerosis, Alzheimer's disease, and diabetes. There has been some evidence to suggest that free radicals and some reactive nitrogen species trigger and increase cell death mechanisms within the body such as **apoptosis** and in extreme cases **necrosis**.

In 1972, **Harman** modified his original theory to what became known as the **mitochondrial theory of aging**. In its current form, this theory proposes that reactive oxygen species that are produced in the mitochondria, causes damage to certain macromolecules including lipids, proteins and most importantly mitochondrial DNA. This damage then causes mutations which lead to an increase of ROS production and greatly enhance the accumulation of free radicals within cells.

Mitochondrial theory of aging was first proposed in 1978, and shortly thereafter the Mitochondrial free radical theory of aging was introduced in 1980. The theory implicates the mitochondria as the chief target of radical damage, since there is a known chemical mechanism by which mitochondria can produce Reactive oxygen species(ROS), mitochondrial components such as mtDNA are not as well protected as nuclear DNA, and by studies comparing damage to nuclear and mtDNA that demonstrate higher levels of radical damage on the mitochondrial molecules.Electrons may escape from metabolic processes in the mitochondria like the Electron transport chain, and these electrons may in turn react with water to form ROS such as the superoxide radical, or via an indirect route the hydroxyl radical. These radicals then damage the mitochondria's DNA and proteins, and these damage components in turn are more liable to produce ROS byproducts. Thus a positive feedback loop of oxidative stress is established that, over time, can lead to the deterioration of cells and later organs and the entire body.



Epigenetic oxidative redox shift (EORS) theory of aging:

Brewer proposed a theory which integrates the free radical theory of aging with the insulin signalling effects in aging. Brewer's theory suggests "sedentary behaviour associated with age triggers an oxidized redox shift and impaired mitochondrial function". This mitochondrial impairment leads to more sedentary behaviour and accelerated aging. (refer note on **Cellular Basis Of Aging**)

Ageing of connective tissue

The biological mechanisms of ageing are poorly understood. One of the few areas in which reasonable progress is being made is in the age-related changes in connective tissues. This is understandable since connective tissues are involved in many of the gross manifestations of ageing such as wrinkling of the skin, physical disabilities of joints, and vascular disease. Even here, however, we are only beginning to

learn of the nature of the age changes in precise biochemical and biophysical terms. Only recently has our knowledge of the connective tissue components collagen, proteoglycans, and elastin progressed to the point where we can frame sound questions about their changes with age. It is important to attempt to define ageing since many studies, supposedly describing age changes, deal with changes that are more reasonably regarded as being due to growth and maturation. There is no consensus on which definition should be adopted but Strehler (1962) has suggested criteria which any change must meet if it is to be regarded as a true ageing event. True ageing changes should be (1) universal-that is, they must occur in all members of the population; (2) intrinsic to the organism that is, a change must not be a function of diet, disease, environment, or other external influences; (3) progressive; and (4) deleterious to the organism. While the applicability of such criteria can be questioned they nevertheless provide a framework for assessing whether changes are true age changes. In certain instances, particularly at a molecular level, it is difficult to determine whether changes might be deleterious. This is because their implications in terms of the function of a tissue or an organ are not always clear. Another problem in studies of ageing is to differentiate between chronological and physiological age. This is particularly difficult in randomly bred (human) populations where individuals vary widely in their physiological capabilities at any particular chronological age. This may be less of a problem in highly inbred animal strains where changes tend to be displayed more uniformly. The difficulties of deciding true physiological age may be insuperable. Chronological age may then be the only measurement that can be made. In general, it may be assumed that ageing starts after growth and maturation have ceased-at, say, 20-25 years of age for humans-and that it will go on progressively. Although geriatricians tend to be more concerned with physical and mental deterioration during the last 10 to 20 years of the human lifespan, this period may not be so important from the point of view of true ageing processes. Of more interest may be the changes from maturity until 60 to 65 years of age. During this period changes due to ageing proper occur, making a person more susceptible to disease and to other injurious factors. Connective tissues such as tendon, skin, bone, and cartilage perform a number of mechanical functions. Other connective tissues may fulfil different roles. The properties of each depend on the nature of its components-whether collagen, proteoglycan, elastin, or glycoprotein (Jackson, 1978; Bailey, 1978; Ireland, 1 978; Muir, 1978)-and on the ways in which they interact. It is generally accepted that the tensile strength of connective tissue depends on the collagen component and that resistance to compression is conferred by the proteoglycans (see Kempson, 1975). Proteoglycans are also important in determining the transport properties of the tissue towards compounds such as salts and water, hormones, waste products, and gases (Preston and Snowden, 1972; Maroudas, 1973; Glatz and Massaro, 1976). Changes in the functional properties of connective tissues play a part in certain disease processes. These changes and the underlying biochemical alterations are therefore of considerable interest. For example, mechanical factors play a part in the osteoarthrotic breakdown of human articular cartilage. Since the incidence of osteoarthrosis increases with age, knowledge of changes with age in the mechanical and biochemical properties of cartilage facilitates the definition of changes of importance in the pathogenesis of osteoarthrosis. Although alterations in the connective tissues, particularly cross-linking in collagen and elastin, were once thought to be causative factors in ageing this concept is now largely abandoned. But this does not diminish the potential importance of age-related alterations in connective tissues in the pathogenesis of disease. The connective tissues are a heterogeneous group in terms of their function, appearance, and biochemical constitution. It would be impracticable to discuss here age changes in every individual component of each tissue. We shall therefore review selected reports of biochemical and other changes in connective tissue components that may indicate general trends. As a specific example of how biochemical and mechanical properties are related and of how they may be related to disease we shall discuss the hypothesis that fatigue failure in the collagen network of articular cartilage may be a primary event in some forms of osteoarthrosis. Biochemical changes in constituents of connective tissues with age COLLAGEN Older studies dealt with the possibility that crosslinking between individual collagen molecules increases with age (Sinex, 1968; Kohn, 1971; Hall, 1976). However, almost all the early evidence is indirect.Manyfindings may be interpreted as evidence of change in the type of cross-link, with stabilisation of pre-existing cross-links, rather than as an increase in their number (Bailey and Robins, 1973; Bailey et al., 1974; Bailey and

Robins, 1976; see A. J. Bailey (Bailey, 1978) at page 49, and D. S. Jackson (Jackson, 1978) at page 44). That excessive cross-linking could be deleterious is illustrated by the mechanical properties of tanned leather. Tanned leather contains extra, artificial cross-links which make the material more rigid than normal; but tanned leather has a lower tensile strength than untanned leather. The tanned collagen fibres cannot adapt to local stresses because of restrictions imposed by the presence of the extra cross-links (Harkness, 1971). Although much is known of the chemical nature of the initial stages in the cross-linking of collagen in a wide variety of tissues (Bailey, 1978) the crosslinks in mature tissues have not yet been characterised. As tissues mature the reducible cross-links, whose structures are nowknown, gradually disappear. There is no fall in the extent of cross-linking so therefore the reducible cross-links must be converted to more stable, non-reducible forms. As yet there is no consensus on the mechanism of stabilisation of the reducible cross-links. Reduction of the aldimine type cross-links may occur in vivo (Mechanic et al., 1971; Deshmukh and Nimni, 1972) although Bailey and his colleagues have been unable to confirm this (Robins et al., 1973). The disappearance of reducible cross-links is more or less complete in the mature animal (Robins et al., 1973). However, there are changes in the properties of collagen between maturity and old age that suggest that its cross-linking characteristics may change during the ageing phase of the lifespan as well as during growth and maturation. For example, Steven (1966a, b) demonstrated changes between the ages of 25 and 65 years in the nature of the crosslinks in human achilles tendon collagen: the changes were determined by a combination of chemical and enzymic techniques. Further, Hamlin and Kohn (1971; 1972) have shown increasing resistance of human diaphragm tendon collagen to attaek by bacterial collagenase between maturity and old age. It is clearly important to establish the chemical nature of the cross-links formed during maturation of collagen and to determine whether changes take place during ageing, after maturity. It is also important to learn whether the collagens of different tissues are affected similarly during ageing. To understand the relationship between possible age changes in collagen cross-linking and the mechanical properties of collagen fibres is an important goal. Hall (1976) and Piez (1969) have suggested the opposite age-related change of increased crosslinking-that is, cleavage of peptide bonds or crosslinks, or both. Although such a mechanism could have important mechanical consequences-for example, in influencing fatigue failure of articular cartilage-there is little supporting biochemical evidence for this view. The racemisation of amino-acids of collagen from the L form to the D form (Helfman et al., 1977) may occur with age. All amino-acids with the exception of glycine possess an asymmetric and hence an optically active carbon atom. Thus there are both D- and L-diastereoisomers of amino-acids. But only L-amino-acids are incorporated into protein in mammalian systems. Once incorporated into proteins racemisation at the alpha-carbon can occur. The rate of racemisation is temperature dependent and it has been shown that for aspartic acid in human dentine and enamel proteins the rate of racemisation is such as to cause an enrichment in the D-aspartic acid content of about 0-1 % per year (Helfman et al., 1977). By the age of 60 to 70 years about 6-7 % of the aspartic acid, in a metabolically stable protein, could be in the D form. Racemisation also occurs in other amino-acids but at a lower rate.

The presence of D-amino-acids would tend to disrupt the hydrogen bonding that stabilises the secondary and tertiary structure of polypeptide chains in proteins, leading to alterations in the conformation of molecules. Presumably such alterations in conformation would result in alterations

in functional properties of the protein although there is no information on the nature of this change.

Although racemisation might occur in any protein significant effects would be observed only in proteins turned over very slowly or not at all. Collagen and elastin could be particularly affected during ageing. Tissues other than enamel and dentine must be examined before the significance of this mechanism can be assessed.

Other changes in collagen with age such as an increase in the amount of hexose bound to the e-NH2 groups of lysine and hydroxylysine residues (Robins and Bailey, 1972), the apparent occurrence of intramolecular pseudopeptide bonds between E-NH2 groups of lysine or hydroxylysine and the side chain

COOH groups of aspartic and glutamic acid (Steven et al., 1972), or the existence of other types of cross-link (Hall, 1976) have been reported. Their functional significance is not clear. The binding of hexose to the E-NH2 groups of lysine and hydroxylysine does not appear to play any part in linking other connective tissue components (glycoproteins or proteoglycans) to collagen (Bailey et al., 1974). Steven et al. (1972) suggested that intramolecular pseudopeptide bonds could reduce the hydrophilic nature of the collagen polymerised into fibrils. The functional significance of this reduction is not clear.

High-angle x-ray diffraction experiments have provided evidence that the amianthoid change (fibrillation) during ageing in costal cartilage corresponds to an increased orientation of collagen fibrils in the affected areas (Hukins et al., 1976). The collagen in such areas appears to have a normal periodicity, as judged by low-angle x-ray diffraction patterns and electron microscopy, but the fibrils have unusually large diameters. These large diameter fibrils are probably a result of fusion of smaller fibrils with their axial banding patterns in register. Studies of tissues such as skin and tendon have also demonstrated an increase in the mean diameter of collagen fibrils with age and a greater spread of values about the mean (Hall, 1976).

PROTEOGLYCANS:

Considerable scope exists for age-related changes inthesecomplex connective tissue components. Much of the present evidence on age-changes in proteoglycans has been derived from cartilage, particularly articular cartilage, because of the increased incidence of osteoarthrosis with age. There are reports of alterations in the relative proportions of different glycosaminoglycans as a function of age in costal cartilage (Mathews and Glagov, 1966), knee joint cartilage (Greiling and Baumann, 1973), and human intervertebral disc (Buddecke and Sziegoleit, 1964; Gower and Pedrini, 1969; Szirmai, 1970). Adams amd Muir (1976) noted progressive changes in chondroitin sulphate to keratan sulphate ratios in the nucleus and annulus of lumbar intervertebral discs but the changes with respect to site did not follow the same pattern at different ages. In the spines from young persons (aged 8 and 16 years) the keratan sulphate to chondroitin sulphate ratio tended to decrease in the annuli of discs taken from progressively lower regions of the spine and this ratio tended to increase in the nuclei of discs from progressively lower regions. In a spine from a person aged 44, however, the ratio decreased progressively down the spine in both annulus and nucleus. The fact that in general the ratio of keratan sulphate to total chondroitin sulphates increases with age may be explained by the possibility that proteoglycan subunits from older specimens are altered. Their molecular size may be reduced owing to a decrease in the number of chondroitin sulphate chains attached to the protein core whereas the number of keratan sulphate chains is unaffected (Inerot et al., 1978). Buddecke et al. (1973) showed that such variations in the relative proportions of different glycosaminoglycans are not features of all connective tissues. They found no change in the relative proportion of different glycosaminoglycans in bovine thoracic aorta up to 13 years of age. It is therefore certain that changes in the proteoglycans occur with age in certain tissues. The ability of the proteoglycans to form the large aggregates, normally present in young tissues, and their capacity to interact with collagen may also alter with age. gimCunek and Muir (1972) showed that the amount of proteoglycan extracted from pig articular cartilage by iso-osmotic sodium acetate decreased considerably over the first 3 years of life but then remained more or less unchanged to 5 years of age. The amount of proteoglycan remaining bound to the collagen after two further extractions with 2M CaCl2 increased up to 3 years of age and then remained constant to 5 years of age. This indicated that the proteoglycans became more tightly bound to collagen up to 3 years of age. Similarly, Quintarelli et al. (1975) showed that less rabbit costal cartilage proteoglycan could be extracted using 4M guanidinium chloride at 4 years of age than at 1 month of age. Adams and Muir (1976) have described similar changes in the extractability of proteoglycans from human lumbar discs between the ages of 8, 16, and 44 years, indicating that the proteoglycans interact more strongly with collagen with advancing age in both the nucleus and the annulus. These observations are important but difficult to interpret. The oldest of the animals tested by SimCunek and Muir (1972) and by Quintarelli et al. (1975) cannot be regarded as being

truly old for the species. The maximum lifespan of the rabbit is about 12 years and of the pig about 20 years (Comfort, 1964). The oldest of the human samples studied by Adams and Muir (1976) was only 44 years, and the 16-year-old sample cannot be regarded as being from a mature adult. Thus the changes described might be attributable to growth and maturation rather than to ageing. Until more detailed experiments are performed it remains difficult to make an accurate assessment. Such problems of interpretation arise in many studies of ageing. It is often impossible to examine enough points in a lifespan and there may be inadequate baseline data for the mature adult. Proteoglycan subunits interact with each other as well as with collagen, and this second type of interaction is affected by age. Adams and Muir (1976), for example, showed that when proteoglycans extracted from human discs of the spine of a 44- year-old were chromatographed on agarose columns much smaller amounts of proteoglycans were excluded from the gel compared with those from discs from a 16-year-old and an 8-year-old. Also, of the proteoglycans that were retarded by the gel those from older discs were smaller than those from younger discs. Thus, while increasing age is associated with an increased interaction between proteoglycans and collagen it is also associated with a diminished capacity to form aggregates among proteoglycans that do not interact with the collagen. Perricone et al. (1977) examined the aggregation behaviour of proteoglycans extracted from cartilages from aged persons. The cartilage appeared morphologically and histochemically normal. They found that whereas proteoglycans from other sources exist to a considerable extent as large aggregates the proteoglycans from aged hip cartilage (pooled tissue from three individuals, aged 69, 76, and 81 years) were not aggregated to any significant extent as judged by chromatography on agarose columns. Furthermore, the size of the proteoglycans was not affected by digestion with hyaluronic acid ,ll-3 hydrolase, indicating that the proteoglycans were not complexed to hyaluronic acid. When isolated proteoglycan subunits from hip cartilage were incubated with hyaluronic acid little change in the elution patterns from the agarose columns was effected, in contrast to the pronounced aggregation seen when hyaluronic acid was mixed with proteoglycan subunits from normal bovine knee joint cartilage. Thus, the proteoglycans from aged human hip joint and older lumbar discs appear unable to aggregate with hyaluronic acid, probably because of a defect in the hyaluronic acid binding region of the protein core of the proteoglycans (Adams and Muir, 1976; Perricone et al., 1977). Proteoglycans extracted from human articular cartilage of different ages were studied by Baylissand Ali (1978). Using density gradient centrifugation techniques, they showed that whereas the bulk of the proteoglycans from young (16-year-old) femoralhead cartilage were of high density and had a low protein to uronic acid ratio those from a 78-year-old specimen were of lower density and had a higher protein to uronic acid ratio. Similar differences were noted between talus cartilage from a 5-year-old and from a 64-year-old. Significant differences were also apparent between the 5-year-old and a 12-year-old but in this case the density gradient pattern for the 12-year-old was intermediate between the two extremes. In contrast to Adams and Muir (1976) and to Perricone et al. (1977), Bayliss and Ali (1978) found evidence for aggregation of proteoglycans in old cartilage, although the high density aggregates observed in young cartilage were absent. The differences in density between proteoglycans from young and old specimens appeared to be a function of the differing protein-to-glycosaminoglycan ratios. However, few specimens were examined in this further study; moreover, no fully mature young adult specimens were included, making it difficult to assess whether the changes described are associated with maturation or with ageing proper. Changes throughout a lifespan in the properties of proteoglycans from canine articular cartilage were studied by Inerot et al. (1978). Samples were examined from animals aged 4 5 months, 6 months, 8 months, 12 months, 17 months, 60 months, 96 months, and 126 months. The size of the proteoglycan subunits decreases with age. The size of the chondroitin sulphate chains did not alter appreciably, indicating that the proteoglycan subunits were smaller in older animals because of a reduction in the number of chondroitin sulphate chains attached to the protein core of each proteoglycan subunit. This reduction in the number of chondroitin sulphate chains was reflected in an increased content of keratan sulphate in the extracted proteoglycans. In keeping with other studies, the amount of proteoglycan extracted from the tissue decreased progressively with age, indicating a tighter binding to the collagen. In keeping with the findings of Bayliss and Ali (1978), however, Inerot et al. (1978) concluded that there was no appreciable change with age in the ability of the extracted proteoglycans to bind to hyaluronic

acid as measured by chromatography on agarose columns. This suggests that proteoglycans subunits at all ages possess an intact hyaluronic acid binding region. Also in keeping with the study of Bayliss and Ali (1978) the buoyant density of the extracted proteoglycans decreased with age and the protein content increased. Changes such as those described above in the relative proportions of different glycosaminoglycans and in the aggregation behaviour of proteoglycans would be expected to affect the mechanical properties of tissues. Thus the decrease with age in the number of chondroitin sulphate chains attached to proteoglycan subunits may account for the increase in the compliance of cartilage with age found by Armstrong et al. (1977) and Armstrong (1977). The chondroitin sulphate-rich region is probably essential for this property because of its high content of negatively charged groups. Changes in the relative proportions of the individual glycosaminoglycans are also likely to alter the transport of substances through the extracellular matrix. ELASTIC FIBRES The elastic fibres of tissues such as the major elastic arteries and lung consist of elastin filaments and a microfibrillar glycoprotein surrounding these filaments (see A. J. Bailey (Bailey, 1978) at page 49). The characterisation of the basic structure of elastin has been greatly hampered by its extreme insolubility even under very harsh conditions, and subsequent studies of changes with age and disease have also been rendered difficult. Elastic fibres are often said to take on a more frayed, split, and fragmented appearance with increasing age and the elastin becomes more brittle. Such changes have led to the concept that wear and tear takes place in elastic fibres and that there must be profound underlying chemical changes. The nature of these changes and their functional implications remain unresolved. The biochemical studies that have been carried out into age changes in elastin have been limited to gross compositional analyses. Another difficulty, which particularly affects studies on human vascular tissue, is that of knowing whether observed changes are actually due to ageing or to changes wrought by the increasing incidence of atheromatous lesions with advancing years (see C. 1. Levene (Levene, 1978) at page 165). Much of the literature on age changes in elastin has been reviewed by Hall (1976) and Sandberg (1976). It seems to be generally accepted that the elasticity of elastic tissues decreases with ageing, although changes in the mechanical properties of whole tissues such as elastic arteries are complex, affecting collagen, elastin, and microfibrillar glycoprotein. The elastin content of tissues can change, as in hypertension, when there is an increased elastin content (as well as an increased collagen content) in the hypertensive state as compared to normotensive controls (Sandberg, 1976). This increase in elastin content in large vessels is associated with an increase in lamellar units. The elastin content of different regions within the same organ may not show identical changes, however. For example, John and Thomas (1972) observed an increase with age in the elastin content of the visceral pleura of human lung expressed as a percentage of the total dry weight of the tissue but the content of elastin in parenchyma remained constant. Studies on changes with age in the composition of elastin have yielded variable results (Sandberg, 1976). The discrepancies between results are, in all likelihood, explained, firstly, because different methods for elastin purification allow the removal of other proteins to a greater or lesser extent and, secondly, because the efficacy of the extraction procedures varies according to the age of the subject. John and Thomas (1972) studied the chemical composition ofelastins from human pulmonary tissues and aorta throughout the lifespan. They observed that in elastin from visceral pleuras the aspartic acid, glutamic acid, lysine, and arginine contents increased from the end of the second decade onwards while the content of serine and threonine increased after the fifth decade. The glycine, alanine, and valine contents also tended to decrease slightly in the very old specimens. The total carbohydrate of these preparations was shown to increase from the third decade onwards. Similar compositional changes were noted in elastins from lung parenchyma and from aorta. Keeley and Partridge (1974) and Spina and Garbin (1976) observed compositional changes similar to those described by John and Thomas (1972) for human aorta as a function of age. However, such compositional changes were shown to be due to contaminating collagen and glycoproteins. Treatment of the tissue (Keeley and Partridge, 1974) or treatment of elastins purified by standard procedures (Spina and Garbin, 1976) with EDTA before applying standard purification procedures resulted in elastin preparations which showed no significant compositional changes with age. Spina and Garbin (1976) determined the number of N-terminal amino-acid residues in elastin preparations before and after EDTA treatment. Treatment did not decrease the number of Nterminal residues in young adult elastin (17-27

years old) but almost halved the number of N-terminal residues in old elastin (54-74 years old). This indicated that EDTA removed polypeptides with exposed N-terminal residues; further characterisation of the extracted material showed that it consisted of elastin degradation products and polar glycoproteins. It was estimated that the contamination of the young (17-27 years old) elastin preparations amounted to about 80/ of the total weight of material. At 32-48 years the contamination was 14%, and at 54-74 years 17%. Removing the non-elastin contaminants from elastin apparently becomes more difficult as age increases. Spina and Garbin (1976) found elastin degradation products (EDP) in their elastin preparations. That might be evidence in support of the concept that cleavage of peptide bonds occurs in connective tissue proteins with increasing age (Piez, 1969; Hall, 1976). However, in view of the drastic treatments (autoclaving in water at 120°C for 24 hours or treatment with 01 N NaOH at 100°C for extended periods) this point requires further investigation. Peptide bond cleavage is almost certain to occur under these conditions. Even if such a mechanism of deterioration can be demonstrated the extent of degradation and its functional significance will have to be determined. John and Thomas (1972) also suggested that the cross-linking of elastin may change as a function of age. Thus the content of the cross-linking aminoacids desmosine plus isodesmosine (Bailey, 1978) in lung visceral pleuras and parenchyma was constant up to about 40 years but declined progressively after that. The content of another cross-linking amino-acid, lysinonorleucine, was constant up to this age but it increased after the third decade and was again constant thereafter. Changes were also observed in the aorta, but in this case desmosine plus isodesmosine did not decrease until after the sixth decade although the lysinonorleucine content increased after the third decade and then remained constant. Although some of the decrease in desmosine plus isodesmosine can be attributed to a dilution caused by the increase in bound glycoprotein, this contamination was insufficient (John and Thomas, 1972) to account for all the decrease. Keeley and Partridge (1974) and Spina and Garbin (1976) concluded that the content of desmosine plus isodesmosine remained constant with age and that any decrease could be entirely accounted for by dilution of the elastin by contaminating glycoproteins. Thus the amino-acid compostion and cross-link patterns of elastin do not change with age, but with increasing age the non-elastin and elastin components of elastic tissues become more difficult to separate. This tighter binding of elastin with other proteins 179 may be responsible for the stiffening and loss of long-range elasticity of elastin with age. Critical evaluation of such a mechanism is still awaited. Clearly there is no increased cross-linking of elastin with advancing age by mechanisms involving the known Jysine-derived cross-links. It has been recognised for some time, however, that elastin preparations contain certain fluorescent compounds and that fluorescence of elastin preparations increases with age (Hall, 1976; John and Thomas, 1972). The fluorescence of elastin may be due to the presence of cross-linking compounds derived from tyrosine, such as di-tyrosine and quinones, although there have been some suggestions that di-tyrosine may be present in contaminating glycoproteins rather than elastin itself (Hall, 1976). The possibility of such cross-links does not appear to have attracted very much attention, however, and more detailed characterisation of these putative cross-links, such as the isolation and characterisation of di-tyrosine or quinone-containing peptides which can be shown to be derived from elastin, will be required before their role in the ageing of elastin can be assessed. A further progressive change with age that affects the physical properties of large arteries, particularly the aorta, is an increase in the degree of calcification occurring preferentially in the elastic lamellae (Hall, 1976). The calcium is deposited as calcium phosphate in the form of apatite crystallites. Studies of purified elastins from aorta have shown that there is an increase in the calcium content of elastin with age (see Kohn, 1971; Hall, 1976; Keeley and Partridge, 1974; Spina and Garbin, 1976). Spina and Garbin (1976) thought that there might be two forms of calcium in elastin preparations. They were unable to remove all the calcium from their elastin preparations even after prolonged extraction with EDTA. The proteins extracted by EDTA contained calcium. The question arises whether the calcium in elastins prepared by the usual methods (which do not include EDTA extraction) is actually bound to the elastin or whether the preparative procedures are simply inadequate to solubilise apatite crystals even if the clacium is not bound to elastin. Keeley and Partridge (1974) argue that elastin preparation methods such as digestion in hot alkali to remove non-elastin contaminants may not in any event be expected to solubilise apatite crystals. Evidently the increase in the calcium content is concomitant with changes in amino-acid composition due

to non-elastin proteins. Keeley and Partridge (1974) conclude that the increase in contamination of elastin preparations may occur because of the presence of apatite crystals which bind the contaminating proteins. They do not favour the view that the presence of contami-nating protein bound to elastin may serve as nucleation centres for calcium phosphate deposition. Another approach used in studying the relationship between calcium and elastin has been that of determining the ability of elastin preparations to undergo mineralisation in vitro. A number of studies have led to the suggestion that the calciumbinding capacity of elastin increases progressively with age. However, such studies are again difficult to interpret owing to the problems of determining whether the elastin preparations are pure or whether they still contain non-elastin proteins. Seligman et al. (1975), for example, carried out experiments to determine the kinetics of mineralisation of human aortic elastin of different ages. The rate of mineralisation of elastin increased with age. In elastin from young people there was a lag period, when the rate of mineralisation was low, followed by a period of sharp increase in the rate of mineralisation. This lag period decreased with age and was virtually abolished in elastins from older persons. However, the elastin preparations from the older subjects had amino-acid compositions which suggested contamination by non-elastin proteins. It is therefore difficult to assess the role of elastin itself in the changes observed in the kinetics of mineralisation. Important questions concerning age changes in elastin remain unresolved. Answers to these problems would be easier to obtain if more satisfactory procedures for the purification of this protein in its native state could be devised. GLYCOPROTEINS The connective tissue glycoproteins are poorly characterised, although significant progress has been made in recent years (Anderson, 1976). It is not surprising that there have been few studies of agerelated changes in the connective tissue glycoproteins. The stabilisation of aggregates between proteoglycan subunits and hyaluronic acid in cartilage requires the participation of glycoprotein 'links' (Muir, 1978). Perricone et al. (1977) studied the proteoglycans extracted from aged human hipjointcartilage. They concluded that the glycoprotein 'links' generally present in the proteoglycans extracted from normal young tissues are absent from the proteoglycans extracted from aged hip joint cartilage. Presumably the region of the protein core which binds the glycoprotein 'links' was also absent in these proteoglycans as well as that region that binds hyaluronic acid. Another possibility is that the cartilage cells in older subjects do not synthesise the glycoprotein links in the same manner as the cells of young cartilage. A study of the glycopeptides released by papain J. D. Schofield and B. Weightman digestion of human lumbar intervertebral discs (Pearson et al., 1972) indicated that the glycoprotein content of both the annulus and the nucleus rose sharply after the fourth decade relative to both collagen and proteoglycans. Galactose, mannose, fucose, and glucose were identified as the major neutral sugars in the disc glycoproteins. The composition of the glycoproteins apparently changed with age in that the relative amounts of these sugars altered. The finding of glucose in the papainreleased glycoproteins is unusual. It is not a common constituent of glycoproteins in animal tissues. However, the glycoprotein fraction in discs was not fully characterised and the exact nature and role of this fraction in discs remains to be demonstrated. The glycoprotein fraction will probably include the glycoprotein 'link' components of proteoglycan complexes. The degree of heterogeneity of the proteins in this fraction was not investigated. The binding between elastin and non-elastin glycoprotein components becomes stronger with ageing. Although a fraction enriched in polar glycoproteins was isolated by EDTA extraction by Spina and Garbin (1976), characterisation of this fraction was incomplete. Probably much of the glycoprotein associated with old elastin is related to the microfibrillar glycoprotein component of elastic fibres, which has been partially characterised (Ross and Bornstein, 1970). Connective tissues relatively rich in glycoproteins include the basement membranes (see J. T. Ireland (Ireland, 1978) at page 59). Although the glycoproteins of these tissues have been analysed their exact nature and functional role are not fully understood. The glycoprotein components change during growth and maturation but little attention has been paid to change during ageing. Morphological and histochemical evidence suggest chemical alterations in basement membranes during ageing, possibly affecting non-collagen glycoproteins. Some basement membranes increase in thickness with age; the range of values about the mean also increases (Kilo et al., 1972; Darmady et al., 1973; Leuenberger, 1973; Regnault and Kern, 1974). The changes are complex. The thickening may be focal, the membrane may reduplicate, and vacuolation is often present in older animals. The lamina densa

increases in thickness with age at the expense of the laminae rarae. The PAS-positive components of renal basement membranes increase in prominence with age; the Alcian blue-staining components decrease (Ashworth et al., 1960; Rosenquist and Bernick, 1971). ThePAS reaction is greatly reduced by prior trypsin but not by collagenase treatment, indicating the noncollagen glycoprotein nature of the PAS-positive material (Rosenquist and Bernick, 1971). Colloidal iron stains the basement membranes of young but not of older animals. These findings together with the observation that neuraminidase treatment reduces staining in young animals indicate that sialic acidcontaining glycoproteins are present in young basement membranes but absent in old. Detailed biochemical investigations of age changes in basement membranes are still required. Changes in connective tissue cells with age The biosynthesis of connective tissue components has been investigated in relation to age. The results for human skin collagen suggest little change in the rate of synthesis after the age of 10 to 20 years although collagen synthesis continues during this period. Uitto (1971) measured the rates of collagen synthesis in human skin by examining the incorporation of 14C-proline into polypeptides and the synthesis of 14C-hydroxyproline. He also used prolyl hydroxylase activity as an index of the rate of collagen synthesis and showed that the rates of synthesis are high in fetal and very young tissues but fall rapidly over the first 10 to 20 years of life; they remain constant thereafter. Similar conclusions have been reached from studies of the activities of prolyl hydroxylase, lysyl hydroxylase, collagen glucosyltransferase, and collagen galactosyltransferase in human skin by Tuderman and Kivirikko (1977), Anttinen et al. (1973), and Anttinen et al. (1977). Risteli and Kivirikko (1976) also examined the activities of these four enzymes involved in collagen synthesis in rat liver up to the age of 420 days. Although 420 days cannot be regarded as truly old the results were similar to those for human skin. They showed that all enzyme activities were constant between 75 days and 420 days, having fallen from relatively higher levels earlier in the lifespan. An interesting aspect of the study by Tuderman and Kivirikko (1977) was the determination of the relative amounts of enzyme activity and immunoreactive enzyme protein. Active prolyl hydroxylase consists of four polypeptides subunits and the enzyme exists in connective tissue cells in the active tetrameric form and also as inactive monomers. Tuderman and Kivirikko (1977) showed that 13-19% of the enzyme protein was in an active form in fetal tissues whereas only 2-4% was in the active form in adult tissues. The ratio of active to inactive enzyme did not change between maturity and old age. There was no change with age in the amount of prolyl hydroxylase in human serum. Presumably there were no profound changes in the rates of collagen synthesis in connective tissues generally. The occurrence of inactive prolyl hydroxylase may be related to the occurrence of inactive enzymes in biochemical systems other than connective tissues, in which it has been observed that the amount of inactive enzyme protein increases with age. This has been interpreted as an indication that abnormal protein molecules accumulate in the tissues of older animals through errors in protein synthesis (see review by Rothstein, 1975). However, Risteli et al. (1976) have shown that in hepatic injury induced by dimethylnitrosamine the ratio of active prolyl hydroxylase to inactive enzyme increases significantly in damaged liver without change in the content of total immunoreactive enzyme protein. This presumably means that the cell is able to control the amount of active enzyme present, possibly by subunit association or by the synthesis of specific enzyme inhibitors (Risteli et al., 1976). Thus, inactive enzymes may be present in old tissues because cells can control the amount of active enzyme present according to their physiological requirements. The presence of inactive enzyme protein may have nothing to do with errors in protein synthesis. The synthesis of glycosaminoglycans is apparently high in very young tissues but decreases as growth and maturation proceed. There is relatively little change with ageing. The rate of incorporation of 35S-sulphate into glycosaminoglycans in human skin and aorta is relatively high in infancy but falls rapidly up to about 20 to 30 years of age. Thereafter, however, there is a relatively slow decline in the rates of 35S-sulphate incorporation up to about 70 years (Junge-Hulsing and Wagner, 1969; Lindner and Johannes, 1973). These workers observed similar changes in rat skin and aorta. The incorporation of 35S-sulphate and 14C-glucose into total and individual bovine aorta glycosaminoglycans has been examined by Buddecke et al. (1973). Here again, incorporation was high in the very young animal but decreased over the first 3 years and was then constant up to 13 years. This was observed for the total glycosaminoglycan fraction and for the individual glycosaminoglycans, heparan sulphate, chondroitin sulphate, and dermatan sulphate. The only exception

was the incorporation of 14C-glucose into hyaluronic acid, which rose over the first 3 years of life and then remained constant. Glycosaminoglycan synthesis in rat xiphoid and costal cartilage follows similar patterns (Lindner, 1973). The rates of 35S-sulphate incorporation fall from high levels over the first 6 months of life and remain constant thereafter up to 24 months. Incorporation of 35S-sulphate into rabbit cartilage chondroitin-4- and chondroitin-6-sulphate (femoral head, humeral head, and costal cartilage) was

observed to be high in very young animals. The rate of incorporation decreased rapidly up to 2 months of age and then was fairly constant up to 12 months (Mankin, 1975). A 12-month-old rabbit is far from old, however. The exception to this pattern was costal cartilage, where incorporation was at the same low level for both glycosaminoglycans at each age studied. Mankin (1975) also showed that the ratio of radioactivity in chondroitin-4-sulphate to that in chondroitin-6-sulphate changed with age. Thus the ratio was over 3 0 in the joint cartilage at birth, declining to below 10 by 12 months. In costal cartilage this ratio rose between birth and two months of age from just over 1 0 to almost 2-0 but then dropped to about 1-5 at 12 months. New knowledge of proteoglycan structure, the finding that proteoglycan subunits from cartilages of old animals do not contain as many chondroitin sulphate chains as those from cartilages of young animals, and advancing knowledge of the mechanisms of proteoglycan synthesis now permit more detailed studies of proteoglycan synthesis to be undertaken with respect to age. It would be of interest to know whether the diminution in the number of chondroitin sulphate chains in cartilage proteoglycan subunits is due to degradation of normally synthesised proteoglycans or to the synthesis of altered proteoglycans. This question could possibly be answered by studying the incorporation of radioactively labelled precursors into different glycosaminoglycan types of isolated, newly-synthesised proteoglycans or by measuring the activities of enzymes participating in glycosaminoglycan synthesis. Similarly, the reason for the failure of proteoglycans from some tissues of older animals to form aggregates might be investigated biosynthetically by determining the ability of newlysynthesised proteoglycans to interact with hyaluronic acid. The finding that the rates of synthesis of collagen and proteoglycans are apparently relatively constant after maturity seems to correlate with studies of the morphological changes in connective tissue cells. For instance, Silberberg and Lesker (1973) observed that chondrocytes in the upper mid-zone of cartilage from the femoral heads of 3-month-old guinea-pigs contained numerous stacked sheets of endoplasmic reticulum densely covered with ribosomes and a well developed Golgi appartus. Such cells seem to be very active in synthesising and secreting products. In contrast, the endoplasmic reticulum in chondrocytes from year-old animals is decreased in amount although the Golgi is still prominent. There then seems to be little change in these subcellular structures up to almost 6 years of age. Another feature noted in cells from older animals was the presence of J. D. Schofield and B. Weightman glycogen deposits and lipid inclusions, not so prominent in younger animals, and increases in the number of lysosomes and mitochondria. The application of morphometric techniques, developed to express morphological data of this kind in quantitative terms, would allow a more accurate assessment of age changes in cellular morphology and would seem to be a potentially fruitful area for further studies on ageing in chondrocytes and other connective tissue cells. Changes in the turnover of connective tissue components as a function of age have attracted less attention. Junge-Hiulsing and Wagner (1969) examined the half lives of 35S-sulphate-labelled glycosaminoglycans in a variety of tissues of rats of different ages. They found that in most tissues the half lives increased with age, indicating a slower rate of breakdown in older animals.Such conclusions have also been expressed by Lindner and Johannes (1973) and Lindner (1973), but how they correlate with observed increases in the activities of various glycosaminoglycan-degrading enzymes in midadult life and decreases in old age (Lindner and Johannes, 1973; Silberberg and Lesker, 1973) is not clear. Ali and Bayliss (1975) studied activities of cathepsin D and cathepsin Bi in human hip joint cartilage and found that the activity of cathepsin D was constant with age while that of cathepsin BI fell with increasing age up to about 40 years of age. Tissue responses to applied stimuli are often delayed in older animals compared with young animals. For example, partial hepatectomy in rodents induces cell division in the remnant so that the tissue regenerates. But there is a lag period before DNA synthesis and cell division begin and this is longer in old animals compared with

young (Bucher et al., 1964). Induction of DNA synthesis in salivary gland cells by injection of isoproterenol (Adelman et al., 1972b) and the induction of certain enzymes in the liver by a variety of meanis (Adelman et al., 1972a) are also delayed in older animals. Attempts have also been made to see whether the reactivity of connective tissue cells is similarly affected by ageing, usually by studying the synthesis of connective tissue components after applying stimuli such as the wounding of skin or the implantation of various granuloma-inducing agents subcutaneously. The effect of age on the kinetics of the formation of granulation tissue in response to the subcutaneous implantation of viscose cellulose sponge in rats was investigated by Heikkinen et al. (1971). They interpreted their results as showing a 'phase difference' between young and old in the parameters used to measure the development and metabolic activity of the granulomas. However, if one examines

the data it is difficult to detect any 'phase difference'. The patterns of development seem to be very much the same for most parameters in both young and old animals. Thus the nitrogen, hydroxyproline, DNA, and RNA contents were very similar at each time point after implantation of the sponges in both young and old animals, the values for each parameter peaking and declining at the same times at both ages. The only real difference between young and old animals is in the rates of incorporation of 14C. proline into protein by the granuloma tissue and in the synthesis of hydroxyproline. Thus granulomas from young animals are much more active than those from old animals at the earliest time points studied (7 days) but those from older animals are more active at later time points. Since the curves for fibrous collagen content, as measured by hydroxyproline levels, are virtually identical in young and old animals at different time points, the results suggest differences between granulomas in young and old animals either in the conversion of newly synthesised collagen into fibrous collagen or in the rates of degradation of collagen. At the earliest time point the conversion of newly synthesised collagen into fibrous collagen appears to be relatively inefficient in the young animal compared to the old animal. Alternatively, the degradation of collagen may be higher in the young animal. The situation is reversed at later times after implantation. Holm-Pedersen et al. (1974) have carried out a similar study on healing, full-thickness incisional wounds in mouse skin. There is no real evidence for any 'phase difference' between old (18 to 24-month) and young (6-week) animals in terms of DNA, RNA, and protein synthesis. There are differences between young and old, however. Cell division, measured by incorporating 3H-thymidine into DNA, peaks four days after wounding in young animals and falls to normal after 21 days. By contrast, DNA synthesis induced by wounding in old mice increases continuously at least up to 21 days. Similar patterns are observed for RNA and protein synthesis. Counts have also been made of the number of fibroblasts per unit area in the wounds. The density of fibroblasts rises to a peak four days after wounding in both young and old animals, to about the same values. The density then falls continuously in wounds in young animals at least up to 21 days after wounding, approaching the values in unwounded skin at this time. In old mice, however, the density of fibroblasts in the wound remains more or less constant between four and 21 days after wounding. How this finding correlates with the apparent continual increase in DNA synthesis is not clear. Studies of glycosaminoglycan synthesis in such experimental systems have been made by JungeHulsing and Wagner (1969), who examined the time course of glycosaminoglycan synthesis in subcutaneous cotton pellet granulomas. The rate of glycosaminoglycan synthesis, measured by incorporating 35S-sulphate, peaked at three days after cotton pellet implantation into young adult rats and then fell quickly. In old rats the rate of synthesis did not peak until seven days after implantation and remained at the same level until 10 days, after which it decreased. In these experiments, therefore, there was a definite 'phase difference' between old and young animals. Experiments such as these are designed partly to investigate whether wound healing is impaired in older individuals. Sussman (1973) has examined the tensile strength and extensibility of wound tissue at different periods after wounding in the skin of adult (8-month-old) and old (20-month-old) rats. He observed that the tensile strength of healing wounds was identical at various time points after wounding in both young and old animals. The thickness of the wounds in old animals was less than that in young animals, however, and hence the total breaking strength of the wounds in old animals was less than that in young animals at 8 weeks and 13 weeks after wounding. This indicates that the younger animal produces a greater amount of fibrous tissue.

Considerable differences were also noted between young and old animals in that the extensibility of the wounds in young animals was significantly greater at 8 weeks and at 13 weeks than in older animals, presumably because the scar tissues being laid down in animals at different ages are qualitatively different. These studies, therefore, show that there are differences between young and old animals in the amount of fibrous tissue produced in response to wounding and in the way in which collagen is laid down in the scar tissue. Presumably these differences reflect differences in the activities of fibroblasts. Ageing of adult articular cartilage: osteoarthrosis As a specific example of how the functional properties of a connective tissue change with age, of how the changes in functional properties might be explained at a molecular level, and of how such events might be related to disease processes we discuss in this section the hypothesis that fatigue failure in the collagen network of articular cartilage may be the initiating event in the development of some forms of osteoarthrosis. It is now generally accepted that mechanical factors play a role in the osteoarthrotic breakdown of human articular cartilage. Since the incidence of osteoarthrosis increases with age, changes in the mechanical properties of articular cartilage with age and the underlying biochemical events are of very considerable interest.

CHANGES IN MECHANICAL PROPERTIES WITH AGE:

Tensile properties Kempson (1978) measured the tensile stiffness and tensile strength of the collagen network by subjecting isolated specimens of cartilage to a gradually increasing stress until fracture occurred. His results for specimens from human femoral condyles (Figs. 1, 2) showed that both of these properties decreased with age after maturity. By subjecting isolated specimens of cartilage to repeated applications of tensile stress until fracture occurs it is possible to measure the tensile fatigue strength of the collagen network. Fig. 3 shows typical fatigue curves (that is, stress versus number of cycles to fracture) for specimens from the superficial layer of human femoral heads (Weightman et al., 1978). Each datum point represents one specimen; the number and letter show the age and sex of the cadaver from which the cartilage was obtained. The solid lines are the best-fit straight lines through the individual sets of data points. The interrupted lines all have the same slope (equal to the mean slope of the 20 individual fatigue curves in the complete study) and illustrate that, within the limits of accuracy of the experiment, the individual fatigue curves are parallel. When the fatigue strength of the cartilage from each femoral head was quantified by the intercept of the interrupted lines with the stress axis (to give projected fracture stress or fatigue strength) the fatigue strength was found to decrease significantly with age (Fig. 4). The decrease in fatigue strength with age is, in fact, more dramatic than indicated by Fig. 4. If the fatigue strengths at 30 and 60 years of age are compared on the basis of projected fracture stress (that is, from Fig. 4) the fatigue strength appears to decrease by just over 20%. Physiologically, however, the decrease in fatigue strength should be assessed by the change in the number of cycles required to produce fracture at the same level of stress. Making this comparison the fatigue strength at 60 is a factor of more than 500 less than it is at 30.

Compressive properties

Armstrong (1977) has recently developed a radiographic technique for measuring the deformation of articular cartilage in loaded human hip joints (Armstrong et al., 1978). Twenty-eight hip joints in the age range 26 to 83 years were injected with a radio-opaque contrast medium to indicate the cartilage-cartilage interface and loaded to five times body weight for 35 seconds. During this time radiographs were taken. Measurements of the thickness of the femoral head cartilage under load were made from the x-ray films and compared with unloaded thickness; the measurements gave cartilage deformation profiles. The average compliance of the cartilage over the whole contact area was determined by dividing the integrated deformation over the contact area by the total force applied to the joint. This study showed that the compliance of femoral head cartilage increases with age (Armstrong et al., 1977). Whereas in young

specimens the cartilage was almost incompressible, reductions in thickness of 15 % were common in old specimens.

FATIGUE HYPOTHESIS :

In unloaded articular cartilage the swell of the proteoglycan gel is limited b3 induces tensile stresses in, the collagen f When a compressive load is transmit synovial joint the hydrated proteog pressurised and the collagen network increased tensile stresses. Expressed in (1) cartilage is a hydrostatic system fluid element is provided by the hydra the proteoglycan gel and the container i the collagen fibres (Wainwright et al., 1976); (2) the function of the collagen is the fixation of the elastic domains of proteoglycans when these experience deforming and displacing stresses (Serafini-Fracassini and Smith, 1974). During activity the position of the load-bearing region within a joint and the magnitude of the transmitted load vary. Clearly, therefore, the collagen fibre network in articular cartilage experiences fluctuating tensile stresses and the proteoglycan gel experiences fluctuating fluid pressure. In general, the strength of a material is defined as 80 100 the minimum stress at which fracture occurs. The strength of a material may vary depending on whether it is subjected to tensile, compressive, or ath (as shear stresses or a combination of these. It also Qge (Weight- depends on whether the load is applied once or repeatedly. In the case of cyclically applied loads fracture can occur at a stress level which is lower than the static stress required to cause fracture. ling tendency This kind of behaviour is known as fatigue, and a y, and hence fatigue-prone material may be considered as being fibre network. 'weaker' when subjected to a cyclically applied tted across a load than when subjected to a load that is applied aycan gel is only once. experiences In theory, mechanical failure in cartilage could i other ways, occur in either the hydrated proteoglycan gel or in in which the the collagen network. However, mechanical failure tion water of in the proteoglycan gel seems unlikely since, if the is provided by mechanism of load-carriage described above is

correct, this component acts as a fluid. While the gel might fail due to shear, the relatively small deformations produced in normal cartilage in vivo seem to make this unlikely. Structural failure affecting primarily the collagen network would seem, on the other hand, to be perfectly possible since the collagen fibres, or the bonds between them, might be overstressed in tension. The collagen network in particular might fracture in the face of a cyclically applied load if the fluctuating tensile stresses produced were sufficient to cause fatigue failure. Collagen would, of course, protect itself from fatigue if its metabolic turnover rate were such that it was replaced before it could experience the number of stress cycles required to produce fracture. Present knowledge, however, indicates that the metabolic turnover rate of collagen in adult human articular cartilage is very low (Muir, 1978). It follows that the material will experience a large number of stress cycles before being replaced. Discussion: fatigue failure of collagen The evidence in support of the hypothesis that fatigue failure in the collagen fibre network is the initiating event in some forms of osteoarthrosis has been reviewed at length elsewhere (Weightman and Chappell, 1978; Freeman and Meachim, 1978). In summary, it includes (1) the increased incidence of osteoarthrosis after meniscectomy (Johnson et al., 1974), a procedure which increases the contact pressure on the cartilage by a factor of three (Seedhom, 1975); (2) the breakdown of cartilage subjected to repetitive loading in the laboratory (Radin and Paul, 1971; Radin et al., 1973; Weightman et al., 1973); (3) the increased thickness (Meachim, 1971) and increased water content (Venn and Maroudas, 1977) of cartilage in very early osteoarthrosis, both of which can be explained by changes in the collagen network which make it more distensible; (4) the abnormally wide separation of the collagen fibres in the cartilage underlying very minor surface disruptions in human knee cartilage (Meachim and Roy, 1969), which again implies increased distensibility of, and, hence mechanical changes in, the fibre network; and (5) the (variably) diminished tensile strength and tensile stiffness of histologically intact cartilage adjacent to areas of fibrillation (Kempson, 1978), which suggest that abnormality of the collagen network is a precursor to spread of fibrillation. The hypothesis is now further supported by the deterioration in

mechanical properties of cartilage with age. That is, since the changes in mechanical properties with age can all be explained by a gradual deterioration of the collagen network (which makes J. D. Schofield and B. Weightman it weaker and more extensible) and the incidence of osteoarthrosis increases with age, a causal connection is suggested (but not proved) between the two. The question now arises. What causes the deterioration of the collagen network with age? Collagen turnover occurs very slowly, if at all, in adult articular cartilage. Furthermore, collagenase is thought to be absent from normal cartilage, although cathepsin B, which is present, does degrade collagen slightly. For these reasons a metabolically induced abnormality in the collagen fibres themselves is unlikely to be responsible. On the other hand, the very inertness of collagen suggests the possibility of its being degraded mechanically, since over the course of a lifetime each fibre will experience a very large number of load cycles and hence may fatigue. Although progressive fatigue failure of the collagen fibres themselves may play a part in the age-related weakening of the fibre network it seems unlikely to be the main factor responsible, since the shortening of the fatigue life in aged compared with young cartilage is too great to be accounted for by the number of load cycles borne by the fibres in the intervening years (Weightman, 1976). One possible explanation concerns the 'linkage' between fibres rather than the fibres themselves. Virtually nothing is known of the way in which individual fibres are linked together to produce a network. Possibly the fibres are simply interlocked physically, or possibly some other constituent of the matrix functions as 'glue' (see A. J. Bailey (1978) at page 49). If the latter proves to be the case changes in the bonding constituent could be responsible for the changes in mechanical properties and might have a metabolic basis. Muir (1978) suggests (1) that the proteoglycans which cannot be extracted with high-strength salt solutions might be attached at 'high affinity' sites on the collagen fibres and act as bonding agents between fibres, spanning distances that would be too great for cross-links to develop; and (2) that the gradual reduction in the number of high-affinity sites with age (and hence the number of proteoglycan bonds) might explain the changes in mechanical properties. Unfortunately, the concept of a gradually decreasing number of bonds between collagen fibres seems to be at variance with another ageing change recently observed in human articular cartilagenamely, a gradual decrease in water content (Venn, 1978, and Fig. 5). Thus if the number of bonds between fibres decreases one would expect the water content to increase not decrease. Similarly, if the number of bonds decreases one would expect an increase in thickness. Meachim (1971) found age to have no effect on the thickness of

the uncalcified matrix of the cartila of the humerus, and Venn (1978) fc in the thickness of cartilage from the of the femoral head with age after Armstrong (1977) and Armstrong (1977) have reported a significant in ness of femoral head cartilage in the zone between the ages of 18 and 45 On balance, we think that the content and thickness point, if a increasing number of bonds be fibres. Perhaps the number of prot decreases while the number of somc 'linkage' increases. The question now arises: Can mechanical properties with ageini on the basis of an increasing nu between collagen fibres? The relati tensile strength and the degree of natural and non-crystallising rut reviewed by Bueche and Berry (materials the tensile strength first in(decreases as the degree of cross-lii One explanation for this is that; point only those chains orientated t4 angle of the direction of the aj supporting the load and that, as t increases beyond a certain degree, chains that can be so orientated dec at least possible that a similar effec articular cartilage if the collagen fil behave in the same way as the n in natural rubber.

Probable questions:

1. Write a short note on ageing of connective tissue
2. What is reactive oxygen species? Mention its role in ageing process.

Suggested readings:

J. Clin. Path, 31, Suppl. (Roy. Coll. Path.), 12, 174-190 Structural and metabolic disease New knowledge of connective tissue ageing J. D. SCHOFIELD AND B. WEIGHTMAN

Unit-IV

Differentiation: i) Processes, determination, induction, competence, mechanism of differentiation

Objective: Differentiation: i) Processes, determination, induction, competence, mechanism of differentiation.

Differentiation :

Cellular differentiation is the process by which a less specialized cell becomes a more specialized cell. Differentiation occurs during the development of a multicellular organisms. The organism develops from a single zygote to a complex system of tissues and cells. Differentiation is also common process in adults: adult stem cells divide to make fully-differentiated daughter cells during tissue repair and during normal cell turnover. Differentiation dramatically changes a cell's size, shape, metabolic activity, and responsiveness to signals. These changes are largely due to changes in gene expression. With a few exceptions, cellular differentiation almost never involves a change in the DNA sequence itself. It does involve switching off many genes not needed in a particular tissue. Thus, cells in different tissues may have very different physical characteristics despite having the same genome.

A cell that is able to differentiate into many cell types is known as pluripotent. Such cells are called stem cells in animals andmeristematic cells in higher plants. A cell that is able to differentiate into all cell types is known as totipotent. In mammals, only the zygote and early embryonic cells are totipotent, while in plants many differentiated cells can become totipotent with simple laboratory techniques.

In developmental biology, cellular differentiation is the process where a cellchanges from one cell type to another. Most commonly the cell changes to a more specialized type. Differentiation occurs numerous times during the development of a multicellular organism as it changes from a simple zygote to a complex system of tissues and cell types. Differentiation continues in adulthood as adult stem cells divide and create fully differentiated daughter cells during tissue repair and during normal cell turnover. Some differentiation occurs in response toantigen exposure. Differentiation dramatically changes a cell's size, shape, membrane potential, metabolic activity, and responsiveness to signals. These changes are largely due to highly controlled modifications in gene expression and are the study of epigenetics. With a few exceptions, cellular differentiation almost never involves a change in the DNA sequence itself. Thus, different cells can have very different physical characteristics despite having the same genome.

A specialized type of differentiation, known as 'terminal differentiation', is of importance in some tissues, for example vertebrate nervous system, striated muscle, epidermis and gut. During terminal differentiation a precursor cell formerly capable of cell division, permanently leaves the cell cycle, dismantles the cell cycle machinery and often expresses a range of genes characteristic of the cell's final function (e.g. myosin and actin for a muscle cell). Differentiation may continue to occur after terminal differentiation if the capacity and functions of the cell undergo further changes.

Among dividing cells, there are multiple levels of cell potency, the cell's ability to differentiate into other cell types. A greater potency indicates a larger number of cell types that can be derived. A cell that can differentiate into all cell types, including the placental tissue, is known as totipotent. In mammals, only the zygote and subsequent blastomeres are totipotent, while in plants many differentiated cells can

become totipotent with simple laboratory techniques. A cell that can differentiate into all cell types of the adult organism is known as pluripotent. Such cells are called meristematic cells in higher plants andembryonic stem cells in animals, though some groups report the presence of adult pluripotent cells. Virally induced expression of four transcription factors Oct4, Sox2, c-Myc, and KIF4 (Yamanaka factors) is sufficient to create pluripotent (iPS) cells from adult fibroblasts. A multipotent cell is one that can differentiate into multiple different, but closely related cell types. Oligopotent cells are more restricted than multipotent, but can still differentiate into a few closely related cell types. Finally, unipotent cells can differentiate into only one cell type, but are capable of self-renewal. In cytopathology, the level of cellular differentiation is used as a measure of cancer progression.

Mammalian cell type:

Three basic categories of cells make up the mammalian body: germ cells, somatic cells, and stem cells. Each of the approximately 37.2 trillion ($3.72x10^{13}$) cells in an adult human has its own copy or copies of the genome except certain cell types, such as red blood cells, that lack nuclei in their fully differentiated state. Most cells are diploid; they have two copies of each chromosome. Such cells, called somatic cells, make up most of the human body, such as skin and muscle cells. Cells differentiate to specialize for different functions.

Germ line cells are any line of cells that give rise to gametes—eggs and sperm—and thus are continuous through the generations. Stem cells, on the other hand, have the ability to divide for indefinite periods and to give rise to specialized cells. They are best described in the context of normal human development.

Development begins when a sperm fertilizes an egg and creates a single cell that has the potential to form an entire organism. In the first hours after fertilization, this cell divides into identical cells. In humans, approximately four days after fertilization and after several cycles of cell division, these cells begin to specialize, forming a hollow sphere of cells, called ablastocyst. The blastocyst has an outer layer of cells, and inside this hollow sphere, there is a cluster of cells called theinner cell mass. The cells of the inner cell mass go on to form virtually all of the tissues of the human body. Although the cells of the inner cell mass can form virtually every type of cell found in the human body, they cannot form an organism. These cells are referred to as pluripotent.

Pluripotent stem cells undergo further specialization into multipotent progenitor cells that then give rise to functional cells. Examples of stem and progenitor cells include:

- Radial glial cells (embryonic neural stem cells) that give rise to excitatory neurons in the fetal brain through the process of neurogenesis.
- Hematopoietic stem cells (adult stem cells) from the bone marrow that give rise to red blood cells, white blood cells, andplatelets
- Mesenchymal stem cells (adult stem cells) from the bone marrow that give rise to stromal cells, fat cells, and types of bone cells
- Epithelial stem cells (progenitor cells) that give rise to the various types of skin cells
- Muscle satellite cells (progenitor cells) that contribute to differentiated muscle tissue.
-

A pathway that is guided by the cell adhesion molecules consisting of four amino acids, arginine, glycine, asparagine, andserine, is created as the cellular blastomere differentiates from the single-layered blastula to the three primary layers of germ cells in mammals, namely the ectoderm, mesoderm and endoderm (listed from most distal (exterior) to proximal (interior)). The ectoderm ends up forming the skin and the nervous system, the mesoderm forms the bones and muscular tissue, and the endoderm forms the internal organ tissues.

Reversibility of differentiated cells :
Shinya Yamanaka shared a Nobel Prize in 2012 for his work which demonstrated that cellular differentiation is reversible.

In 2006, Takahashi and Yamanaka showed that a combination of 4 transcription factors were sufficient to reprogram fibroblasts into less differentiated stem cells, termed **induced pluripotent stem cells** or **iPSCs**. But could this methodology be applied to other cells in the body? Could we now reverse their differentiation as well? And may be more importantly, could we control their differentiation to make any other cell we are interested in?

Indeed, many new combinations of factors have been discovered since Yamanaka's work in 2006 that can be used to de-differentiate various other cell types as well as reprogram them, either by first inducing pluripotency or by taking a shortcut and directly turning one mature cell type into another mature cell type (termed transdifferentiation).

All of this work has huge implications for medicine! One very promising therapeutic application of cardiomyocyte transdifferentiation is cardiovascular disease, a major killer in the U.S. caused by loss or dysfunction of cardiomyocytes. It turns out that the walls of the heart are filled with fibroblasts. One of the bold ideas is to take the factors that turn fibroblasts into cardiomyocytes in a dish and infuse them into people to reprogram fibroblasts in the human heart to become healthy, functional cardiomyocytes - in essence treating CVD.

Dedifferentiation:

Dedifferentiation, or integration is a cellular process often seen in more basal life forms such as worms and amphibians in which a partially or terminally differentiated cell reverts to an earlier developmental stage, usually as part of a regenerativeprocess. Dedifferentiation also occurs in plants. Cells in cell culture can lose properties they originally had, such as protein expression, or change shape. This process is also termed dedifferentiation. Some believe dedifferentiation is an aberration of the normal development cycle that results in cancer, whereas others believe it to be a natural part of the immune response lost by humans at some point as a result of evolution. A small molecule dubbed reversine, a purine analog, has been discovered that has proven to induce dedifferentiation in myotubes. These dedifferentiated cells could then redifferentiate into osteoblasts and adipocytes.

Mechanism:
Each specialized cell type in an organism expresses a subset of all the genes that constitute the genome of that species. Each cell type is defined by its particular pattern of regulated gene expression. Cell differentiation is thus a transition of a cell from one cell type to another and it involves a switch from one pattern of gene expression to another. Cellular differentiation during development can be understood as the result of a gene regulatory network. A regulatory gene and its cis-regulatory modules are nodes in a gene regulatory network; they receive input and create output elsewhere in the network. The systems biology approach to developmental biology emphasizes the importance of investigating how developmental mechanisms interact to produce predictable patterns (morphogenesis). (However, an alternative view has been proposed recently. Based onstochastic gene expression, cellular differentiation is the result of a Darwinian selective process occurring among cells. In this frame, protein and gene networks are the result of cellular processes and not their cause.

A few evolutionarily conserved types of molecular processes are often involved in the cellular mechanisms that control these switches. The major types of molecular processes that control cellular differentiation involve cell signaling. Many of the signal molecules that convey information from cell to cell during the control of cellular differentiation are called growth factors. Although the details of

specific signal transduction pathways vary, these pathways often share the following general steps. A ligand produced by one cell binds to a receptor in the extracellular region of another cell, inducing a conformational change in the receptor. The shape of the cytoplasmic domain of the receptor changes, and the receptor acquires enzymatic activity. The receptor then catalyzes reactions that phosphorylate other proteins, activating them. A cascade of phosphorylation reactions eventually activates a dormant transcription factor or cytoskeletal protein, thus contributing to the differentiation process in the target cell. Cells and tissues can vary in competence, their ability to respond to external signals.

Signal induction refers to cascades of signaling events, during which a cell or tissue signals to another cell or tissue to influence its developmental fate. Yamamoto and Jeffery investigated the role of the lens in eye formation in cave- and surface-dwelling fish, a striking example of induction. Through reciprocal transplants, Yamamoto and Jeffery found that the lens vesicle of surface fish can induce other parts of the eye to develop in cave- and surface-dwelling fish, while the lens vesicle of the cave-dwelling fish cannot.
Other important mechanisms fall under the category of asymmetric cell divisions, divisions that give rise to daughter cells with distinct developmental fates. Asymmetric cell divisions can occur because of asymmetrically expressed maternal cytoplasmic determinants or because of signaling. In the former mechanism, distinct daughter cells are created during cytokinesis because of an uneven distribution of regulatory molecules in the parent cell; the distinct cytoplasm that each daughter cell inherits results in a distinct pattern of differentiation for each daughter cell. A well-studied example of pattern formation by asymmetric divisions is body axis patterning in Drosophila. RNA molecules are an important type of intracellular differentiation control signal. The molecular and genetic basis of asymmetric cell divisions has also been studied in green algae of the genus Volvox, a model system for studying how unicellular organisms can evolve into multicellular organisms. In *Volvox carteri*, the 16 cells in the anterior hemisphere of a 32-cell embryo divide asymmetrically, each producing one large and one small daughter cell. The size of the cell at the end of all cell divisions determines whether it becomes a specialized germ or somatic cell.

Epigenetic control:
Since each cell, regardless of cell type, possesses the same genome, determination of cell type must occur at the level of gene expression. While the regulation of gene expression can occur through cis- and trans-regulatory elements including a gene's promoter and enhancers, the problem arises as to how this expression pattern is maintained over numerous generations of cell division. As it turns out, epigenetic processes play a crucial role in regulating the decision to adopt a stem, progenitor, or mature cell fate. This section will focus primarily on mammalian stem cells. In systems biology and mathematical modeling of gene regulatory networks, cell-fate determination is predicted to exhibit certain dynamics, such as attractor-convergence (the attractor can be an equilibrium point, limit cycle or strange attractor) or oscillatory.

Importance of epigenetic control :
The first question that can be asked is the extent and complexity of the role of epigenetic processes in the determination of cell fate. A clear answer to this question can be seen in the 2011 paper by Lister R, et al. on aberrant epigenomic programming in human induced pluripotent stem cells. As induced pluripotent stem cells (iPSCs) are thought to mimicembryonic stem cells in their pluripotent properties, few epigenetic differences should exist between them. To test this prediction, the authors conducted whole-genome profiling of DNA methylation patterns in several human embryonic stem cell (ESC), iPSC, and progenitor cell lines.

Female adipose cells, lung fibroblasts, and foreskin fibroblasts were reprogrammed into induced pluripotent state with theOCT4, SOX2, KLF4, and MYC genes. Patterns of DNA methylation in ESCs, iPSCs, somatic cells were compared. Lister R,et al. observed significant resemblance in methylation levels between embryonic and induced pluripotent cells. Around 80% of CG dinucleotides in ESCs and

iPSCs were methylated, the same was true of only 60% of CG dinucleotides in somatic cells. In addition, somatic cells possessed minimal levels of cytosine methylation in non-CG dinucleotides, while induced pluripotent cells possessed similar levels of methylation as embryonic stem cells, between 0.5 and 1.5%. Thus, consistent with their respective transcriptional activities, DNA methylation patterns, at least on the genomic level, are similar between ESCs and iPSCs.

However, upon examining methylation patterns more closely, the authors discovered 1175 regions of differential CG dinucleotide methylation between at least one ES or iPS cell line. By comparing these regions of differential methylation with regions of cytosine methylation in the original somatic cells, 44-49% of differentially methylated regions reflected methylation patterns of the respective progenitor somatic cells, while 51-56% of these regions were dissimilar to both the progenitor and embryonic cell lines. In vitro-induced differentiation of iPSC lines saw transmission of 88% and 46% of hyper and hypo-methylated differentially methylated regions, respectively.

Two conclusions are readily apparent from this study. First, epigenetic processes are heavily involved in cell fate determination, as seen from the similar levels of cytosine methylation between induced pluripotent and embryonic stem cells, consistent with their respective patterns of transcription. Second, the mechanisms of de-differentiation (and by extension, differentiation) are very complex and cannot be easily duplicated, as seen by the significant number of differentially methylated regions between ES and iPS cell lines. Now that these two points have been established, we can examine some of the epigenetic mechanisms that are thought to regulate cellular differentiation.

Mechanisms of epigenetic regulation :

Three transcription factors, OCT4, SOX2, and NANOG – the first two of which are used in induced pluripotent stem cell (iPSC) reprogramming, along with Klf4 and c-Myc – are highly expressed in undifferentiated embryonic stem cells and are necessary for the maintenance of their pluripotency. It is thought that they achieve this through alterations in chromatinstructure, such as histone modification and DNA methylation, to restrict or permit the transcription of target genes. While highly expressed, their levels require a precise balance to maintain pluripotency, perturbation of which will promote differentiation towards different lineages based on how the gene expression levels change. Differential regulation of Oct-4and SOX2 levels have been shown to precede germ layer fate selection.[27] Increased levels of Oct4 and decreased levels of Sox2 promote a mesendodermal fate, with Oct4 actively suppressing genes associated with a neural ectodermal fate. Similarly, Increased levels of Sox2 and decreased levels of Oct4 promote differentiation towards a neural ectodermal fate, with Sox2 inhibiting differentiation towards a mesendodermal fate. Regardless of the lineage cells differentiate down, suppression of NANOG has been identified as a necessary prerequisite for differentiation.

Polycomb repressive complex (PRC2) :

In the realm of gene silencing, Polycomb repressive complex 2, one of two classes of the Polycomb group (PcG) family of proteins, catalyzes the di- and tri-methylation of histone H3 lysine 27 (H3K27me2/me3). By binding to the H3K27me2/3-tagged nucleosome, PRC1 (also a complex of PcG family proteins) catalyzes the mono-ubiquitinylation of histone H2A at lysine 119 (H2AK119Ub1), blocking RNA polymerase II activity and resulting in transcriptional suppression. PcG knockout ES cells do not differentiate efficiently into the three germ layers, and deletion of the PRC1 and PRC2 genes leads to increased expression of lineage-affiliated genes and unscheduled differentiation. Presumably, PcG complexes are responsible for transcriptionally repressing differentiation and development-promoting genes.

Trithorax group proteins (TrxG) :

Alternately, upon receiving differentiation signals, PcG proteins are recruited to promoters of pluripotency transcription factors. PcG-deficient ES cells can begin differentiation but cannot maintain the differentiated phenotype. Simultaneously, differentiation and development-promoting genes are activated by Trithorax group (TrxG) chromatin regulators and lose their repression. TrxG proteins are recruited at regions of high transcriptional activity, where they catalyze the trimethylation of histone H3 lysine 4 (H3K4me3) and promote gene activation through histone acetylation. PcG and TrxG complexes engage in direct competition and are thought to be functionally antagonistic, creating at differentiation and development-promoting loci what is termed a "bivalent domain" and rendering these genes sensitive to rapid induction or repression.

DNA methylation:

Regulation of gene expression is further achieved through DNA methylation, in which the DNA methyltransferase-mediated methylation of cytosine residues in CpG dinucleotides maintains heritable repression by controlling DNA accessibility. The majority of CpG sites in embryonic stem cells are unmethylated and appear to be associated with H3K4me3-carrying nucleosomes. Upon differentiation, a small number of genes, including OCT4 and NANOG, are methylated and their promoters repressed to prevent their further expression. Consistently, DNA methylation-deficient embryonic stem cells rapidly enter apoptosis upon in vitro differentiation.

Nucleosome positioning :

While the DNA sequence of most cells of an organism is the same, the binding patterns of transcription factors and the corresponding gene expression patterns are different. To a large extent, differences in transcription factor binding are determined by the chromatin accessibility of their binding sites through histone modification and/or pioneer factors. In particular, it is important to know whether a nucleosome is covering a given genomic binding site or not. This can be determined using a chromatin immunoprecipitation (ChIP) assay.

Histone acetylation and methylation :

DNA-nucleosome interactions are characterized by two states: either tightly bound by nucleosomes and transcriptionally inactive, called heterochromatin, or loosely bound and usually, but not always, transcriptionally active, called euchromatin. The epigenetic processes of histone methylation and acetylation, and their inverses demethylation and deacetylation primarily account for these changes. The effects of acetylation and deacetylation are more predictable. An acetyl group is either added to or removed from the positively charged Lysine residues in histones by enzymes called histone acetyltransferases or histone deacteylases, respectively. The acetyl group prevents Lysine's association with the negatively charged DNA backbone. Methylation is not as straightforward, as neither methylation nor demethylation consistently correlate with either gene activation or repression. However, certain methylations have been repeatedly shown to either activate or repress genes. The trimethylation of lysine 4 on histone 3 (H3K4Me3) is associated with gene activation, whereas trimethylation of lysine 27 on histone 3 represses genes.

In stem cells

During differentiation, stem cells change their gene expression profiles. Recent studies have implicated a role for nucleosome positioning and histone modifications during this process. There are two components of this process: turning off the expression of embryonic stem cell (ESC) genes, and the activation of cell fate genes. Lysine specific demethylase 1 (KDM1A) is thought to prevent the use of enhancer regions of pluripotency genes, thereby inhibiting their transcription. It interacts with Mi-2/NuRD

complex (nucleosome remodelling and histone deacetylase) complex, giving an instance where methylation and acetylation are not discrete and mutually exclusive, but intertwined processes.

Role of signaling in epigenetic control :

A final question to ask concerned the role of cell signaling in influencing the epigenetic processes governing differentiation. Such a role should exist, as it would be reasonable to think that extrinsic signaling can lead to epigenetic remodeling, just as it can lead to changes in gene expression through the activation or repression of different transcription factors. Little direct data is available concerning the specific signals that influence the epigenome, and the majority of current knowledge about the subject consists of speculations on plausible candidate regulators of epigenetic remodeling. We will first discuss several major candidates thought to be involved in the induction and maintenance of both embryonic stem cells and their differentiated progeny, and then turn to one example of specific signaling pathways in which more direct evidence exists for its role in epigenetic change.

The first major candidate is Wnt signaling pathway. The Wnt pathway is involved in all stages of differentiation, and the ligand Wnt3a can substitute for the overexpression of c-Myc in the generation of induced pluripotent stem cells. On the other hand, disruption of ß-catenin, a component of the Wnt signaling pathway, leads to decreased proliferation of neural progenitors.

Growth factors comprise the second major set of candidates of epigenetic regulators of cellular differentiation. These morphogens are crucial for development, and include bone morphogenetic proteins, transforming growth factors (TGFs), and fibroblast growth factors (FGFs). TGFs and FGFs have been shown to sustain expression of OCT4, SOX2, and NANOG by downstream signaling to Smad proteins. Depletion of growth factors promotes the differentiation of ESCs, while genes with bivalent chromatin can become either more restrictive or permissive in their transcription.

Several other signaling pathways are also considered to be primary candidates. Cytokine leukemia inhibitory factors are associated with the maintenance of mouse ESCs in an undifferentiated state. This is achieved through its activation of the Jak-STAT3 pathway, which has been shown to be necessary and sufficient towards maintaining mouse ESC pluripotency. Retinoic acid can induce differentiation of human and mouse ESCs, and Notch signaling is involved in the proliferation and self-renewal of stem cells. Finally, Sonic hedgehog, in addition to its role as a morphogen, promotes embryonic stem cell differentiation and the self-renewal of somatic stem cells.

The problem, of course, is that the candidacy of these signaling pathways was inferred primarily on the basis of their role in development and cellular differentiation. While epigenetic regulation is necessary for driving cellular differentiation, they are certainly not sufficient for this process. Direct modulation of gene expression through modification of transcription factors plays a key role that must be distinguished from heritable epigenetic changes that can persist even in the absence of the original environmental signals. Only a few examples of signaling pathways leading to epigenetic changes that alter cell fate currently exist, and we will focus on one of them.

Expression of Shh (Sonic hedgehog) upregulates the production of BMI1, a component of the PcG complex that recognizes H3K27me3. This occurs in a Gli-dependent manner, as Gli1 and Gli2 are downstream effectors of the Hedgehog signaling pathway. In culture, Bmi1 mediates the Hedgehog pathway's ability to promote human mammary stem cell self-renewal. In both humans and mice, researchers showed Bmi1 to be highly expressed in proliferating immature cerebellar granule cell precursors. When Bmi1 was knocked out in mice, impaired cerebellar development resulted, leading to significant reductions in postnatal brain mass along with abnormalities in motor control and behavior. A separate study showed a significant decrease in neural stem cell proliferation along with increased astrocyte proliferation in Bmi null mice.

In summary, the role of signaling in the epigenetic control of cell fate in mammals is largely unknown, but distinct examples exist that indicate the likely existence of further such mechanisms.

Effect of matrix elasticity:

In order to fulfill the purpose of regenerating a variety of tissues, adult stems are known to migrate from their niches, adhere to new extracellular matrices (ECM) and differentiate. The ductility of these microenvironments is unique to different tissue types. The ECM surrounding brain, muscle and bone tissues range from soft to stiff. The transduction of the stem cells into these cells types is not directed solely by chemokine cues and cell to cell signaling. The elasticity of the microenvironment can also affect the differentiation of mesenchymal stem cells (MSCs which originate in bone marrow.) When MSCs are placed on substrates of the same stiffness as brain, muscle and bone ECM, the MSCs take on properties of those respective cell types. Matrix sensing requires the cell to pull against the matrix at focal adhesions, which triggers a cellular mechano-transducer to generate a signal to be informed what force is needed to deform the matrix. To determine the key players in matrix-elasticity-driven lineage specification in MSCs, different matrix microenvironments were mimicked. From these experiments, it was concluded that focal adhesions of the MSCs were the cellular mechano-transducer sensing the differences of the matrix elasticity. The non-muscle myosin IIa-c isoforms generates the forces in the cell that lead to signaling of early commitment markers. Nonmuscle myosin IIa generates the least force increasing to non-muscle myosin IIc. There are also factors in the cell that inhibit non-muscle myosin II, such as blebbistatin. This makes the cell effectively blind to the surrounding matrix. Researchers have obtained some success in inducing stem cell-like properties in HEK 239 cells by providing a soft matrix without the use of diffusing factors. The stem-cell properties appear to be linked to tension in the cells' actin network. One identified mechanism for matrix-induced differentiation is tension-induced proteins, which remodel chromatin in response to mechanical stretch. The RhoA pathway is also implicated in this process.

Cell determination:

Within the field of developmental biology one goal is to understand how a particular cell (or embryo) develops into the final cell type (or organism), essentially how a cell's fate is determined. Within an embryo, 4 processes play out at the cellular and tissue level to essentially create the final organism. These processes are cell proliferation, cell specialization, cell interaction and cell movement. Each cell in the embryo receives and gives cues to its neighboring cells and retains a cell memory of its own cell proliferation history. Almost all animals undergo a similar sequence of events during embryogenesis and have, at least at this developmental stage, the three germ layers and undergo gastrulation. While embryogenesis has been studied for more than a century, it was only recently (the past 15 years or so) that scientists discovered that a basic set of the sameproteins and mRNAs are involved in all of embryogenesis. This is one of the reasons that model systems such as the fly (Drosophila melanogaster), the mouse (Muridae), and the leech (Helobdella), can all be used to study embryogenesis and developmental biology relevant to other animals, including humans. The fate map of the nematode (Caenorhabditis) can be analyzed down to the cellular level. This is due no cell mixing during development. What continues to be discovered and investigated is how the basic set of proteins (and mRNAs) are expressed differentially between cells types, temporally and spatially; and whether this is responsible for the vast diversity of organisms produced. This leads to one of the key questions of developmental biology of how is cell fate determined.

Cell fate:

The development of new molecular tools including GFP, and major advances in imaging technology including fluorescence microscopy, have made possible the mapping of the cell lineage of Caenorhabditis elegans including its embryo. This technique of fate mapping is used to study

cells as they differentiate into their final cell fates. Merely observing a cell as it becomes differentiated during embryogenesis provides no indication of the mechanisms that drive the specification. Therefore, the addition of molecular manipulation techniques, including gene and protein knock downs, knock outs and overexpression, along with live cell imaging techniques has been transformational in understanding what mechanisms are involved with cell fate determination. Transplantation experiments are commonly used in conjunction with the genetic manipulation and lineage tracing. Transplantation experiments are the only way to determine what state the cell is in on its way to being differentiated.

For a number of cell cleavages (the specific number depends on the type of organism) all the cells of an embryo will be morphologically and developmentally equivalent. This means, each cell has the same development potential and all cells are essentially interchangeable, thus establishing an equivalence group. The developmental equivalence of these cells is usually established via transplantation and cell ablation experiments.

The determination of a cell to a particular fate can be broken down into two states where the cell can be specified (committed) or determined. In the state of being committed or specified, the cell type is not yet determined and any bias the cell has toward a certain fate can be reversed or transformed to another fate. If a cell is in a determined state, the cell's fate cannot be reversed or transformed. In general, this means that a cell determined to differentiate into a brain cell cannot be transformed into a skin cell. Determination is followed by differentiation, the actual changes in biochemistry, structure, and function that result in specific cell types. Differentiation often involves a change in appearance as well as function.

Modes of specification:
There are three general ways a cell can become specified for a particular fate; they are autonomous specification, conditional specification and syncytial specification.

Autonomous specification
This type of specification results from cell-intrinsic properties; it gives rise to mosaic development. The cell-intrinsic properties arise from a cleavage of a cell with asymmetrically expressed maternal cytoplasmic determinants (proteins, small regulatory RNAs and mRNA). Thus, the fate of the cell depends on factors secreted into its cytoplasm during cleavage. Autonomous specification was demonstrated in 1887 by a French medical student, Laurent Chabry, working on tunicate embryos. This asymmetric cell division usually occurs early in embryogenesis.

Positive feedback can create asymmetry from homogeneity. In cases where the external or stimuli that would cause asymmetry are very weak or disorganized, through positive feedback the system can spontaneously pattern itself. Once the feedback has begun, any small initial signaling is magnified and thus produces an effective patterning mechanism. This is normally what occurs in the case of lateral inhibition in which neighboring cells induce specification via inhibitory or inducing signals. This kind of positive feedback at the single cell level and tissue level is responsible for symmetry breaking, which is an all-or-none process whereas once the symmetry is broken; the cells involved become very different. Symmetry breaking leads to a bistable or multistable system where the cell or cells involved are determined for different cell fates. The determined cells continue on their particular fate even after the initial stimulatory/inhibitory signal is gone, giving the cells a memory of the signal.

Conditional specification
In contrast to the autonomous specification, this type of specification is a cell-extrinsic process that relies on cues and interactions between cells or from concentration-gradients of morphogens. Inductive interactions between neighboring cells are the most common mode of tissue patterning. In this mechanism, one or two cells from a group of cells with the same developmental potential are exposed to a signal (morphogen) from outside the group. Only the cells exposed to the signal are induced to follow a

different developmental pathway, leaving the rest of the equivalence group unchanged. Another mechanism that determines the cell fate is regional determination. As implied by the name, this specification occurs based on where within the embryo the cell is positioned, it is also known as positional value. This was first observed when mesoderm was taken from the prospective thigh region of a chick embryo, was grafted onto the wing region and did not transform to wing tissue, but instead into toe tissue.

Syncytial specification:
This type of a specification is a hybrid of the autonomous and conditional that occurs in insects. This method involves the action of morphogen gradients within the syncytium. As there are no cell boundaries in the syncytium, these morphogens can influence nuclei in a concentration-dependent manner.

Embryonic induction:

In amphibian embryos, the dorsal ectodermal cells in a mid-longitudinal region differentiate to form a neural plate, only when the chorda-mesoderm is below it. Chorda-mesoderm is the layer formed by invagination cells from the region of the dorsal blastoporal lip, which form the roof of archenteron. Mangold (1927) selected a small part of dorsal blastoporal lip from an early gastrula of Triturus cristatus and grafted it at a place near the lateral lip of the blastopore of the host gastrula of *T. taeniatus*. The graft cells grew in number and spread inside the host gastrula to form an additional chorda-mesoderm at this place. This chorda-mesoderm, subsequently induced the ectoderm of the host gastrula to form an additional neural tube. The graft cells themselves formed an additional notochord. As the host gastrula developed further, it grew into a double embryo joined together. One of the embryos was the regular one, while the second was the induced one. The latter did not develop a complete head.

This experiment clearly showed that the dorsal blastoporal lip of the blastula had the ability to induce the formation of the neural plate in the ectoderm of the host. This phenomenon is called neural induction. Other parts of an embryo can similarly induce the formation of other structures. This influence of one structure in the formation of another structure is called embryonic induction. In fact, the entire development of an organism is due to a series of inductions. The structure, which induces the formation of another structure, is called the inductor or organizer. The chemical substance that is emitted by an inductor is called an evacuator. The tissue on which an evacuator or inductor acts is called the responsive tissue.

Historical Background of Embryonic Induction:

For the discovery of neural induction, the German embryologist, Hans Spemann and his student, Hilde Mangold (1924) worked a lot and for his work Spemann received Nobel Prize in 1935.

These two scientists performed certain heteroblastic transplantations between two species of newt, i.e., *Triturus cristatus* and *Triturus taeniatus* and reported that the dorsal lip of their early gastrula has the capacity of induction and organization of presumptive neural ectoderm to form a neural tube and also the capacity of evocation and organization of ectoderm, mesoderm and endoderm to form a complete secondary embryo.

They called the dorsal lip of the blastopore the primary organizer since it was first in the sequence of inductions and as it had the capacity to organize the development of a second embryo. Later on, the primary organizer was reported to exist in many animals, e.g. in frogs (Daloq and Pasteels, 1937); in

cyclostomes (Yamada, 1938); in bony fishes (Oppenheimer, 1936); in birds (Waddington, 1933) and in rabbit (Waddington, 1934).

Primary organizer and neural induction have been reported in certain pre-vertebrate chordates, such as ascidians and Amphioxus (Tung, Wu and Tung, 1932). In 1960 and 1963 Curtis investigated and reported that the organizer of gastrula of *Xenopus laevis* can be distinguished in the cortex of gray crescent of a fertilized egg.
Holtfreter (1945) gave an account of how an enormous variety of entirely unspecific substances-organic acids, steroids, kaolin, methylene blue, sulphhydryl compounds, which had nothing in common except the property of being toxic to sub-ectodermal cells-produced neurulation in explants. Barth and Barth (1968, 69) provided further information about the chemical nature of embryonic induction.

Types of embryonic induction:
Lovtrup (1974) classified different types of embryonic induction into two basic categories-endogenous and exogenous inductions.

1. Endogenous induction:
Certain embryonic cells gradually assume new diversification pattern through the inductors that are produced by them endogenously. Due to these inductors, these cells undergo either self-transformation or self-differentiation. Examples of such induction were reported in Mesenchymal cells of ventral pole of Echinoid and in small sized, yolk-laden cells of dorsal lip of amphibian blastopore.

2. Exogenous induction:
When some external agent or a cell or a tissue is introduced into an embryo, they exert their influence by a process of diversification pattern upon neighbouring cells through contact induction. This phenomenon is called exogenous induction. It may be homotypic or heterotypic depending on the fact that whether the inductor provokes the formation of same or different kind of tissues respectively (Grobstein, 1964).

In homotypic induction, a differentiated cell produces an inductor. The inductor not only serves to maintain the state of the cell proper, but also induces adjacent cells to differentiate according to it, after crossing the cell boundaries. Best example of the heterotypic exogenous induction is the formation of a secondary embryonic axis by an implanted presumptive notochord in amphibians.

Experimental evidences to induction:
Spemann and Mangold (1924) transplanted heteroplastically a piece of the dorsal lip of the blastopore of an early gastrula of pigmented newt, *Triturus cristatus* and grafted it near the ventral or lateral lip of the blastopore of the early gastrula of pigmented newt *T. taeniatus*.

Most of the graft invaginated into the interior and developed into notochord and somite's and induced the host ectoderm to form a neural tube, leaving a narrow strip of tissue on the surface.

With the development of host embryo, an additional whole system of organs was induced at the graft – placement area.
Except for the anterior part of the head, almost a complete secondary embryo comprising of the additional organs was formed. Posterior part of the head was present as indicated by a pair of ear rudiments.

Since in this experiment the type of transplantation involved was heteroplastic, it was found that notochord of secondary embryo consisted exclusively of graft cells; the somites consisted partly of graft and partly of host cells.

Few cells, which did not invaginate during gastrulation, were left in the neural tube. The bulk of the neural tube, part of the somites, kidney tubules and the ear rudiments of the secondary embryo consisted of host cells.

The graft becomes self-differentiated and at the same time induces the adjoining host tissue to form spinal cord and other structures including somites and kidney tubules.

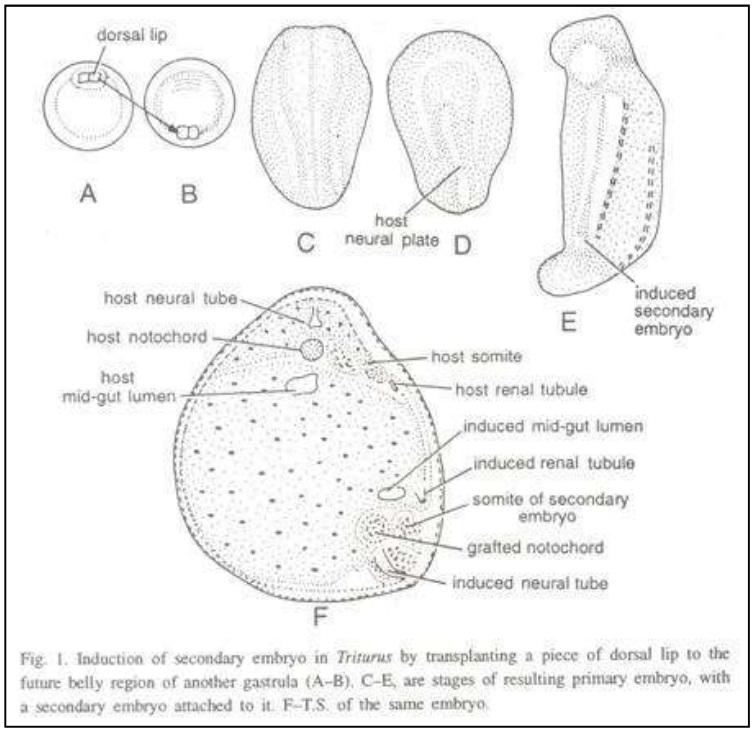


Fig. 1. Induction of secondary embryo in *Triturus* by transplanting a piece of dorsal lip to the future belly region of another gastrula (A–B). C–E, are stages of resulting primary embryo, with a secondary embryo attached to it. F–T.S. of the same embryo.

Spemann (1938) described dorsal lip of the early gastrula as a "primary organizer" of the gastrulative process. However, organization of the secondary embryo results from a series of both inductive interactions and self-differentiative changes in the host and donor tissues. Hence, now a days the term "embryonic induction" or "inductive interactions" is preferred. The part, which is the source of induction, is called "inductor".

Characteristics of the organizer:

Organizer has the ability for self-differentiation and organization. It also has the power to induce changes within the cell and to organize surrounding cells, including the induction and early organization of neural tube. Primary organizer determines the main features of axiation and organization of the vertebrate embryo.

Induction is a tool-like process, utilized by this center of activity through which it affects changes in surrounding cells and as such influences organization and differentiation. These surrounding cells, changed by the process of induction, may in turn act as secondary inductor centers with abilities to organize specific sub-areas.

Thus, the transformation of the late blastula into an organized condition of the late gastrula appears to be dependent upon a number of separate inductions, all integrated into one coordinated whole by the "formative stimulus" of the primary organizer located in the pre-chordal plate area of the endodermal - mesodermal cells and adjacent chorda-mesodermal material of the early gastrula.

Regional specificity of the organizer:
Vital-staining experiments of Vogt with newt eggs have shown that the material successively forming the dorsal blastoporal lip moves forward as the archenteron roof. Transplants taken from this region are also able to induce a secondary embryo or the belly of a new host i.e. the archenteron roof acts as a primary inductor in essentially the same way as does the dorsal lip tissue proper. The inductions of neural inductor are found to be regionally specific and the regional specificity is imposed on the induced organ by the inductor.

Therefore, the inductive capacity of the blastoporal lip varies both regionally and temporally. Most of the dorsal and dorso-lateral blastoporal material is necessary for a graft to induce a more or less complete secondary embryo. Spemann (1931) demonstrated that during gastrulation anterior part of the archenteric roof invaginates over the dorsal lip of the blastopore earlier.

Dorsal blastopore lip of the early gastrula contains the archenteric and deuterocephalic organizer and the dorsal blastopore lip of the late gastrula contains the spinocaudal organizer. Inductions produced by the dorsal lip of the blastopore taken from the early and the late gastrula differ in accordance with exception; the first tends to produce head organs and the second tends to produce trunk and tail organs (Fig. 2).

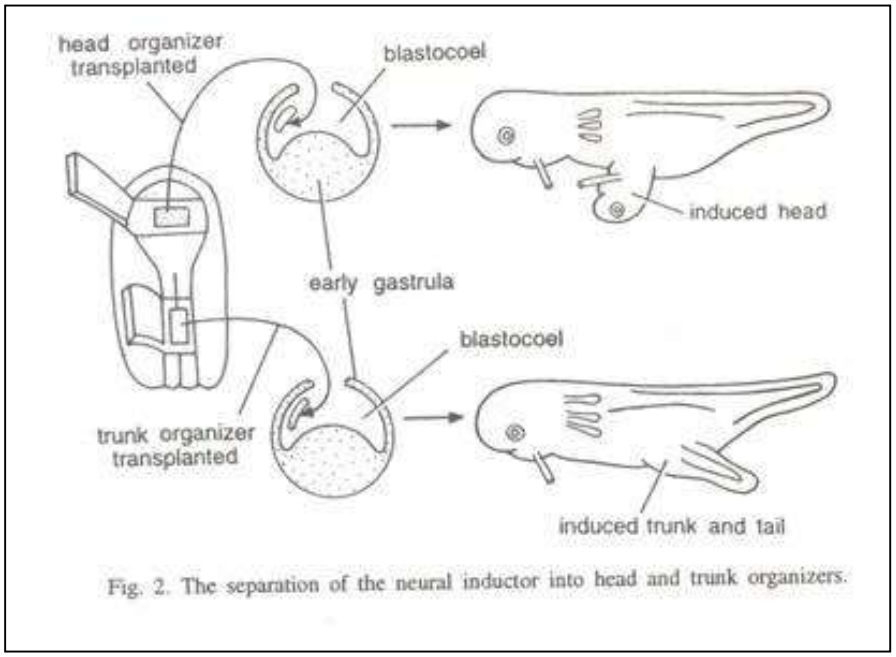


Fig. 2. The separation of the neural inductor into head and trunk organizers.

As invagination continues and the dorsal lip no longer consists of prospective head endo-mesoderm but progressively becomes prospective trunk mesoderm; it acts as a trunk-tail inductor. The most caudal region of the archenteron roof, in fact, specifically induces tail somites and probably other mesodermal tissues. The archenteron roof induces entirely different class of tissues; various neural and meso-ectodermal tissues by its anterior region and various mesodermal tissues by its most posterior region.

Therefore, differences in specific induction capacities exist between head and trunk level of archenteron roof and are related to the regional differentiation of the neural tissue into archencephalic (including fore-brain, eye, nasal pit), deuterencephalic (including hind-brain, ear vesicle) and spinocaudal components. Thus, archenteron roof consists of an anterior head inductor including an archencephalic inductor and a deuterencephalic inductor and a trunk or spinocaudal inductor.

Primary induction and gray crescent:

The dorsal lip region of the blastopore at the onset of gastrulation can be traced back to the gray-crescent of the undivided fertilized amphibian egg. It was conceived by some developmental biologists that the crescent material of egg cortex initiated gastrulation and has the capacity of neural induction. A.S.G. Curtis (1963) performed a series of experiments of transplanting parts of the cortex of the fertilized egg of the clawed toad, *Xenopus laevis* at the beginning of cleavage. In one experiment, the gray-crescent cortex was excised from the fertilized egg and it was observed that the cell division though proceeded undisturbed, the gastrulation failed to take place (Fig. 3A). In another experiment, the gray crescent cortex of uncleaved fertilized egg was excised and transplanted into a ventral position of a second egg, so that the egg receiving the graft had two gray crescents on opposite sides.

As a result, egg cleaved to form a blastula, which underwent two separate gastrulation movements to produce two separate primary nervous systems, notochord and associated somites (Fig. 3D). Similar experiments conducted on the eight-cell stage showed that something had happened during the short – interval represented by the first three cleavages.

Gray crescent cortex of the eight-cell stage still retained its inductive capacity when grafted to younger stages (Fig. 3C). Removal of the gray crescent at this stage no longer inhibits subsequent gastrulation and normal development, the missing crescent properties being replaced from adjacent cortical regions (Fig. 3B).

According to Curtis, a change in cortical organization spreads across the surface of the egg during the second and third cleavages, starting from the gray crescent; when this change is completed, interactions, probably of a biophysical nature, can take place among various parts of the cortex.

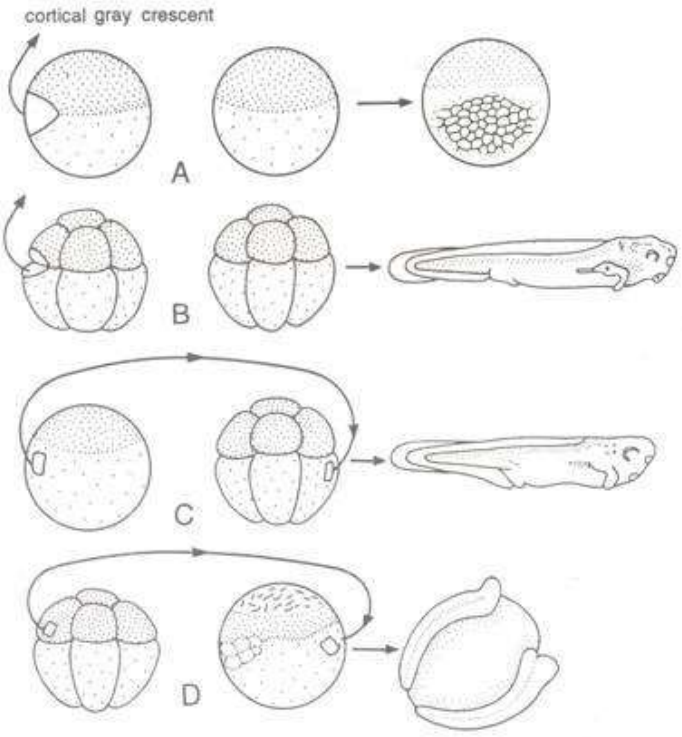


Fig. 3. Experiment of Curtis on *Xenopus*. A–Excision of the cortical gray crescent area at one cell stage : *no gastrulation*. B–Same experiment at the eight-cell stage : *normal embryo*. C–Graft of the gray-crescent cortex of one-cell stage to the ventral part of the eight-cell stage does not result in the induction of a secondary embryonic axis. D–Graft of the gray crescent cortex from the eight-cell stage to the ventral margin of the one-cell stage induces a secondary embryonic axis.

Mechanism of neural induction:
Development of the ectoderm overlying the roof of the archenteron into neural tissue suggests a direct action upon the ectodermal cells, either by surface interaction or by chemical mediation.

(1) One of the broad possibility is surface interaction of the cells at the inductive interface. The contact of the two cellular layers may provide a device whereby the structural pattern or geometry or behaviour of the ectodermal cell membranes is altered directly by the underlying chorda mesodermal cells.

Thus, the spatial configuration of the latter membranes might induce a change in the spatial configuration of the ectodermal cell membranes, this in turn producing in the interior of the cell changes that determine its development into neural plate. A morphological arrangement of this kind could account for quick and effective transmission of the inductive effect.

(2) Another broad possibility is a chemical mediation of the inductive effect. Therefore, a chemical substance or substances produced and released by inducing chorda mesoderm cells at the archenteron - ectoderm interface may act upon or enter the ectodermal cells to initiate cellular activities leading to neural development. A great deal of evidence favours the idea of an exchange of material between cells and also suggests that a diffusible substance may act as effective inductive stimulus.

Chemical basis of neural induction:
The results of numerous studies to elucidate the mechanism of induction and to identify the chemical substance or substances presumed to be involved have not yielded good results. It was found that many different tissues, embryonic or adult, from a great variety of different species, were capable of inducing nervous tissue in amphibian embryos. Moreover, some foreign tissues were found to be much more potent inductors after they had been killed by heat or alcohol treatment.

This fact remains against the concept of a universally present 'masked organizer', released in the primary inductor region. Few inorganic agents as iodine and kaolin, local injury, exposure to saline solutions of excessively high or low pH, cause neural differentiation in ectoderm. These findings establish the early grand concept of master-chemical embryonic organizer of Holtfreter's sublethal cytolysis. It has the concept of reversible cell injury liberating neural inductor.

Different chemical substances of either gray crescent or dorsal lip or chordamesoderm are separated by different biochemical methods to find out the molecule which causes the neural induction and then the inductive capacity of each molecule was tested separately. Few experiments show that evocator or inducing substance is a protein.

Exhaustive attempts were made by different embryologists to understand the real mechanism of neural induction. Some theories have been put forward to understand the mechanism of neural induction, out of which the most important are as follows:

1. Protein denaturation theory of neural induction:
According to Ranzi (1963) neural induction and notochord formation are related to protein denaturation. Site of notochord formation is amphibian gray crescent, which is a center of high metabolic activity. Such centers of greater metabolic activity correspond to sites of protein denaturation.

2. Gradient theory of neural induction:
Toivonen (1968) and Yamada (1961) stated that two chemically distinct factors are involved in the action of the primary inductor. Out of these two factors, one is neuralizing agent and the other is mesodermalizing agent. These experiments were conducted with denatured bone marrow and liver as the inductors.

Regional specificity of the embryonic axis arises from the interaction between two gradients: neutralizing principle has its highest concentration in the dorsal side of the embryo and diminishes laterally, while the mesodermalizing principle is present as an antero-posterior gradient with its peak in the posterior region. Anteriorly the neutralizing principle acts alone to induce forebrain structures, more posteriorly the mesodermalizing principle acts along with the neutralizing one to induce mid-brain and hind-brain structures, while even more posteriorly the high concentration level of the mesodermal gradient produces spino-caudal structures (Fig. 4).

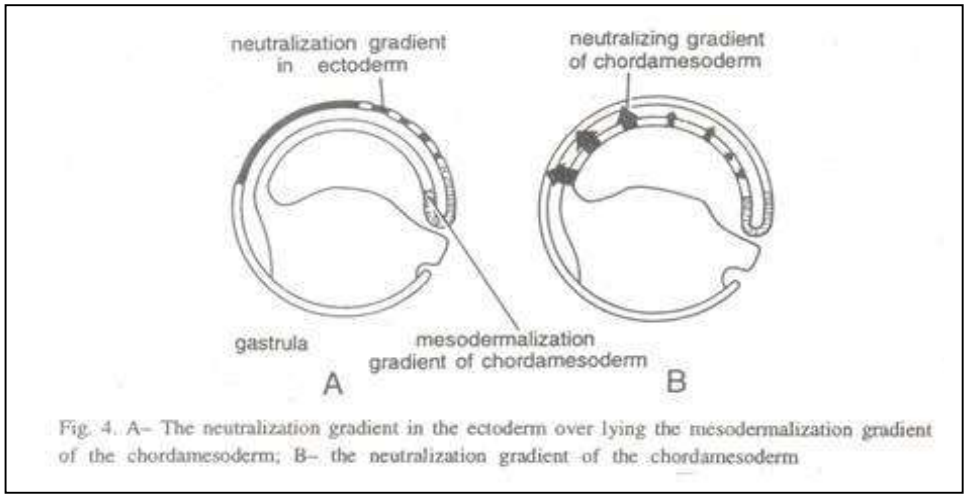


Fig. 4. A– The neutralization gradient in the ectoderm over lying the mesodermalization gradient of the chordamesoderm; B– the neutralization gradient of the chordamesoderm

3. One factor hypothesis of neural induction:

Nieuwkoop (1966) using living notochord as the inductor, postulated that only one factor which first evokes ectoderm to form neural tissue and later causes ectoderm to transform into more posterior and mesodermal structure (Fig. 5) is involved.

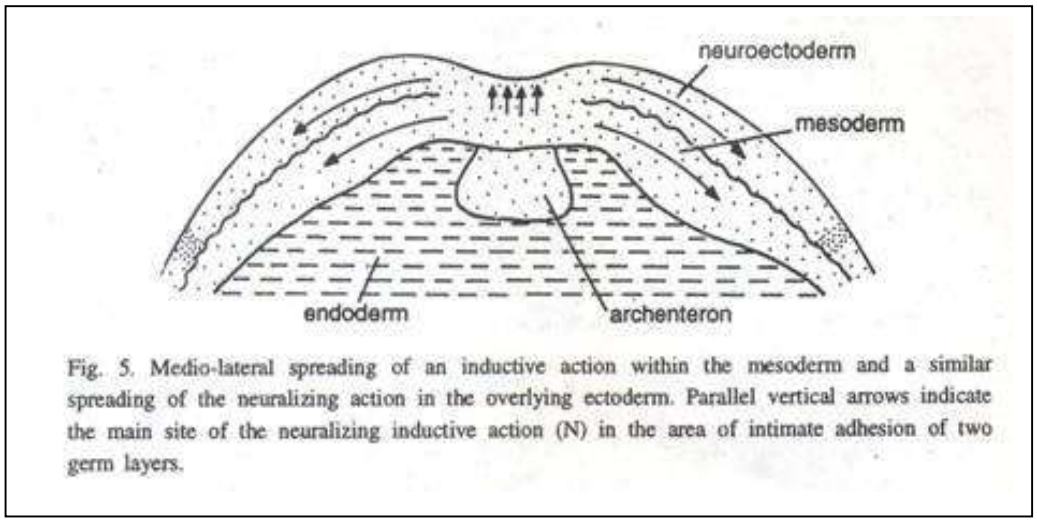


Fig. 5. Medio-lateral spreading of an inductive action within the mesoderm and a similar spreading of the neuralizing action in the overlying ectoderm. Parallel vertical arrows indicate the main site of the neuralizing inductive action (N) in the area of intimate adhesion of two germ layers.

In one experiment, consisting of combining isolated gastrular ectoderm with a piece of notochord and then removing the notochord tissue after varying lengths of time, it was found that only 5 minutes exposure to inductor caused a part of the ectoderm to transform into brain and eye structures.

4. Ionic theory of neural induction:

According to Barth and Barth (1969), the actual process of induction may be initiated by release of ions from bound form, representing a change in the ratio between bound to free ions within the cell of the early

gastrula. Induction of nerve and pigment cells in small aggregates of prospective epidermis of the frog gastrula were found to be dependent on the concentration of the sodium ions.

Normal induction of nerve and pigment cells by mesoderm in small explants from the dorsal lip and lateral marginal zones of the early gastrula is dependent on the external concentration of sodium. Thus, normal embryonic induction depends on an endogenous source of ions and that an intracellular release of such ions occurs during late gastrulation.

Genic basis of neural induction:
There are evidences that the component tissues of neural inductor become differentiated prior to ectodermal cells. During this process, the rate of transcription of mRNA and differential activation of genes becomes many fold, while the differentiation of ectodermal cells is set in only after mid-gastrulation.

According to experiments conducted by Tiedemann (1968), after 2 to 7 days of cultivation of dorsal blastopore lip of young Triturus gastrula with adjacent ectoderm in a medium containing sufficient quantities of Actinomycin-D to inhibit RNA synthesis, induction could not take place, but some differentiation of muscle and notochord occurred. It shows that mRNA by transcription from the DNA was required, which also requires the presence of Actinomycin-D. Therefore, no neural induction could be detected in this experiment.

Time of neural induction:
Neural induction occurs at the time when the material of chordamesoderm moves from the dorsal lip of blastopore inward and forward. The inductive stimuli exhibit a time gradient, which may be crucial with regard to action and reaction events.

Embryonic induction in different chordates:
Although neural induction was first discovered in urodele amphibians, it was found that the dorsal lip of the blastopore and the roof of the archenteron of other vertebrates have the same function. The chordamesoderm in all vertebrates induces the nervous system and sense organs. Neural inductor has been investigated in the following chordates:

(1) In Cyclostomes, especially in lampreys, the property of neural induction lies in the presumptive chorda mesodermal cells of dorsal lip of the blastopore.

Prior to cyclostomes, in Ascidians different blastomeres of eight cell stage have the following presumptive fates-(i) the two anterior animal pole blastomeres produce head epidermis, palps and the brain with its two pigmented sensory structures, (ii) two posterior animal pole blastomeres produce epidermis, (iii) two anterior vegetal blastomeres produce notochord, spinal cord and part of the intestine (iv) two posterior vegetal cells produce mesenchyme, muscles and part of the intestine.

From these experiments, Raverberi (1960) concluded that the formation and differentiation of brain by two anterior animal blastomeres is dependent on the induction of two anterior vegetal blastomeres, which act as neural inductors. It was further concluded that the two anterior vegetal blastomeres gave rise to diverse tissues, namely, endoderm, notochord and spinal cord.

(2) Wu and Tung (1962) proved the existence of the primary organizer and neural induction in Amphioxus. They transplanted pieces of tissues from the inner surface of the dorsal blastopore lip of an early gastrula of Amphioxus into the blastocoel of another embryo in the same stage (Fig. 6) and observed that secondary embryo developed in the ventral region of the host with a notochord and mesoderm produced by the graft and the neural tube from host tissue.

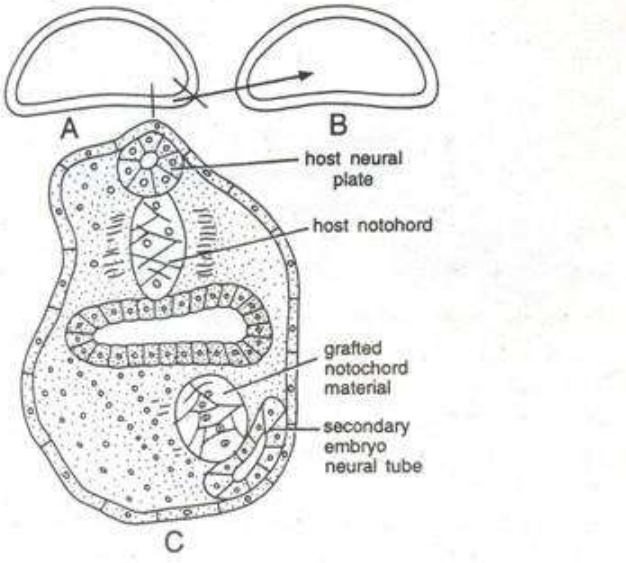


Fig. 6. Neural induction in *Amphioxus*. A–Diagram of operation-cells lying inside dorsal lip of the blastopore transplanted into the blastocoel of an embryo; B–in the same stage of development; C–T.S. of host embryo with the secondary neural tube.

Thus, the chordal tissue of Amphioxus gastrula possesses the power of neural induction, while mesodermal and endodermal tissues have little such inductive power.

(3) In bony fishes, induction of secondary well developed embryos were produced by transplanting the posterior edge of the blastodisc which corresponds to the dorsal lip of the blastopore, into the blastocoel of another embryo (Fig. 7) or by transplanting the chordamesoderm and ectoderm. Neural inductions were also obtained by transplanting the dorsal lip of the blastopore in the sturgeon.

(4) In frogs, the induction of secondary embryo can be produced by the dorsal lip of the blastopore transplanted into the blastocoel of a young gastrula, in very much the same way as in newts and salamandars.

(5) In reptiles archenteron has the same inducing activity as in other vertebrates but there is no experimental proof of occurrence of neural inductor.

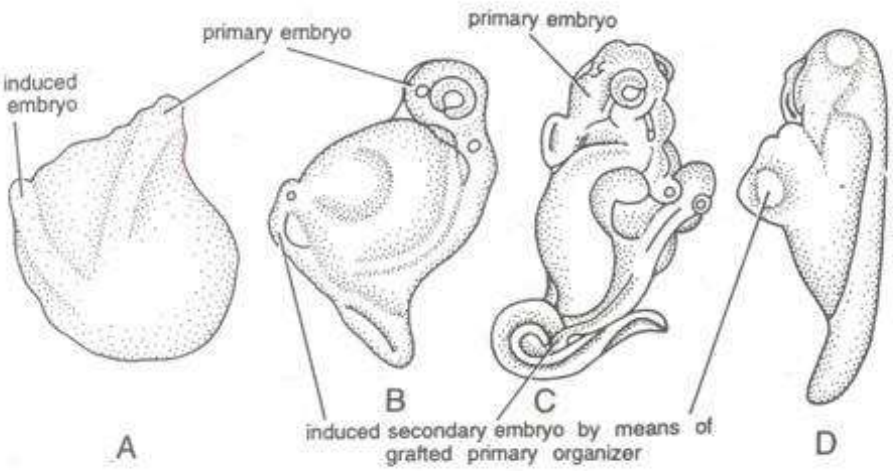


Fig. 7. Induction of secondary embryos by means of grafted primary organizers in the-A-lamprey; B–and C–perch; D–frog.

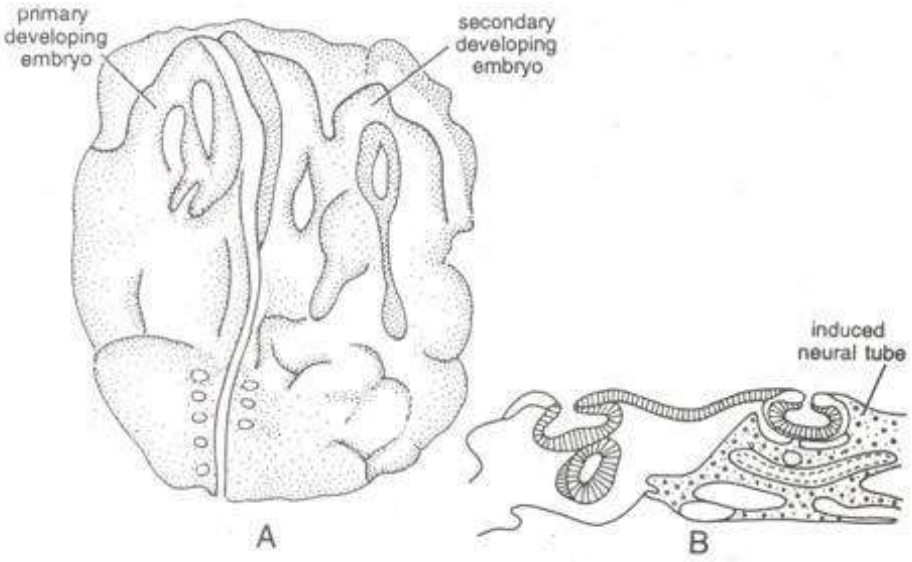


Fig. 8. Induction of a secondary embryo by means of a grafted primitive streak in a bird. A–surface view; the secondary embryo is on the right. B–Section through the same embryo showing the host axial system (right) and the induced neural tube and mesoderm developed from the graft (left).

(6) In birds the existence of primary organizer was established by Waddington and co-workers. Anterior half of the primitive streak was the inducing part similar to the lips of the blastopore in amphibians. In the experiment whole blastoderms were removed from the egg in early gastrulation and cultivated in vitro on the blood plasma clot.

From another embryo, parts of the primitive streak were then inserted between epiblast and hypoblast, inductions of secondary embryos obtained. Primitive streak was found dependent on the underlying hypoblast for its formation (Fig. 8).

(7) A successful neural induction was performed in a rabbit embryo by cultivating the early blastodisc on a plasma clot and implanting the primitive streak of the chick as inductor. Tissues of the mammalian gastrula were found having competence for neural induction. Anterior end of a rabbit embryo, with two pairs of somites, induced a neural plate in a chick embryo when placed under a chick blastoderm.

Other types of embryonic inductions:
Along with gastrulation growth, various organ systems of the embryo begin to differentiate and acquire the power of inducing the differentiation of later formed structures or organs such as eyes, ears, limbs and lungs, etc. These organs develop organizing property and become the source of induction.

Therefore, this series of organizers can be called as secondary, tertiary and quaternary organizers. Progressive development of embryonic organs is dependent on sequential induction. One embryonic tissue interacts with the adjacent one and induces it to develop and this process continues in sequence.

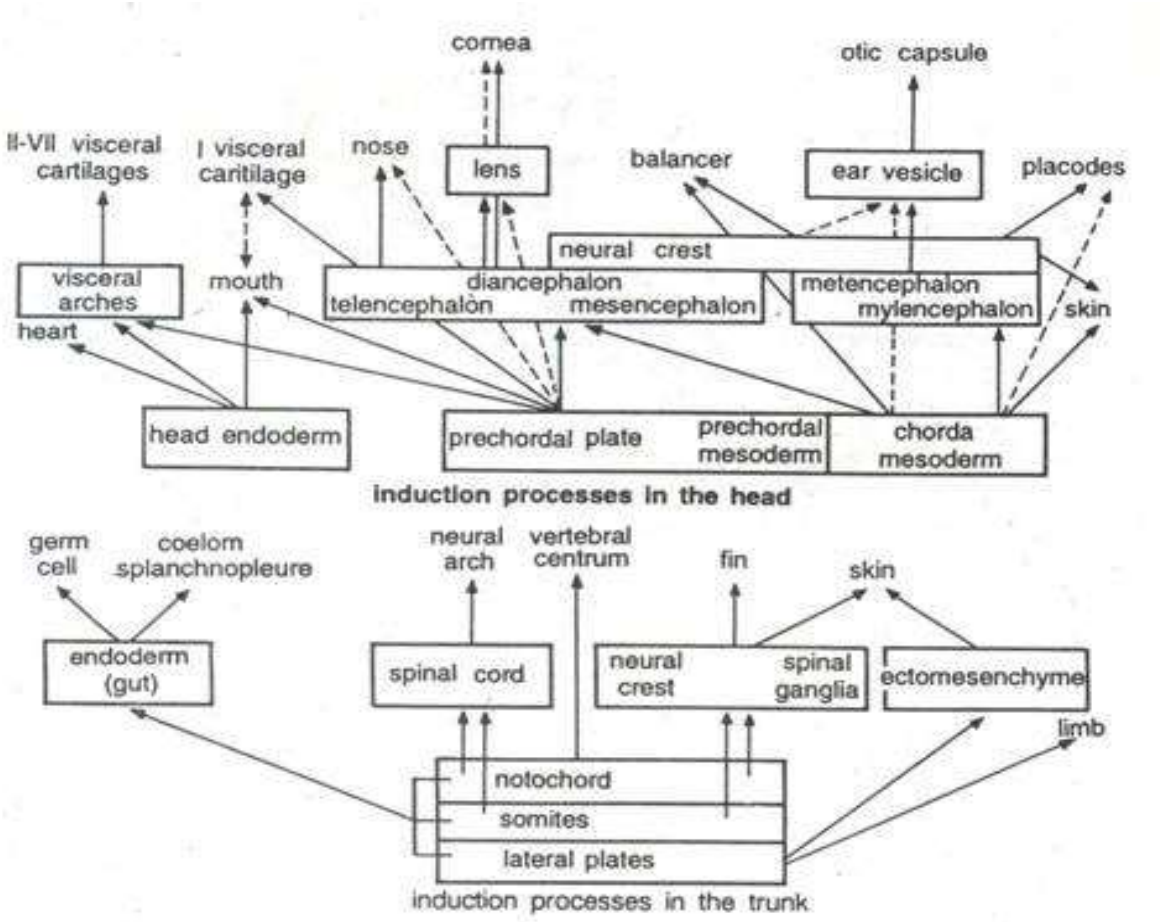


Fig. 9. The induction process during early amphibian development in head and in trunk regions. The inductive action starts from the archenteron roof (inductor of the first degree–primary inductor); the inductors of second, third and following degrees affect the process by their own actions. The different strengths of the inductive action are indicated by thick and broken arrows.

Development of eye:
Chorda mesoderm, the primary organizer induces the formation of fore-brain and optic area in the anterior part of the embryo. The optic area evaginates forming the optic vesicle. By invagination it changes into a double walled cup-like structure, the optic cup which acts as secondary organizer to induce the formation of tertiary organizer to form cornea. The layer of mesenchyme left in front of the anterior chamber of eye combines with the overlying somatic ectoderm (epidermis) and forms cornea, choroid and sclera (Fig. 9). Thus the whole process of development seems to be a cause of induction and interaction only. Number of inductions are secondary or tertiary such as nasal-groove, optic vesicle, lens, cornea and so on involve ectodermal reactions.

Induction and Competence
Organs are complex structures composed of numerous types of tissues. In the vertebrate eye, for example, light is transmitted through the transparent corneal tissue and focused by the lens tissue (the diameter of which is controlled by muscle tissue), eventually impinging on the tissue of the neural retina. The precise arrangement of tissues in this organ cannot be disturbed without impairing its function. Such coordination in the construction of organs is accomplished by one group of cells changing the behavior of an adjacent set of cells, thereby causing them to change their shape, mitotic rate, or fate. This kind of interaction at close range between two or more cells or tissues of different history and properties is called proximate interaction, or induction. There are at least two components to every inductive interaction. The first component is the inducer: the tissue that produces a signal (or signals) that changes the cellular behavior of the other tissue. The second component, the tissue being induced, is the responder.

Not all tissues can respond to the signal being produced by the inducer. For instance, if the optic vesicle (presumptive retina) of *Xenopus laevis* is placed in an ectopic location (i.e., in a different place from where it normally forms) underneath the head ectoderm, it will induce that ectoderm to form lens tissue. Only the optic vesicle appears to be able to do this; therefore, it is an inducer. However, if the optic vesicle is placed beneath ectoderm in the flank or abdomen of the same organism, that ectoderm will not be able to respond. Only the head ectoderm is competent to respond to the signals from the optic vesicle by producing a lens[†]

Cascades of induction: Reciprocal and sequential inductive events
Another feature of induction is the reciprocal nature of many inductive interactions. Once the lens has formed, it can then induce other tissues. One of these responding tissues is the optic vesicle itself. Now the inducer becomes the induced. Under the influence of factors secreted by the lens, the optic vesicle becomes the optic cup, and the wall of the optic cup differentiates into two layers, the pigmented retina and the neural retina. Such interactions are called reciprocal inductions.

Instructive and permissive interactions
Howard Holtzer (1968) distinguished two major modes of inductive interaction. In instructive interaction, a signal from the inducing cell is necessary for initiating new gene expression in the responding cell. Without the inducing cell, the responding cell would not be capable of differentiating in that particular way. For example, when the optic vesicle is experimentally placed under a new region of the head ectoderm and causes that region of the ectoderm to form a lens, that is an instructive interaction. Wessells (1977) has proposed three general principles characteristic of most instructive interactions:

1. In the presence of tissue A, responding tissue B develops in a certain way.
2. In the absence of tissue A, responding tissue B does not develop in that way.
3. In the absence of tissue A, but in the presence of tissue C, tissue B does not develop in that way.

The second type of induction is permissive interaction. Here, the responding tissue contains all the potentials that are to be expressed, and needs only an environment that allows the expression of these

traits.‡ For instance, many tissues need a solid substrate containing fibronectin or laminin in order to develop. The fibronectin or laminin does not alter the type of cell that is to be produced, but only enables what has been determined to be expressed.

Epithelial-mesenchymal interactions:
Some of the best-studied cases of induction are those involving the interactions of sheets of epithelial cells with adjacent mesenchymal cells. These interactions are called epithelial-mesenchymal interactions. Epithelia are sheets or tubes of connected cells; they can originate from any germ layer. Mesenchyme refers to loosely packed, unconnected cells. Mesenchymal cells are derived from the mesoderm or neural crest. All organs consist of an epithelium and an associated mesenchyme, so epithelial-mesenchymal interactions are among the most important phenomena in nature.

Regional specificity of induction :
Using the induction of cutaneous structures as our examples, we will look at the properties of epithelial-mesenchymal interactions. The first of these properties is the regional specificity of induction. Skin is composed of two main tissues: an outer epidermis, an epithelial tissue derived from ectoderm, and a dermis, a mesenchymal tissue derived from mesoderm. The chick epidermis signals the underlying dermal cells to form condensations (probably by secreting Sonic hedgehog and TGF-β2 proteins, which will be discussed below), and the condensed dermal mesenchyme responds by secreting factors that cause the epidermis to form regionally specific cutaneous structures. These structures can be the broad feathers of the wing, the narrow feathers of the thigh, or the scales and claws of the feet. Researchers can separate the embryonic epithelium and mesenchyme from each other and recombine them in different ways (Saunders et al. 1957). As Figure 6.7demonstrates, the dermal mesenchyme is responsible for the regional specificity of induction in the competent epidermal epithelium. The same type of epithelium develops cutaneous structures according to the region from which the mesenchyme was taken. Here, the mesenchyme plays an instructive role, calling into play different sets of genes in the responding epithelial cells.

Genetic specificity of induction
The second property of epithelial-mesenchymal interactions is the genetic specificity of induction. Whereas the mesenchyme may instruct the epithelium as to what sets of genes to activate, the responding epithelium can comply with these instructions only so far as its genome permits. This property was discovered through experiments involving the transplantation of tissues from one species to another. In one of the most dramatic examples of interspecific induction, Hans Spemann and Oscar Schotté (1932) transplanted flank ectoderm from an early frog gastrula to the region of a newt gastrula destined to become parts of the mouth. Similarly, they placed presumptive flank ectodermal tissue from a newt gastrula into the presumptive oral regions of frog embryos. The structures of the mouth region differ greatly between salamander and frog larvae. The salamander larva has club-shaped balancers beneath its mouth, whereas the frog tadpole produces mucus-secreting glands and suckers. The frog tadpole also has a horny jaw without teeth, whereas the salamander has a set of calcareous teeth in its jaw. The larvae resulting from the transplants were chimeras. The salamander larvae had froglike mouths, and the frog tadpoles had salamander teeth and balancers. In other words, the mesodermal cells instructed the ectoderm to make a mouth, but the ectoderm responded by making the only kind of mouth it “knew” how to make, no matter how inappropriate.

Metaplasia:
Metaplasia (Greek: "change in form") is the reversible transformation of onedifferentiated cell type to another differentiated cell type. The change from one type of cell to another may be part of a normal maturation process, or caused by some sort of abnormal stimulus. In simplistic terms, it is as if the original cells are not robust enough to withstand their environment, so they transform into another cell type better suited to their environment.

If the stimulus causing metaplasia is removed or ceases, tissues return to their normal pattern of differentiation. Metaplasia is not synonymous with dysplasia, and is not considered to be an actual cancer. It is also contrasted with heteroplasia, which is the spontaneous abnormal growth of cytologic and histologicelements.

Today, metaplastic changes are usually considered to be an early phase of carcinogenesis, specifically for those with a history of cancers or who are known to be susceptible to carcinogenic changes. Metaplastic change is often viewed as a premalignant condition that requires immediate intervention, either surgical or medical, because metaplasia is associated with cancer.

When cells are faced with physiological or pathological stresses, they respond by adapting in any of several ways, one of which is metaplasia. It is a benign (i.e. non-cancerous) change that occurs as a response to change of milieu (physiological metaplasia) or chronic physical or chemical irritation (pathological metaplasia). One example of pathological irritation is cigarette smoke that causes the mucus-secreting ciliated pseudostratified columnar respiratory epithelial cells that line the airways to be replaced by stratified squamous epithelium, or a stone in the bile duct that causes the replacement of the secretory columnar epithelium with stratified squamous epithelium (Squamous metaplasia).

Metaplasia is an adaptation that replaces one type of epithelium with another that is more likely to be able to withstand the stresses it is faced with. It is also accompanied by a loss of endothelial function, and in some instances considered undesirable; this undesirability is underscored by the propensity for metaplastic regions to eventually turn cancerous if the irritant is not eliminated.

The cell of origin for many types of metaplasias is controversial or unknown.
For example, there is evidence supporting several different hypotheses of origin in Barrett's esophagus. They include directtransdifferentiation of squamous cells to columnar cells, the stem cell changing from esophageal type to intestinal type, migration of gastric cardiac cells, and a population of resident embryonic cells which are present through adulthood.

Significance:
Normal physiological metaplasia, such as that of the endocervix, is highly desirable. The medical significance of metaplasia is that in some sites where pathological irritation is present, cells may progress from metaplasia, to develop dysplasia, and then malignant neoplasia (cancer).

Thus, at sites where abnormal metaplasia is detected, efforts are made to remove the causative irritant, thereby decreasing the risk of progression to malignancy. The metaplastic area must be carefully monitored to ensure that dysplastic change does not begin to occur. A progression to significant dysplasia indicates that the area could need removal to prevent the development of cancer.

Example:
Barrett's esophagus is an abnormal change in the cells of the lower esophagus, thought to be caused by damage from chronic stomach acid exposure.

The following table lists some common tissues susceptible to metaplasia, and the stimuli that can cause the change:

Tissue	Normal	Metaplasia	Stimulus
Airways	Pseudo stratified columnar epithelium	Squamous epithelium	Cigarette smoke
Urinary bladder	Transitional epithelium	Squamous epithelium	Bladder stone
Esophagus	Squamous epithelium	Columnar epithelium (Barrett's Esophagus)	Gastro-esophageal reflux
Cervix	Glandular epithelium	Squamous epithelium	Low pH ofvagina

Probable questions:

1. What is differentiation? Describe the mechanism of differentiation.
2. Define induction and competence.
3. Elucidate the process of determination.

Suggested readings:

GILBERT, S. F., & BARRESI, M. J. F. (2016). *Developmental biology*.

Unit-V

Reversibility of differentiated state, criteria for dedifferentiation, metaplasia and transdifferentiation, modulation

Objective: In this unit, you will learn about Reversibility of differentiated state, criteria for dedifferentiation, metaplasia and transdifferentiation, modulation.

Transdifferentiation, also known as **lineage reprogramming**, is a process in which one mature somatic cell transforms into another mature somatic cell without undergoing an intermediate pluripotent state or progenitor cell type. It is a type ofmetaplasia, which includes all cell fate switches, including the interconversion of stem cells. Current uses of transdifferentiation include disease modeling and drug discovery and in the future may include gene therapy andregenerative medicine. The term 'transdifferentiation' was originally coined by Selman and Kafatos in 1974 to describe a change in cell properties as cuticle producing cells became salt-secreting cells in silk moths undergoing metamorphosis.

Discovery

Davis et al. 1987 reported the first instance of transdifferentiation where a cell changed from one adult cell type to another. Forcing mouse embryonic fibroblasts to express MyoD was found to be sufficient to turn those cells into myoblasts.

Natural examples

There are no known instances where adult cells change directly from one lineage to another except *Turritopsis dohrnii*. Rather, cells dedifferentiate and then redifferentiate into the cell type of interest. In newts when the eye lens is removed, pigmented epithelial cells de-differentiate and then redifferentiate into the lens cells. In the pancreas, it has been demonstrated that alpha cells can spontaneously switch fate and transdifferentiate into beta cells in both healthy and diabetic human and mouse pancreatic islets. While it was previously believed that oesophageal cells were developed from the transdifferentiation of smooth muscle cells, that has been shown to be false.

Induced and therapeutic examples

The first example of functional transdifferentiation has been provided by Ferber et al. by induce a shift in the developmental fate of cells in liver and convert them into 'pancreatic beta-cell-like' cells. The cells induced a wide, functional and long-lasting transdifferentiation process that reduced the effects of hyperglycemia in diabetic mice. Moreover, the trans-differentiated beta-like cells were found to be resistant to the autoimmune attack that characterizes type 1 diabetes.

The second step was to undergo transdifferentiation in human specimens. By transducing liver cells with a single gene, Sapir et al. were able to induce human liver cells to transdifferentiate into human beta cells. This approach has been demonstrated in mice, rat, xenopus and human tissues.

Schematic model of the hepatocyte-to-beta cell transdifferentiation process:
Hepatocytes are obtained by liver biopsy from diabetic patient, cultured and expanded ex vivo, transduced with a PDX1 virus, transdifferentiated into functional insulin-producing beta cells, and transplanted back into the patient.

Methods:

Lineage-instructive approach
In this approach, transcription factors from progenitor cells of the target cell type are transfected into a somatic cell to induce transdifferentiation. There exists two different means of determining which transcription factors to use: by starting with a large pool and narrowing down factors one by one or by starting with one or two and adding more. One theory to explain the exact specifics is that ectopic Transcriptional factors direct the cell to an earlier progenitor state and then redirects it towards a new cell type. Rearrangement of the chromatin structure via DNA methylation or histone modification may play a role as well. Here is a list of in vitro examples and in vivo examples. In vivo methods of transfecting specific mouse cells utilize the same kinds of vectors as in vitro experiments, except that the vector is injected into a specific organ. Zhou et al. (2008) injected Ngn3, Pdx1 and Mafa into the dorsal splenic lobe (pancreas) of mice to reprogram pancreaticexocrine cells into β-cells in order to ameliorate hyperglycaemia.

Initial epigenetic activation phase approach
Somatic cells are first transfected with pluripotent reprogramming factors temporarily (Oct4, Sox2, Nanog, etc.) before being transfected with the desired inhibitory or activating factors. Here is a list of examples in vitro.

Pharmacological agents
The DNA methylation inhibitor, 5-azacytidine is also known to promote phenotypic transdifferentiation of cardiac cells to skeletal myoblasts.

Mechanism of action
The transcription factors serve as a short term trigger to an irreversible process. The transdifferentiation liver cells observed 8 months after one single injection of pdx1.
The ectopic transcription factors turn off the host repertoire of gene expression in each of the cells. However, the alternate desired repertoire is being turned on only in a subpopulation of predisposed cells. Despite the massive dedifferentiation – lineage tracing approach indeed demonstrates that transdifferentiation originates in adult cells.

Mogrify algorithm
Determining the unique set of cellular factors that is needed to be manipulated for each cell conversion is a long and costly process that involved much trial and error. As a result, this first step of identifying the key set of cellular factors for cell conversion is the major obstacle researchers face in the field of cell reprogramming. An international team of researchers have developed an algorithm, called Mogrify, that can predict the optimal set of cellular factors required to convert one human cell type to another. When tested, Mogrify was able to accurately predict the set of cellular factors required for previously published cell conversions correctly. To further validate Mogrify's predictive ability, the team conducted two novel cell conversions in the laboratory using human cells, and these were successful in both attempts solely using the predictions of Mogrify. Mogrify has been made available online for other researchers and scientists.

Issues

Evaluation
When examining transdifferentiated cells, it is important to look for markers of the target cell type and the absence of donor cell markers which can be accomplished using green fluorescent protein or immunodetection. It is also important to examine the cell function, epigenome, transcriptome,

and proteome profiles. Cells can also be evaluated based upon their ability to integrate into the corresponding tissue in vivo and functionally replace its natural counterpart. In one study, transdifferentiating tail-tip fibroblasts into hepatocyte-like cells using transcription factors Gata4, Hnf1α and Foxa3, and inactivation of p19(Arf) restored hepatocyte-like liver functions in only half of the mice using survival as a means of evaluation.

Transition from mouse to human cells

Generally transdifferentiation that occurs in mouse cells does not translate in effectiveness or speediness in human cells. Pang et al. found that while transcription factors Ascl1, Brn2 and Myt1l turned mouse cells into mature neurons, the same set of factors only turned human cells into immature neurons. However, the addition of NeuroD1 was able to increase efficiency and help cells reach maturity.

Order of transcription factor expression

The order of expression of transcription factors can direct the fate of the cell. Iwasaki et al. (2006) showed that in hematopoietic lineages, the expression timing of Gata-2 and (C/EBPalpha) can change whether or not a lymphoid-committed progenitors can
differentiateinto granulocyte/monocyte progenitor, eosinophil, basophil orbipotent basophil/mastcell progenitor lineages.

Immunogenicity

It has been found for induced pluripotent stem cells that when injected into mice, the immune system of the synergeic mouse rejected the teratomas forming. Part of this may be because the immune system recognized epigenetic markers of specific sequences of the injected cells. However, when embryonic stem cells were injected, the immune response was much lower. Whether or not this will occur within transdifferentiated cells remains to be researched.

Method of transfection

In order to accomplish transfection, one may use integrating viral vectors such as lentiviruses or retroviruses, non-integrating vectors such as Sendai viruses or adenoviruses, microRNAs and a variety of other methods including using proteins and plasmids; one example is the non-viral delivery of transcription factor-encoding plasmids with a polymeric carrier to elicit neuronal transdifferentiation of fibroblasts. When foreign molecules enter cells, one must take into account the possible drawbacks and potential to cause tumorous growth. Integrating viral vectors have the chance to cause mutations when inserted into the genome. One method of going around this is to excise the viral vector once reprogramming has occurred, an example being Cre-Lox recombination. Non-integrating vectors have other issues concerning efficiency of reprogramming and also the removal of the vector. Other methods are relatively new fields and much remains to be discovered.

Pluripotent reprogramming

Almost all factors that reprogram cells into pluripotency have been discovered and can turn a wide variety of cells back into induced pluripotent stem cells (iPSCs). However, many of the reprogramming factors that can change a cell's lineage have not been discovered and these factors apply only for that specific lineage.

- The final products of transdifferentiated cells are capable of being used for clinical studies, but iPSCs must be differentiated.
- It may become possible in the future to use transdifferentiation in vivo, whereas pluripotent reprogramming may cause teratomas in vivo.

- Transdifferentiated cells will require less epigenetic marks to be reset, whereas pluripotent reprogramming requires nearly all to be removed, which may become an issue during redifferentiation.
- Transdifferentiation is geared towards moving between similar lineages, whereas pluripotent reprogramming has unlimited potential.
- Pluripotent cells are capable of self-renewal and often go through many cell passages, which increases the chance of accumulating mutations. Cell culture may also favor cells that are adapted for surviving under those conditions, as opposed to inside an organism. Transdifferentiation requires fewer cell passages and would reduce the chance of mutations.
- Transdifferentiation can also be much more efficient than pluripotency reprogramming due to the extra step involved in the latter process.
- Both pluripotent and transdifferentiated cells use adult cells, thus starting cells are very accessible, whereas human embryonic stem cells require that one navigate legal loopholes and delve into the morality of stem cell research debate.

Probable questions:

1. Differentiate between dedifferentiation and transdifferentiation.
2. Describe the mechanism of dedifferentiation.
3. Elucidate the significance of transdifferentiation.

Suggested readings:

GILBERT, S. F., & BARRESI, M. J. F. (2016). *Developmental biology*.

Unit-VI

Neural crest cell migration based differentiation

Objective: In this unit, you will learn about neural crest cell migration based differentiation.

Neural crest cell migration based differentiation

The first important morphogenetic change following gastrulation is the deve-lopment of the central nervous system. The central nervous system starts as a simple tubular nerve tube which, in course of development, transforms into brain, spinal cord and their associated structures.

The morphogenetic processes involved in this process are designated as neuralisation. It includes the separation of neural materials from the embryonic ectoderm, their migration inward to form a hollow nerve tube together with the segregation of neural crest cells. The nerve tube differentiates into the brain and spinal cord, while neural crest cells develop into neuroblasts and many other structures.

Methods of Neuralisation:

Neuralisation occurs by two ways in different vertebrates.

These are:

(a) Thickened Keel Method: In teleost, ganoid fishes and cyclostomes the neural materials become aggregated to form a thickened keel or ridge extending along the mid-dorsal axis of the body. This ridge separates itself from the overlying ectoderm and develops a lumen within to form a tube.

(b) Neural Fold Method: It occurs in most of the vertebrates where neural cells become aggregated to form a neural plate. This plate folds inward to form neural groove. The neural groove transforms into a neural tube which sinks from the overlying ectoderm.

Events in Neural Morphogenesis:

After the completion of gastrulation, the ectoderm of the future dorsal side of the developing embryo tends to condense to form a thick and compact neural plate with elevated margins. This thickened part is called the neural (or medullary) plate or neural placode.

The plate is formed by two simultaneous processes:

(a) Elongation of prospective neural cells in the direction perpendicular to the surface of the developing embryo, and

(b) Shrinking the exposed surfaces both dorsally and ventrally.

The neural plate is pear-shaped, i.e. it is broader at the anterior part but gradually narrows towards the posterior end. This particular shape of the neural plate is crucial for shaping the future structures. The shaping of the neural plate is resulted as the consequence of regional differences in the cell contration. Fig. 5.25 relates the stages of neural morphogenesis in frog.

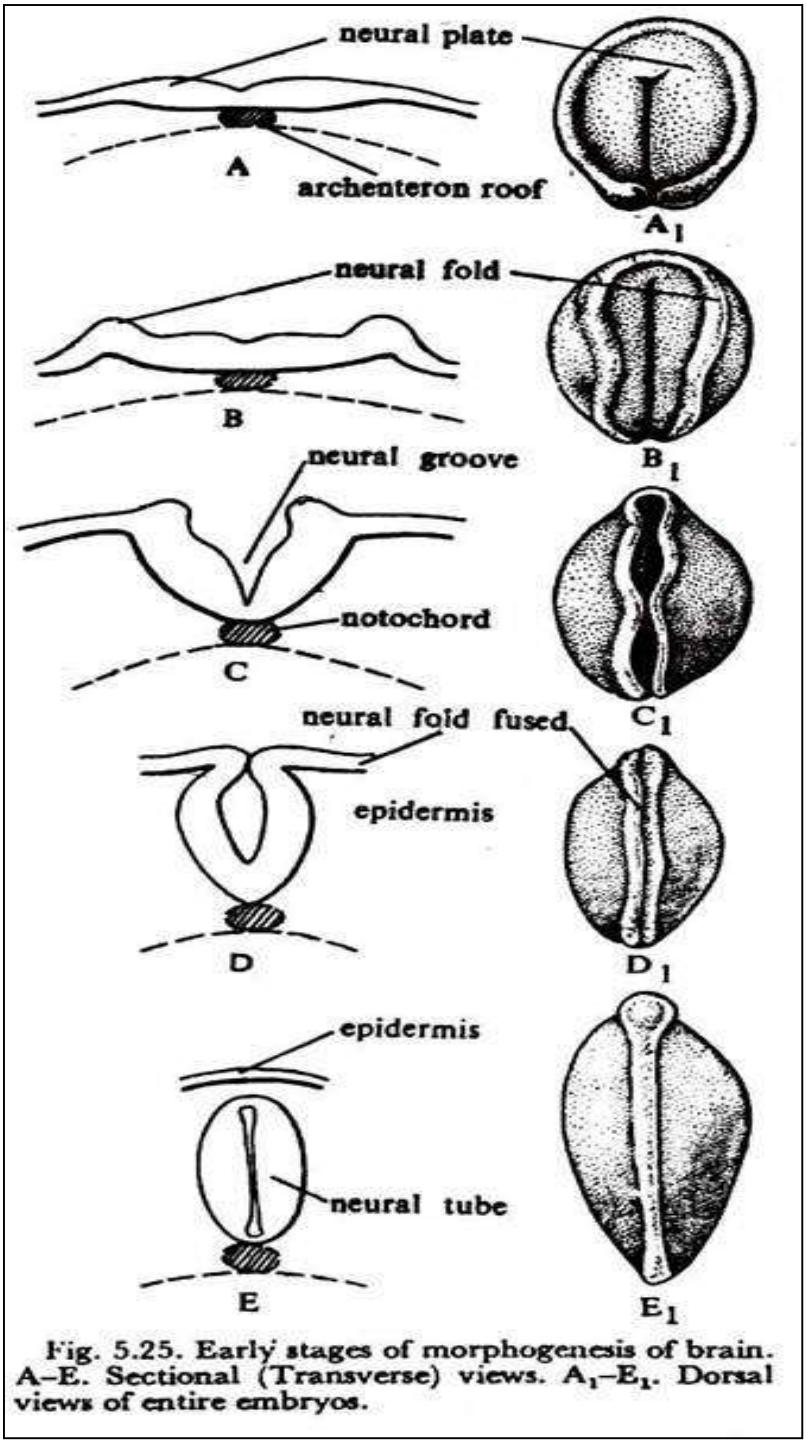


Fig. 5.25. Early stages of morphogenesis of brain. A–E. Sectional (Transverse) views. A_1–E_1. Dorsal views of entire embryos.

A depression appears along the entire length of the neural plate which folds downward to form a neufal or medullary groove. The formation of the neural groove is associated with the median and dorsal movement of the ectodermal layer attached to the lateral edges of the neural plate.

Thus the raised or folded margin of the neural groove is called the neural fold. The downward movement of the neural plate to form the neural groove depends largely on the lateral shifting of somatic mesoderm from the notochordal area to accommodate the invaginating neural groove. The lateral neural folds rise and meet along the middle line. This union begins from the anterior end and runs posteriorly.

With the union-of the folds, the outer ectodermal layers become continuous and the inner nervous layer, after fusion with the corresponding part, forms a tube and separates itself from the upper ectodermal layer. This tube-like structure is called the neural tube. The cavity of the neural tube is called the neurocoel which is broader at the anterior end and opens to the exterior through an opening called the neuropore. The neuropore ultimately closes at the later stage of development.

Associated with the formation of neural tube neural crest cells become segregated on the two sides of the neural tube. These neural crest cells lie as two longitudinal strips of cells, one on each dorsal side of the neural tube.

Neural Crest and its Fate:

At the corners of the fusing neural fold during brain formation, groups of neural crest cells become detached to occupy a position over the neural tube. In course of development these cells leave their position and migrate to other parts of the embryo.

These cells are versatile in their developmental fate and develop neuroblasts of the spinal and sympathetic ganglia, Schwann sheath cells producing the myelin sheath and neurilemma of the nerve fibres, melanoblasts, chromaffin tissue of adrenal medulla, meninges, cartilages of the jaw, etc. Weston (1963) has shown the migration of neural crest cells.

The neural tube and neural crest cells labelled with radioactive isotopes are excised from the trunk of a developing chick embryo and transplanted to a normal (non-labelled) host in place of its counterparts.

It has been shown that the neural crest cells migrate along two ways:

(i) Dorsolaterally along the skin and

(ii) Ventrolaterally in relation to the neural tube.

Structural Differentiation of the Neural Tube:

The differentiation of the neural tube into the brain and spinal cord depends upon many intrinsic and extrinsic factors. The anterior part of the neural tube transforms into the brain while the posterior narrow part becomes elongated to form the spinal cord.

The broad anterior part is demarcated from the narrow posterior part by isthmus. Remarkable changes occur in the anterior part during its conversion into the brain.

This is caused by:

(a) Unequal thickening of the neural tube wall,

(b) Invaginations or evaginations of the wall and

(c) Various types of bending or folding (flexure formation).

Immediately after the formation of the neural tube, the anterior part swells up and two constrictions develop to divide the anterior part into three general regions: Prosencephalon, Mesencephalon and Rhombencephalon.

In course of development, prosencephalon and rhombencephalon become further subdivided thus giving rise to five parts: Telencephalon, Diencephalon, Mesencephalon, Metencephalon and Myelen- cephalon (Fig. 5.26).

Many factors are responsible in brain morphogenesis. Differential growth and intraventricular pressure are regarded to be the important morphogenetic factors in brain development, especially in flexure formation. Fig. 5.26 relates the development of flexures and different regions of the brain.

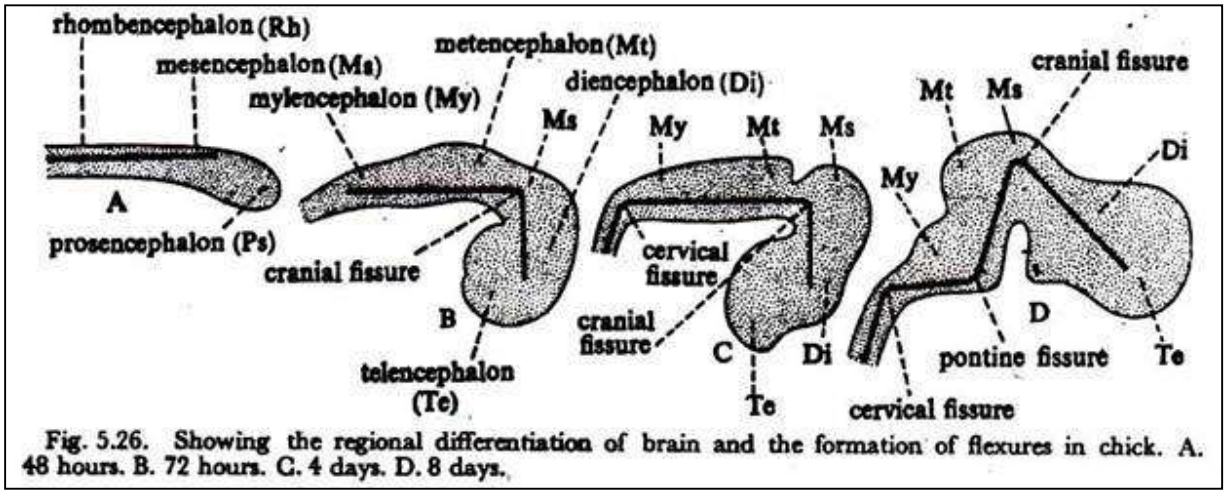


Fig. 5.26. Showing the regional differentiation of brain and the formation of flexures in chick. A. 48 hours. B. 72 hours. C. 4 days. D. 8 days.

Histogenesis in Brain Development:

The early neural tube is fairly uniform in structure. The walls are composed of neural epithelial cells which eventually differentiate into: (1) neuroblasts and (2) spongioblasts. The neuroblasts develop into nerve cells and fibres while the spongioblasts give origin to ependymal and neuroglial cells.

The neural epithelium is composed of pseudostratified columnar epithelial cells which form the primitive ependymal layer or matrix layer. Gradually the cells of the matrix layer migrate to cach lateral side to form a cellular layer called the mantle layer.

And lateral to the matle layer lies a cell-free marginal layer. The cells of primitive ependymal layer are usually called the germinal cells, some of which after a day or two following the closure of neural groove, develop neuroblasts and migrate first to the mantle layer (Fig. 5.27).

In the mantle layer, the cells differentiate into

(a) Neuroblasts and neurons and

(b) Spongioblasts and neuroglial cells.

The neuroglial cells give rise to astrocytes and oligodendrocytes. The neuroblasts do not remain evenly distributed but are aggregated into clusters. From mature neuroblasts, nerve cells and fibres grow out in a distinct pattern and turn the brain into a ‘working unit’.

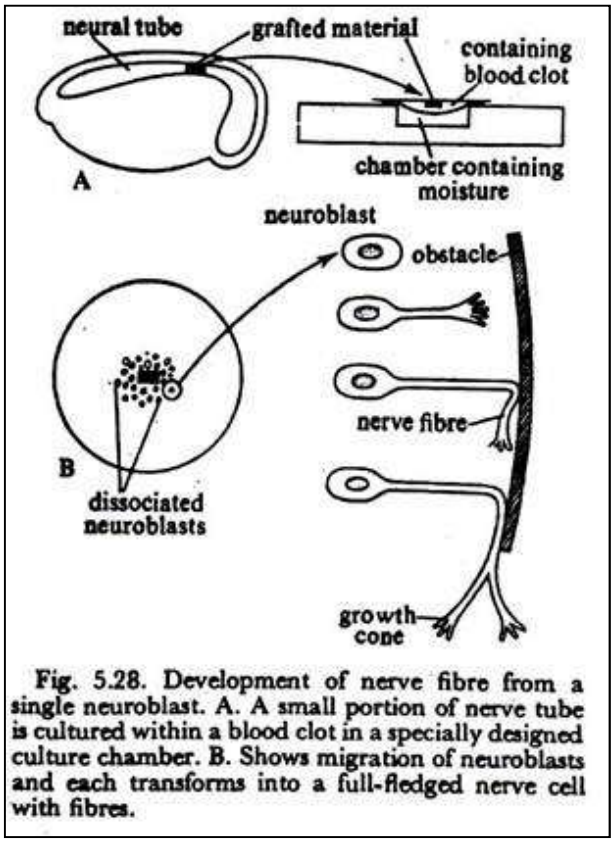


Fig. 5.28. Development of nerve fibre from a single neuroblast. A. A small portion of nerve tube is cultured within a blood clot in a specially designed culture chamber. B. Shows migration of neuroblasts and each transforms into a full-fledged nerve cell with fibres.

Development of Nerve Cells and Fibres:

Nerve cells originate from neuroblasts which develop from the neural tube, neural crests and cranial placodes. The actual stages of conversion of a small (neuroblast to a large cell-body of a nerve cell can be seen in tissue culture method reported first by Harrison in 1907. As in Fig. 5.28, a small fragment of neural tube is transplanted in a blood clot and kept sealed in a moist chamber.

Dissociation and dispersion of cells are the first observable events in tissue culture. The origin of nerve fibres is the most notable event in this process of conversion. Three theories are extant on this particular issue.

They are:
(a) Cell-chain theory. This theory relates that the fibre is laid down by chains of cells which surround the nerve fibre.

(b) Plasmodism theory. According to this theory the nerve fibre is laid down on preformed protoplasmic bridge.

(c) Outgrowth theory. The theory advocates that the fibre is formed as an outgrowth of a single neuroblast.

The tissue culture experiment gives support to the last concept and setties the long standing controversy regarding the issue. At the beginning, a thin strand of protoplasm emerges as outgrowth from one side of the neuroblast.

This outgrowth becomes amoeboid and creeps along the solid object. The outgrowth has developed a growth cone at the terminal end which may branch to form two or more growth cones. The growth of nerve fibre exhibits streotropism, i.e. it moves along solid object.

Causal Analyses in Brain Morphogenesis:
In the entire process of nervous system formation, a number of inductive events occur. In the amphibian eggs, the dorsal lip of blastopore acts as primary organizer to induce the inward moving cells to form chordamesoderm which in turn induces the dorsal ectoderm to be neuralised. The formation of the neural tube is also guided by the influence of regionally specific inductions.

The neural plate at the beginning is an oval, flattened plate and is formed by the ectodermal cells which have come from lateral regions to the dorsal side. The neural plate elongates rapidly, which is caused by the movement of cells. The cells first move towards the middle and then run in two directions: anteriorly and posteriorly. The transformation of neural plate to neural tube which is called neurulation is also known to occur in vitro. It begins with a depression in the centre and curving of the edges which fuse together to form the tube. To search the motive force behind the formation of tube, the behaviour of cells in the centre and periphery is intimately studied. Certain suggestions, like differential water uptake, differential cell divisions have been negativated. It is now claimed that elongation of the plate is due to migration of cells but curvature is caused by changes in the cell adhesion. In further development, the anterior part of the tube swells up considerably to form brain vesicles. Considerable amounts of cell division and cell movement occur during the process. The different parts of the brain in course of its development induce the formation of structures like optic, auditory and nasal placodes on the outer ectodermal covering. It must be remembered that mesodermal cells which immediately remain around neural tube are believed to play most important role in the epigenetic profess.

The formation of brain establishes:
(a) many histological features remain determined at neural plate stage and

(b) all the cells do not transform into neural element at the same time. On the contrary a gradient exists in the anterior-posterior plane.

Probable questions:

1. What is neural crest?
2. Write a short note on neural crest cell migration.

Suggested readings:

GILBERT, S. F., & BARRESI, M. J. F. (2016). *Developmental biology*.

Unit- VII

Cartilage: a. Structure, differentiation

Objective: In this unit you will learn about the structure and differentiation of cartilage.

The Structure (Physical Description) of cartilage tissue in general: Cartilage is a connective tissue consisting of a dense matrix of collagen fibres and elastic fibres embedded in a rubbery ground substance. The matrix is produced by cells called chondroblasts, which become embedded in the matrix as chondrocytes, i.e. mature cartilage cells are called **chondrocytes**.

They occur, either singly or in groups, within spaces called **lacunae** (sing. *lacuna*) in the matrix. The surface of most of the cartilage in the body is surrounded by a membrane of dense irregular connective tissue called perichondrium. This is important to remember especially because, unlike other connective tissues, cartilage contains no blood vessels or nerves except in the perichondrium.

There are three different types (structures) of cartilage that have slightly different structures and functions. They are hyaline cartilage, fibrocartilage, and elastic cartilage. Each of these is described separately in the sections below.

1. **Hyaline Cartilage:** Hyaline cartilage is the most abundant of the three types of cartilage. It is found in many locations in the body, including:
    - Bronchi, bronchial tubes, costal cartilages, larynx (voice-box), nose, trachea
    - Covering the surface of bones at joints - especially in areas where damage due to wear may lead to osteoarthritis incl. e.g. the ends of the long bones, and also the anterior ends of the ribs.
    - Embryonic skeleton (i.e. in the fetus).

The Structure of hyaline cartilage tissue: Hyaline cartilage consists of a bluish-white, shiny ground elastic material with a matrix of chondroitin sulphate into which many fine collagen fibrils are embedded. It contains numerous chondrocytes.

The Functions of hyaline cartilage tissue: Hyaline cartilage tissue provides smooth surfaces, enabling tissues to move/slide easily over each other, e.g. facilitating smooth movements at joints. It is also provides flexibility and support.

Fibrocartilage: Examples include:

- Calli (sing. *callus*), which is the tissue formed between the ends of the bone at the site of a healing fracture (blood clot -> granulation tissue -> cartilage -> bone);
- Intevertebral discs (i.e. the discs between the vertebrae of the spine);
- Menisci (cartilage pads) of the knee joint.
- Pubic symphysis, which is the position at which the hip bones join at the front of the body.
- Also in the portions of the tendons that insert into the cartilage tissue, especially at joints.

The Structure of fibrocartilage tissue: Fibrocartilage is a tough form of cartilage that consists of chondrocytes scattered among clearly visible dense bundles of collagen fibres within the matrix. Fibrocartilage lacks a perichondrium.

The Functions of fibrocartilage tissue: Fibrocartilage tissue provides support and rigidity to attached/surrounding structures and is the strongest of the three types of cartilage.

Elastic Cartilage:

- **Auditory (Eustachian) Tubes**;
- **External Ear (Auricle)**;
- **Epiglottis** (the lid on the top of the larynx).

The Structure of elastic cartilage tissue: In elastic cartilage, which is yellowish in colour, the cartilage cells (chondrocytes) are located in a threadlike network of elastic fibres within the matrix of the cartilage. A perichondrium is present.

The Functions of elastic cartilage tissue: Elastic cartilages provides support to surrounding structures and helps to define and maintain the shape of the area in which it is present, e.g. the external ear.

- **Differentiation of cartilage:**
  Differentiation of cartilage cells from embryonic precursor cells is characterized by the onset of biosynthesis of at least two cartilage-specific gene products, type II collagen and cartilage-specific chondroitin sulfate proteoglycan (CSPG). Biochemical and immunological assays for these compounds now allow rapid, quantitative, and specific determination of the onset of cartilage differentiation, and present several advantages over assays that use histochemical stains or [35S]-sulfate incorporation into glycosaminoglycans. Chondrogenic differentiation also is associated with the formation of extracellular, high MW proteoglycan (CSPG) aggregates containing hyaluronic acid and the loss of fibronectin, or LETS protein, a cell surface glycoprotein found on presumptive chondroblasts, fibroblasts, and several other cell types. Comparatively little insight has been gained recently regarding the mechanism of cartilage cell differentiation. A number of factors or "inducers" of cartilage differentiation, such as chondroitin sulfate proteoglycan, notochord, spinal cord, low oxygen tension, and collagen substrates, increase the amount of glycosaminoglycan synthesis per cell, but the question remains open as to whether these factors also selectively increase the number of cells differentiating from precursor cells into chondroblasts, or whether they only increase cell viability. Other factors, such as conditioned medium from chondrocyte cultures, increase significantly the number of chondrocyte colonies arising in mass cultures of limb bud mesenchyme, but differentiation of nonchondrogenic cells is stimulated as well. Similarly, many inhibitors of cartilage differentiation, such as BrdUrd and 6-amino nicotinamide, also inhibit myogenic differentiation. It is possible that a unique and specific inducer or regulating factor of cartilage cell differentiation may not exist, for cartilage differentiation of normal embryonic mesenchyme can be triggered by a variety of environmental conditions, such as cell density, pH, potassium ion concentration, and fetal calf serum. These results imply that the temporal and spatial controls of cartilage differentiation are governed by environmental influences that are each of rather low specificity, but which together synergistically generate a morphogenetic control of high specificity. Signals which appear able to mimic those controlling normal cartilage differentiation seem to be exchanged during formation of ectopic cartilage. Muscle tissue and periosteum can be triggered to form cartilage by demineralized bone matrix. Chick limb bud epithelium induces type II collagen synthesis in embryonic mouse tooth germ, whereas homologous, oral epithelium induces the formation of dentin (type I collagen). Thus, the type of response elicited from mesenchyme cells can be determined by nearby epithelia, and that response frequently can be the formation of cartilage.

Proteoglycans:
Proteoglycans (PGs) consist of a protein portion and long, unbranched polysaccharides (glycosaminoglycans or GAGs). The latter have a high negative charge, owing to the presence of acidic sugar residues and/or modification by sulphate groups. The acidic sugar alternates with an amino sugar in

repeated disaccharide units. The GAGs adopt an extended conformation, attract cations, and bind water. Hydrated GAG gels enable joints and tissues to absorb large pressure changes. In addition to buffering pressure changes, PGs play important roles in control of growth and differentiation. Particular sulphation patterns in the GAG chains allow interactions, normally of ionic nature, with growth factors, for example. Recent studies have identified ~30 PG protein cores. These cores are not just scaffolds for GAGs: they contain domains that have particular biological activities. Many PGs are thus multifunctional molecules that engage in several different specific interactions at the same time. After synthesis PGs are transported from the Golgi to their destinations: the extracellular matrix (ECM), the cell surface or intracellular organelles. Such vectorial transport requires mechanisms for recognition, sorting and delivery, which are especially important in cells such as epithelial cells and neurons, where the cell membrane comprises separate domains. Recognition and sorting must require determinants in the GAG chains and/or in the PG protein cores. Here we discuss how and where chondroitin sulphate (CS)/dermatan sulphate (DS) and heparan sulphate (HS)/heparin GAGs are synthesised, and how these GAGs influence the sorting of PGs to the sites at which they act.

Probabale questions:

1. What is cartilage?
2. Describe the structure of cartilage.
3. Write a short note on differentiation of cartilage.

Suggested readings:

GILBERT, S. F., & BARRESI, M. J. F. (2016). *Developmental biology*.

Unit-VIII

Experimental induction of cartilage and proteoglycan synthesis

Objective: In this unit, you will learn about the experimental induction of cartilage and proteoglycan synthesis.

Proteoglycan synthesis (^{35}S incorporation into GAGs)
For studying proteoglycans synthesis, ^{35}S-sulfate is the radioisotope of choice as it is incorporated preferentially (> 90%) into chondroitin sulfate and keratan sulfate of the newly synthesized proteoglycans. This can be performed both on cartilage in explant culture or on isolated chondrocytes in monolayer or three-dimensional culture. The general protocol is as follows.
Cultures are incubated for 4–24h in presence of ^{35}S (20 μCi ml^{-1}). Slices, monolayer or gels are then rinsed and extracted at 4°C with 4M guanidine HCI, 0.05 M sodium acetate, pH 6.0, containing protease inhibitors. Extracted samples and/or culture medium are subjected to sieve chromatography on Sephadex G25 (PD 10 columns) equilibrated and eluted with 4M guanidine HCI, 0.05M sodium acetate, 0.1M sodium sulfate, 0.50% Triton X-100, pH 7.5. Radioactivity in the excluded peak, representing newly synthesized proteoglycan, is measured by scintillation counting.
A rapid filtration assay for the quantification of ^{35}S-labelled proteoglycan or ^{35}S-labelled GAGs in a large number of samples has been recently described.[79] In this assay, separation of ^{35}S-labelled proteoglycan and ^{35}S-labelled GAGs from unincorporated ^{35}S is effected by forming insoluble complexes between alcian blue and the GAG moieties of the proteoglycan and then filtering the solutions through Durapore membrane discs (0.45 μM pore size) fitted in a 96-well plate. Following brief rinsing steps, the discs are punched out and ^{35}S-labelled macromolecules are then quantified by scintillation counting.

Increased proteoglycan synthesis in cartilage in experimental canine osteoarthritis does not reflect a permanent change in chondrocyte phenotype

OBJECTIVE:
To determine whether chondrocytes in early experimental osteoarthritic (OA) cartilage continue to show increased synthesis and turnover of proteoglycans (PGs) during explant culture. A comparison was also made between the responsiveness of experimental OA and control cartilage to interleukin-1 beta (IL-1 beta) and tumor necrosis factor alpha (TNF alpha) after 1 day and 3 days in culture.

METHODS:
OA was induced in mature animals by sectioning of the anterior cruciate ligament followed by 3 months of normal exercise. PG synthesis in the articular cartilage was determined by measuring 35S-sulfate incorporation during explant culture over 1-3 days. Inhibition of PG synthesis was also determined with various concentrations of IL-1 beta and TNF alpha after 1 and 3 days in culture. PGs extracted from the articular cartilage over 1-3 days in culture were examined by agarose-polyacrylamide gel electrophoresis.

RESULTS:
Up to 24 hours after excision from the joint, PG synthesis was higher in experimental OA cartilage than in control cartilage. It was also less sensitive to inhibition by TNF alpha. These differences were no longer detected after 48-72 hours in culture. There were no changes in the relative proportions of aggrecan and decorin/biglycan extracted from and synthesized by control and experimental OA cartilage over the 3 days in culture.

CONCLUSION:
Previous results indicated that PG synthesis and turnover in articular cartilage was increased for many months after induction of experimental OA. Our present results show that the enhanced rate of PG synthesis and turnover were evident in freshly explanted tissue, but the differences were lost over 3 days in culture. A decreased responsiveness to TNF alpha was also lost. The hypermetabolic activity of experimental OA chondrocytes was thus reversible and not a permanent change in chondrocyte phenotype.

Suggested readings:

1. Write a short note on Experimental induction of cartilage and proteoglycan synthesis.

Suggested readings:

GILBERT, S. F., & BARRESI, M. J. F. (2016). *Developmental biology*.

Disclaimer:

The study materials of this book have been collected from various books, e-books, journals and other e sources.

Post-Graduate Degree Programme (CBCS)

in

ZOOLOGY

SEMESTER-III

ELECTIVE THEORY PAPER

CYTOGENETICS AND MOLECULAR BIOLOGY

ZET-302

SELF LEARNING MATERIAL

DIRECTORATE OFOPEN AND DISTANCE LEARNING

UNIVERSITY OF KALYANI

KALYANI, NADIA,

W.B., INDIa

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Director's Message

Satisfying the varied needs of distance learners, overcoming the obstacle of distance and reaching the unreached students are the threefold functions catered by Open and Distance Learning (ODL) systems. The onus lies on writers, editors, production professionals and other personnel involved in the process to overcome the challenges inherent to curriculum design and production of relevant Self Learning Materials (SLMs). At the University of Kalyani a dedicated team under the able guidance of the Hon'ble Vice-Chancellor has invested its best efforts, professionally and in keeping with the demands of Post Graduate CBCS Programmes in Distance Mode to devise a self-sufficient curriculum for each course offered by the Directorate of Open and Distance Learning (DODL), University of Kalyani.

Development of printed SLMs for students admitted to the DODL within a limited time to cater to the academic requirements of the Course as per standards set by Distance Education Bureau of the University Grants Commission, New Delhi, India under Open and Distance Mode UGC Regulations, 2017 had been our endeavour. We are happy to have achieved our goal.

Utmost care and precision have been ensured in the development of the SLMs, making them useful to the learners, besides avoiding errors as far as practicable. Further suggestions from the stakeholders in this would be welcome.

During the production-process of the SLMs, the team continuously received positive stimulations and feedback from Professor (Dr.) Sankar Kumar Ghosh, Hon'ble Vice- Chancellor, University of Kalyani, who kindly accorded directions, encouragements and suggestions, offered constructive criticism to develop it within proper requirements. We gracefully, acknowledge his inspiration and guidance.

Sincere gratitude is due to the respective chairpersons as well as each and every member of PGBOS (DODL), University of Kalyani. Heartfelt thanks is also due to the Course Writers-faculty members at the DODL, subject-experts serving at University Post Graduate departments and also to the authors and academicians whose academic contributions have enriched the SLMs. We humbly acknowledge their valuable academic contributions. I would especially like to convey gratitude to all other University dignitaries and personnel involved either at the conceptual or operational level of the DODL of University of Kalyani.

Their persistent and co-ordinated efforts have resulted in the compilation of comprehensive, learner-friendly, flexible texts that meet the curriculum requirements of the Post Graduate Programme through Distance Mode.

All the Self Learning Materials are self writing and collected from e-book, journals and websites.

Prof. Manas Mohan Adhikary
Director
Directorate of Open and Distance Learning
University of Kalyani

ELECTIVE PAPER (ZET-302)

Cytogenetics and Molecular Biology

	Unit	Content	Credit	Class	Time (h)	Page No.
ZET - 302 (Cytogenetics and Molecular Biology)	I	Cancer monoclonal origin; differences of normal cells and cancer cells; cell transformation and factors for cell proliferation; DNA and RNA tumour viruses	2.0	1	1	
	II	Concept of oncogene and their role in cancer; tumour suppressor and apoptotic genes. Chromosomal basis of human cancer		1	1	
	III	Mutations and mutagenesis types of mutation; biochemical basis of mutations; mutagenesis; spontaneous and induced mutation; reversion as a means of detecting mutagens and carcinogens.		1	1	
	IV	DNA repair and retrieval; repair of spontaneous and induced mutations; mechanism of DNA repair; repair by direct reversion; excision repair; SOS response.		1	1	
	V	Human genetics: karyotype and sex chromosomes; sex mosaics; sex chromosome anomalies; sex influenced and sex limited genes.		1	1	

	VI	Behavioural genetics influence of single defects on behaviour; Genetic analysis of behaviour in experimental animals, chromosome anomalies and insight into human behaviour.		**1**	**1**	
	VII	Environmental effects and gene expression: effects of external and internal environment; phenocopies.		**1**	**1**	
	VIII	Sex determination in Human and role of Y-chromosome.Twin studies; concordance and discordance; identical and fraternal twins.		**1**	**1**	

Unit-I

Cancer monoclonal origin; differences of normal cells and cancer cells; cell transformation and factors for cell proliferation; DNA and RNA tumour viruses

Objective: In this unit you will learn about Cancer disease. Differences of normal cells and cancer cells, cell transformation and factors for cell proliferation and role of DNA and RNA tumour viruses in cancer .

Introduction:

In multicellular organisms, cell division is a normal process. Cells divide for growth, for the development of organs, for healing of wounds and also for the replacement of older and damaged cells. Cell division is a very complex process which is controlled by a regulatory mechanism at both molecular and cellular level. Again, in higher multicellular organism, each and every cell belongs to a particular type of tissue like epithelial tissue, connective tissue muscular tissue etc.

Hence, when a cell of a specific tissue divides, it normally produces its own kinds of cell of the tissue to which it belongs. It never produces the cells of other tissues. Therefore, the process by which cells achieve this specification and specialisation is known as cellular differentiation. Differentiation of cell begins during embryonic gastrulation stage and continues through tissue formation.Actually differentiation has a genetic basis and the process results from the interaction of the nucleus and the cytoplasm. After the cells become well- differentiated, they cannot go back normally to the undifferentiated stage unless disturbed internally or externally.

Therefore, in multicellular organism, the cell division, differentiation and survival of individual cells are carefully regulated to meet the needs of the organism as a whole. When this regulation is lost due to any reason, the cells behave unusually and defy their control mechanism.Then the cells grow and divide in an uncontrolled manner ultimately spreading throughout the body and interfering with the functions of normal tissues and organs. As a whole, this condition leads to cancer. Cancer develops from defects in fundamental regulatory mechanisms of the cell.

Meaning of Cancer:

Cancer is a non-infectious disease. It starts at the molecular level of the cell and, ultimately affects the cellular behaviour. Generally, it can be defined as uncontrolled proliferation of cells without any differentiation.

Differences of Normal cell and Cancer cells:

A malignant tumor, or cancer**,** is an aggregate of cells, all descended from an initial aberrant founder cell. In other words, the malignant cells are all members of a single clone, even in advanced cancers having multiple tumors at many sites in the body. Cancer cells typically differ from their normal neighbors by a host of phenotypic characters, such as rapid division rate, ability to invade new cellular territories (metastasis), high metabolic rate, and abnormal shape. For example, when cells from normal epithelial cell sheets are placed in cell culture, they can grow only when anchored to the culture dish itself. In addition, normal epithelial cells in culture divide only until they form a single continuous layer. At that point, they somehow recognize that they have formed a single epithelial sheet and stop dividing. In contrast, malignant cells derived from epithelial tissue continue to proliferate, piling up on one another. Clearly, the factors

regulating normal cellular physiology have beenaltered.

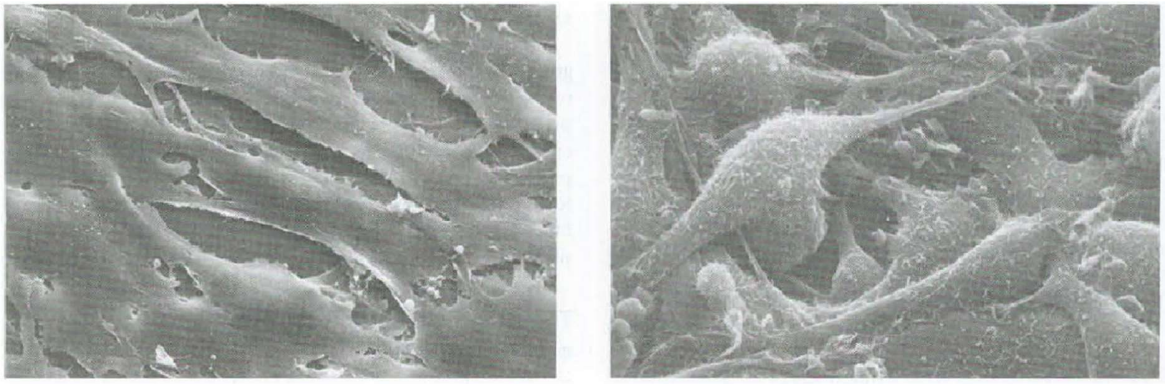


Fig 1: Electron microscopic picture of normal (left) and cancer (right) cells

The most striking feature of tumor cells is that their entire genetic makeup differs dramatically from that of normal cells. Characteristic of nearly all tumor cells is aneuploidy, the presence of an aberrant number of chromosomes generally too many. Normal differentiated cells rely on mitochondrial oxidative phosphorylation to satisfy their energy needs. Cells metabolize glucose to carbon dioxide by oxidation of pyruvate through the tricarboxylic acid (TCA) cycle in the mitochondria. Only under anaerobic conditions do cells undergo anerobic glycolysis and produce large amounts of lactate. In contrast to normal cells, most cancer cells relyon glycolysis forenergy production irrespective of whether oxygen levels arc high or low, producing large amounts of lactate. The use of glycolysis to produce energy even in the presence of oxygen, also called aerobic glycolysis, was first discovered in cancer cells by the cell biologist Otto Warburg and is therefore called the Warburg effect.

Cell transformation and factors for cell proliferation:

The study of tumor induction by radiation, chemicals, or viruses requires experimental systems in which the effects of a carcinogenic agent can be reproducibly observed and quantitated. Although the activity of carcinogens can be assayed in intact animals, such experiments are difficult to quantitate and control. The development of *in vitro* assays to detect the conversion of normal cells to tumor cells in culture, a process called cell transformation, therefore represented a major advance in cancer research. Such assays are designed to detect transformed cells, which display the *in vitro* growth properties of tumor cells, following exposure of a culture of normal cells to a carcinogenic agent. Their application has allowed experimental analysis of cell transformation to reach a level of sophistication that could not have been attained by studies in whole animals alone.

The first and most widely used assay of cell transformation is the focus assay, which was developed by Howard Temin and Harry Rubin in 1958. The focus assay is based on the ability to recognize a group of transformed cells as a morphologically distinct “focus” against a background of normal cells on the surface of a culture dish. The focus assay takes advantage of three properties of transformed cells: altered morphology, loss of contact inhibition, and loss of density-dependent inhibition of growth. The result is the formation of a colony of morphologically altered transformed cells that overgrow the background of normal cells in the culture. Such foci of transformed cells can usually be detected and quantified within a week or two after exposure to a carcinogenic agent. In general, cells transformed *in vitro* are able to form tumors following inoculation into susceptible animals, supporting *in vitro* transformation as a valid indicator of the formation of cancer cells.

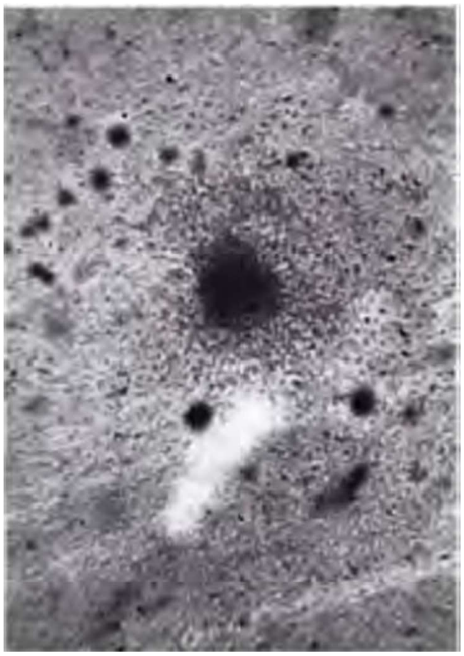


Fig 2 :A focus of chicken embryo fibroblast induced by Rous sarcoma virus

Figure 3 shows transformed 3T3 cells with DNA from a human bladder cancer and cloning the specific DNA segment that causes transformation. It was remarkable that a single small piece of DNA had this capability; if more than one incorporated DNA fragment had been needed to induce transformation, the experiment would have failed. Subsequent studies showed that the cloned segment included a mutant version of the cellular *ras* gene, where the glycine normally found in position 12 is replaced with a valine. This mutant was designated *rasD,* where the *D* stands for "dominant." The mutation is genetically dominant because the active protein has an effect even in the presence of the other, normal *ras* allele.

Types of Cancer:

Cancer is a large class of diverse disease. All types of cancer can result from uncontrolled cell growth and division of any of the different kinds of cells in the body. So there are more than a hundred distinct types of cancer which vary in their behaviour and response to treatment.The uncontrolled cell growth produces a mass of cells which are called tumours or neoplasm tumours may be benign or malignant. A benign tumor remains confined to its original location. They do not invade the surrounding normal tissues. They do not spread to distant body sites.

The most common example of tumour is the skin wart. A benign tumour consists of closely resembles normal cells and may function like normal cells. Generally benign tumours are harmless and can usually be removed surgically. However, these tumours may sometimes become quite harmful if they are located in organs like brain and liver.A malignant tumour does not remain confined to its original location. They are capable of both invading surrounding normal tissue and spreading throughout the body via the circulatory or lymphatic systems. Malignant tumours become life-threatening if, they spread throughout the body.

Only malignant tumours are properly designated as cancers. The cells of malignant tumour are derived from single cell, thus they are monoclonal in character. Malignant tumour is composed of aberrant cells. They behave like embryonic type, undifferentiated, having irregular, large nucleus, and deficient of cytoplasm. Malignant tumours are generally classified into four main types on the basis of cell type from which they arise.

(i) Carcinomas:

It includes approximately 90% of human cancer. This type is principally derived from epithelial cells of ectoderm and endoderm. The solid tumours in nerve tissue and in tissues of body surfaces or their attached glands are example of carcinomas. Cervical, breast, skin and brain carcinomas are developed from malignant tumour.

(ii) Sarcomas:

Sarcomas are solid tumours of connective tissues such as muscle, bone, cartilage and fibrous tissue. This type of malignant tumours are rare in human (about 2% of human cancer).

(iii) Lymphomas:

It is a type of malignancy in which there is excessive production of lymphocytes by the lymph nodes and spleen. It accounts for approximately 8% of human cancers. Hodgkin's disease is an example of human lymphoma.

(iv) Leukemia's:

This type of malignancy arises from the blood forming cell. Leukemia's are commonly known as blood cancer. Leukemia's are neoplastic growth (uncontrolled cell growth at the cost of remaining cells) of leucocytes or WBC.They are characterised by excessive production of WBC of the blood. The name leukemia is derived from Greek leukos (white) + haima (blood) the massive proliferation of leukemia cells can cause a patient's blood to appear milky.

In addition to the types of cancer mentioned above, cancers are further classified according to tissue of origin, for example lung cancer, breast cancer, and the type of cells involved, for example fibro sarcoma arises from fibroblasts, erythromoidleukemia's from precursor of erythrocytes. Although there are many kinds of cancer, the four most common cancers are those of prostrate, breast, lung and colon/rectum.

Development of Cancer:

The development of cancer is a multistep process in which cells gradually become malignant through a progressive series of alternations. This process involves mutation and selection for cells with progressively increasing capacity for cell division, survival, invasion and metastasis (spread of cancer cells through the blood or lymphatic system to other organ sites).

The first step in the process is when a single cell within a tissue of the organ concerned is genetically modified. The modified cell divides rapidly, although surrounding cells do not— and a mass of tumour cells forms.These cells constitute a clone where cells are identical in terms of structure, characteristics and function. Rapid cell proliferation leads to the tumorous outgrowth or adenoma or polyp. This tumour is still benign.

Tumour progression continues as additional mutation occur within cells of tumour population. Some of these mutations give a selective advantage to the cell such as rapid growth and the descendants of a cell bearing such a mutation will consequently become dominant within the tumour population.This process is known as clonal selection. Clonal selection continues throughout tumour development and, consequently, tumour become more and more rapid, growing and increasingly malignant. The tumour cells, by their rapid proliferation, invades the basal lamina that surrounds the tissue.

Then tumour cells spread into blood vessels that will distribute them to other sites in the body. This is known as metastasis. If the tumour cells can exit from the blood vessels and grow at distant site, they are considered malignant (Fig. 23.1).

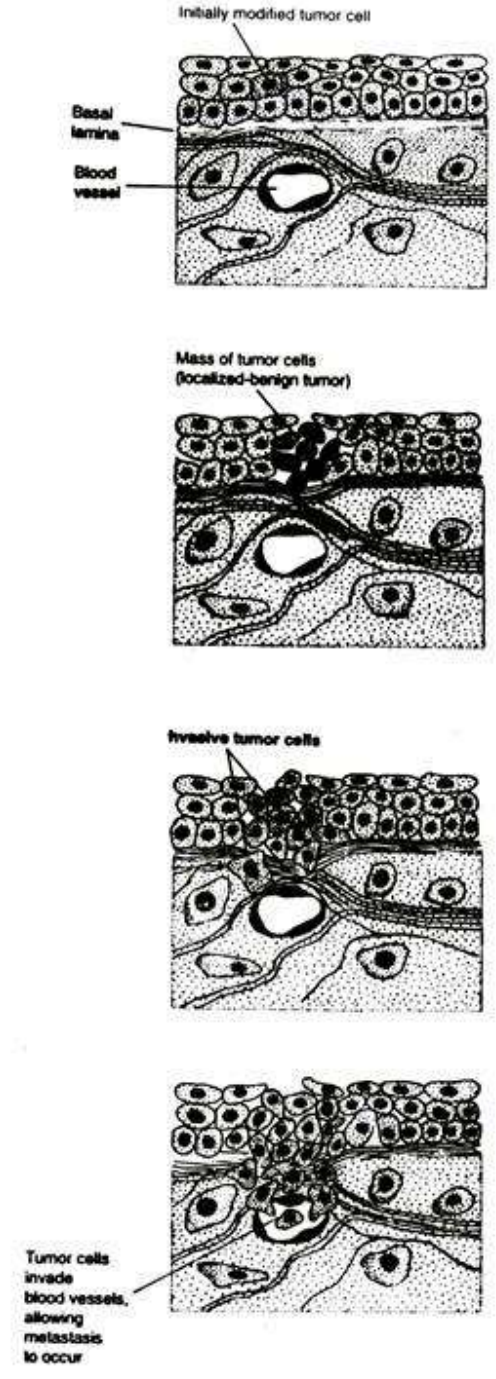


Fig. 23.1: Stages in tumour growth and metastasis.

Characteristics of Cancer Cells:

The uncontrolled growth of cancer cells results from accumulated abnormalities affecting many of the cell regulatory mechanisms. The process of cell change in which a normal cell loses its ability to control its rate of division and thus becomes a tumour cell is called cell transformation.

Cancer cells shows some typical characteristic properties that are absent in normal cells. Sometimes cancer cell properties are just opposite to the properties of normal cell. Cancer cells in vivo differ from their normal counterparts in several respects. Some characteristic properties of cancer cells can also be demonstrated by cell culture in vitro.

(i) Immortalization:

Normal cell culture do not survive indefinitely For example, human cell culture die after about 50 generations. On the other hand, transformed cell cultures can go on indefinitely and remain immortal if the nutrition is provided and overcrowding avoided.

(ii) Loss of Contact Inhibition:

Normal cells growing in tissue culture tend to make cell contacts by adhesion to neighbouring cells. At the points of adhesion some kind of electron-dense plaque is formed in both contacting cells. At the same time there is a slowing down of the amoeboid process which results in contact inhibition of movement. In contrast, cancer cells are unable to form adhesive junctions and do not show this type of contact inhibition.

Experimentally, it has been observed that when normal cells have become completely surrounded by other cells, their mobility stops and they form a monolayer. At the same time there is inhibition of growth and the number of cells in the Petridish remains practically constant. On the other hand, cancer cells continue to multiply and pile up forming irregular masses several layers deep. Cancerous cells undergo a change in property of their cell membranes and cell coat such as disappearance of gap junction, loss of coupling changes in glycolipid and glycoprotein and a reduction in gangliosides.In the cell coat fibronectin, a large glycoprotein found in footprints of moving cultured cells is reduced in cancerous cells. These changes enable the cells to dissociate from neighbouring cells and show loss of contact inhibition.

(iii) Reduced Cellular Adhesion:

Most cancer cells are less adhesive than the normal cells due to reduced expression of cell surface adhesive molecules. When normal cells are transformed into cancer cells, then a change of stickiness of their cell membrane results. Normal cells show stickiness or adhesiveness.

If normal cells are grown in a liquid nutrient medium kept in a glass vessel, the cells stick to glass wall rather than float in the medium. But when cancer cells are allowed to grow in nutrient medium, they stick to each other less than do normal cells.Adhesiveness shows considerable specificity. For example, a liver cell tends to stick with another liver cell and not to other types of cell such as kidney cell. Cancerous cells do not show this property. They are able to mix and stick to any type of normal cell. For example, a malignant liver cell can mix and stick to normal kidney cell. Hence this unusual behaviour of cancer cell explains that cancer cells can invade several normal organs.

(iv) Invasiveness:

One of the most important characteristics of cancer cells is their invasiveness. It is the ability to invade other tissues. Malignant cells generally secrete proteases that digest extracellular matrix components, allowing the cancer cells to invade adjacent normal tissues. For example, secretion of collagenase by the cancer cells helps to digest and penetrate through basal laminae to invade the underlying connective tissue.

Cancer cells also secrete growth factors that promote the formation of new blood vessels. This is known as angiogenesis. Angiogenesis is necessary to support the growth of tumour beyond the size of about a million cells at which point new blood vessels are needed to supply oxygen and nutrients to the multiplying tumour cells.Actually the growth factor secreted by the tumour cells stimulates the endothelial cells present in the wall of capillaries.

As a result, new outgrowth of the capillaries is formed into the tumour. These outgrowths of capillaries are also helpful for metastasis of malignant cells. Therefore, angiogenic stimulation induces the growth of new blood capillaries which penetrate easily in the tumour tissue and provide the opportunity for the cancer cells to enter the circulatory system. As a result, metastasis process begins.

(v) Failure to Differentiate:

Another general characteristic of most of the cancer cells is that they fail to differentiate. This property is closely related with the abnormal proliferation. Normal cells are fully differentiated. In most fully differentiated cells, cell division ceases. In case of cancer-cells, normal differentiation program is blocked at the early stages of differentiation. The relationship between defective differentiation and rapid proliferation is clearly noted in case of leukaemia.

All of the different types of blood cells develop from a common pluripotent stem cell in the bone marrow. Some of the descended cells develop erythrocytes but others differentiate to form lymphocytes, granulocytes and macrophages. Cells of each of these types become round as they differentiate but once they become fully differentiated cell division ceases But leukaemia cells fail to undergo terminal differentiation. Instead, they become blocked at early stage of maturation at which they retain their capacity for proliferation and continue to divide.

(vi) Auto stimulation of Cell Division:

Cancer cells produce growth factor that stimulates their own cell division. Such abnormal production of a growth factor by the cancer cell leads to continuous auto stimulation of cell division. This is known as autocrine growth stimulation. Hence the cancer cells are less dependent on general growth factor produced within the body physiologically from normal source for inducing growth of all normal cells. It is also noted that the reduced growth factor dependence of cancer cell results from abnormalities in intracellular signalling system.

(vii) Apoptosis:

For every cell, there is a fixed span of life, i.e., time to live and time to die. This cell death is a very orderly process and so it is called Programmed Cell Death or PCD or Apoptosis. Apoptosis is a mechanism of programmed cell death or cell suicide which is essential for the survival of the organism, for the normal development of the organism as the programmed destruction of the organism as the programmed destruction of cells is found during embryo-genesis. It also protects the organism by removing damaged cells which may be due to viral infection or due to exposure to radiations. It also inhibits the tumour development and so any defect in the control of apoptosis may lead to cancer.

There are two methods by which cells may die such as:

1. Death by injury that is through mechanical damage or due to toxic chemicals.

2. By Apoptosis, i.e., through programmed cell death.

(a) Characteristic charges during apoptosis:

The following distinct morphological changes are found during apoptosis:

1. Shrinkage of cells.

2. Cell forms tight sphere.

3. Cell membrane forms bubble-like blebs on the outer surface.

4. Occurrence of nuclear membrane break.

5. Endonucleolytic clearance of DNA at inter-nucleosomal sites occurs leading to the degradation of chromatin.

6. Breakdown of mitochondria is found with the release of cytochrome C.

7. Breakage of cells into small fragments.

8. Engulfment of cells fragments by phagocytic cells:

(b) Genetic Control of Apoptosis:

Some apoptosis genes have already been identified which are responsible for switching on or off apoptosis. These genes include ICE (Interleukin-lb-Converting Enzyme) and P53. There are other factors that also regulate the process of apoptosis.

One of them is the signal protein which is released either due to some cell injury or through cytokine medicated pathways. There are some critical proteins or modulating factors which determine whether a cell will be repaired or undergo death.

These genes or factors may initiate some stimuli for cell death or induces cellular susceptibility to apoptosis or initiates some effector mechanisms for apoptosis. Some of the genes or factors responsible for apoptosis are listed in the Table 23.1.

Table 23.1: Gene/Factors

(a) Initiating Stimuli	**Function**
Tumour Necrosis Factor α receptor family (TNF)	Death signal
Ceramide	gives signal for apoptosis induction.
FAS/Apo–1	Death signal like TNF; For peripheral deletion of T lymphocytes.
Nur 77 (Zinc finger containing steroid receptor)	Death signal in thymocytes.
(b) Inducing Cellular susceptibility	
c-mye	produces myc protein which gives cell susceptibility for apoptosis
Rb–1	Deficiency of Rb–1 gives susceptibility.
	Rb protein may inhibit P 53 mediated apoptosis
E2F1	induces susceptibility
P 53	apoptosis in response to cell injury is dependent on P 53.
(c) Modulating factors	
DAD 1 gene	gives signal for cell death
BCI–2 gene family	Some members inhibit cell death, such as bcl–2, BCI–X. Members which promote death like bax, bid and bad.
(d) Effector mechanisms	
Caspases, ICE, Ich–1	Genes encoding cysteine proteases which are involved in the effector pathway of apoptosis.

(c) Mechanism of Apoptosis:

There are generally three different mechanisms for apoptosis. These are:

1. Triggered by internal signals, i.e., signals arising within the cell.

2. Triggered by external signals.

3. By Apoptosis-Inducing Factor (AIF).

1. By Internal Signals:

In a normal cell, the protein (BC1-2) produced from a gene BC1-2 remains on the outer surface of the mitochondria. The protein BC1-2 holds the apoptotic protease activating factor- 1 (Apaf-1). But when the damage occurs in the cell internally due to some reactive oxygen, the Apaf-1 factor is released from BC1- 2-Apaf-l complex.

This allows the protein Bax to penetrate the mitochondrial membrane causing a leakage of cytochrome C from the mitochondria.

Then the released cytochrome C and Apaf-1 bind to molecules of caspase 9. The complex containing cytochrome C, Apaf- 1, caspase 9 and ATP is called Apoptosome. Caspase 9 is actually one form of protease which cleaves proteins at Aspartic acid residues.

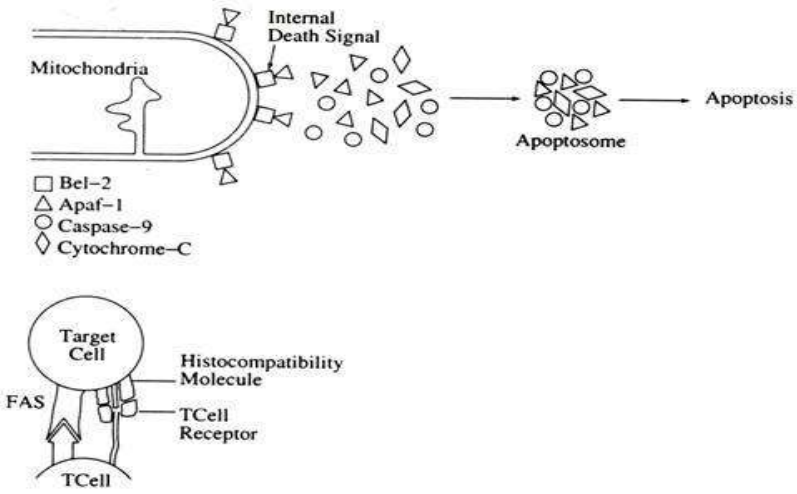
The caspase 9 activates other caspases creating a cascade of proteolytic activity which leads to the lysis of cell through digestion of structural proteins of the cytoplasm and degradation of chromosomal DNA.

2. External Signals:

Some receptor proteins (FAS and TNF) and other molecules residing on the surface of the cell are responsible for apoptosis. when cytotoxic T cells containing complementary factor FASL bind to the target cell, FASL binds with the FAS of the target cell leading to the death of the cell by apoptosis.

3. Apoptosis-Inducing Factor (AIF):

This AIF is a protein located in the inter-membrane space of mitochondria. When the cell receives the signal for its death, AIF is released from the mitochondria to the cytoplasm. AIF then goes to the nucleus and binds to DNA causing destruction of the DNA and finally the death of the cell.



In case of cancer, there are some virus like Human Papilloma Virus (HPV), Epstein-Barr Virus (EBV) produce a special type of protein E6 or BC1-2 which inactivate apoptosis promoter P 53, leading to the proliferation of cancer.

Again those cancer cells without the intervention of viruses also have some techniques to inactivate apoptosis. some B-cell leukemia's Melanoma (one type of skin cancer), lung cancer cells, colon cancer cells, etc. produce some proteins or factors like BC1-2 "decoy" molecule, Fas L can avoid apoptosis by inhibiting Apaf-1, or binding to Fas leading to proliferation of cancer.

(viii) Density-Dependent Inhibition:

One of the primary distinguishing characteristic features between cancer cell and normal cell is that normal cells show density-dependent inhibition of cell division in culture but cancer cells continue to proliferate independent of cell density.

Proliferation of normal cell continues until they reach a finite cell density. Normal cells are very sensitive to cell density. So when they reach a finite density they enter the G_0 state of the cell cycle. But cancer cells continue to divide to high cell density.

(ix) Cellular Characteristics:

Cancer cells can be distinguished from normal cells by microscopic examination. Cancer cells have a high nucleus to cytoplasm ratio, prominent nucleoli, many mitosis, and relatively little specialised structure. Normal cells have a cytoskeleton which consists of microtubules and microfilaments. But the cytoskeleton of cancer cells undergo de-polymerisation and the microtubules disaggregate.

(x) Chromosomal Change:

Normal cell contains normal chromosome number, e.g., normal cells of human beings contain 46 or 23 pairs chromosomes. But in cancer cell the chromosomes can undergo both structural and numerical changes. In human being the parent cell of any cancer has 46 chromosomes Later, after a series of abnormal divisions the cancer cells contain series of chromosome numbers and karyotype.

The chromosomes swell up and the number of chromosome sets increase owing to the growth of cancer cells. This condition is known as aneuploidy. Earlier workers have suggested that in different cancer cell populations there are chromosomal stem lines involving a particular spectrum of chromosome structure and number.An established cancer cell population will have a modal number in most of the cells over quite long periods and it is relatively stable. Generally speaking, no two karyotypes are identical in cancer cell and no typical chromosome group has been found to be involved. Therefore, the occurrence of any aneuploid cells in a particular tissue may have the possibility to become cancerous cell.

(xi) Interaction With Immune System:

A few normal cells may be transformed in pre-cancer cells every day in each of us in response to radiation, certain viruses or chemical carcinogens in the environment. Because they are abnormal cells, some of their surface proteins are different from those of normal body cells. Such proteins act as antigens and stimulate an immune response that generally destroys these abnormal pre-cancer cells.

If the pre-cancer cells are destroyed by the immune system, then how does cancer occur? Further investigation demonstrates that there are some transformed cancer cells whose surface proteins are not so changed.Hence such cancer cells may remain anti-genetically similar to normal cells. As a result, the immune system cells may fail to distinguish the cancer cell from normal cell. Some workers suggest that sometimes cells of the immune system do recognise cancer cells but are not able to destroy them.

In such case, cancer cells can stimulate B cells to produce IgG antibodies that combine with antigens on the surface of the cancer cells.These blocking antibodies may block the T cells so that they are unable to adhere to the surface of the cancer cells and destroy them. For some unknown reason, the blocking antibodies are not able to activate the complement system that would destroy the cancer cells.

Causes of Cancer:

Many agents including radiation, chemicals and viruses have been found to induce cancer in both experimental animals and humans. Agents which cause cancers are called carcinogens. Radiation (Solar ultraviolet ray, X-ray) and chemical carcinogens act by damaging DNA and inducing somatic mutations.These carcinogens are generally called initiating agent because the induction of mutations in key target genes is supposed to be the initial event leading to cancer development.

Some of the initiating agents that cause human cancers include solar ultraviolet radiation—the major cause of skin cancer. The exposure of the thyroid gland to X-rays greatly increases the incidence of thyroid cancers.Varieties of chemical carcinogen including tobacco smoke (containing benzo(a)pyrene, dimethyl nitrosamine and nickel compound) and aflatoxin produced by some moulds are the major identified cause of human cancer. Other carcinogens induce the cancer development by stimulating cell proliferation rather than inducing mutations. Such compounds axe called tumour promoters.

The first suggestion that chemicals can cause cancer dates back to 1761, when a doctor noted that people who use snuff suffer from nasal cancer. A few years later a British physician observed a high incidence of cancer of the scrotum among the chimney-sweepers iii their youth.He explained the fact that the chimney soot became dissolved in the natural oil of the scrotum, irritating the skin and, consequently, initiates the development of cancer. On the basis of two separate observations it became evident that certain chemicals (Table 23.1) can cause cancer.Later, as the industrial revolution moved into twentieth century, more and more incidence of cancer were reported among the workers who were continuously exposed to industrial chemicals.

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c-mye	produces myc protein which gives cell susceptibility for apoptosis
Rb–1	Deficiency of Rb–1 gives susceptibility,
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E2F1	induces susceptibility
P 53	apoptosis in response to cell injury is dependent on P 53.
(c) Modulating factors	
DAD 1 gene	gives signal for cell death
BCl–2 gene family	Some members inhibit cell death, such as bcl–2, BCl–X. Members which promote death like bax, bid and bad.
(d) Effector mechanisms	
Caspases, ICE, Ich–1	Genes encoding cysteine proteases which are involved in the effector pathway of apoptosis.

In the early 1940s Peyton Rous observed that repeated application of coal tar to rabbit skin causes tumour to develop, but the tumour disappears when application of the coal tar is stopped. It is also noted that when the skin is treated with turpentine, tumour again reappears.Normally turpentine does not cause cancer itself. Therefore the coal tar and turpentine are playing two different roles. Some carcinogens induce some normal cells to become irreversibly altered to a pre-neoplastic state.

This is known as initiation and the carcinogens are known as initiation agents. Here coal tar is an initiating agent. On the other hand, some carcinogens stimulate the pre-neoplastic cells to divide and form tumour. This is known as promotion and the carcinogens are termed promoting agents. Here turpentine behaves as promoting agents. Berenblum observed that painting the skin of a mouse a single time with methylcholanthrene rarely causes the development of tumours. But subsequently application of castor oil (an oil derived from seeds of *Croton tiglium*) triggers the formation of multiple tumours on the skin which has been exposed previously to methylcholanthren is acting as an initiator whereas castor oil acts as a promoter.

Initiation is a quick, irreversible process that causes a permanent change in a cell's DNA. The carcinogenic chemicals that act as initiating agent are capable to bind with DNA. Hence they interfere with the normal function of DNA and induce somatic mutation and, consequently, bring about stable, inheritable changes in the cell's properties.

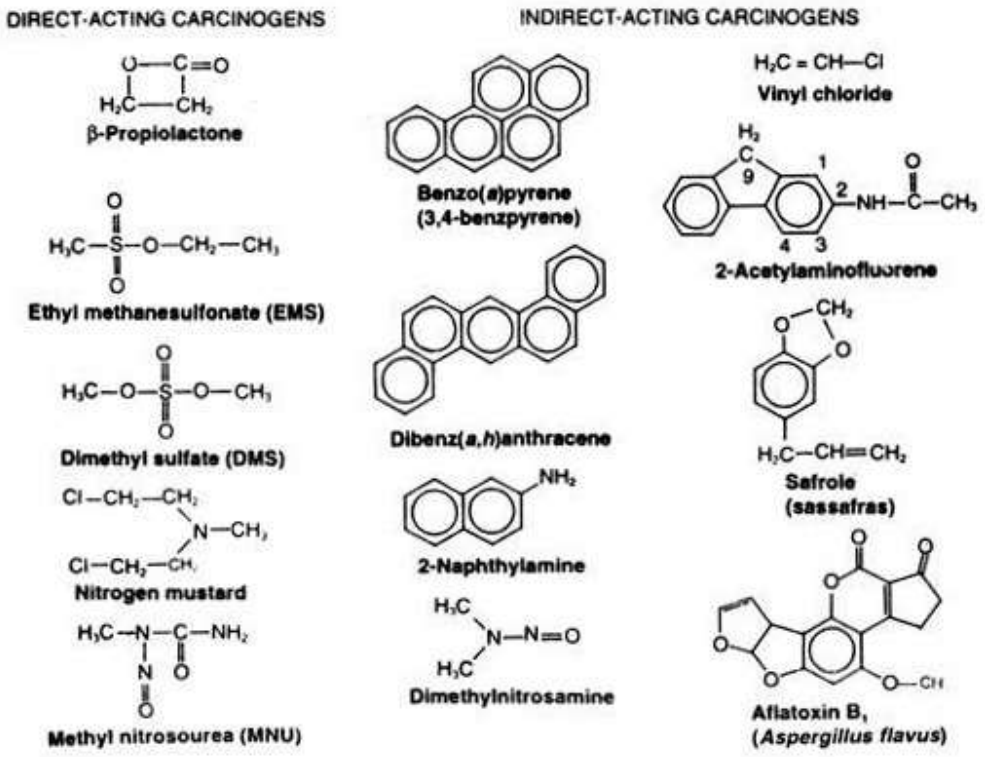


Fig. 23.2: Structure of some direct acting and indirect acting chemical carcinogens.

On the basis of action of chemical carcinogens on DNA, there are two broad categories of carcinogens—direct acting and indirect acting (Fig. 23.2). Direct acting carcinogens are highly electrophilic compounds that react with DNA.Indirect acting carcinogens are converted to ultimate carcinogens by introduction of electrophilic centres. In other words, indirect acting carcinogens must be metabolised before they can react with DNA.

The steps of metabolic activation of benzo(a)pyrene—a polycyclic aromatic hydrocarbon—are shown in Fig. 23.3.:

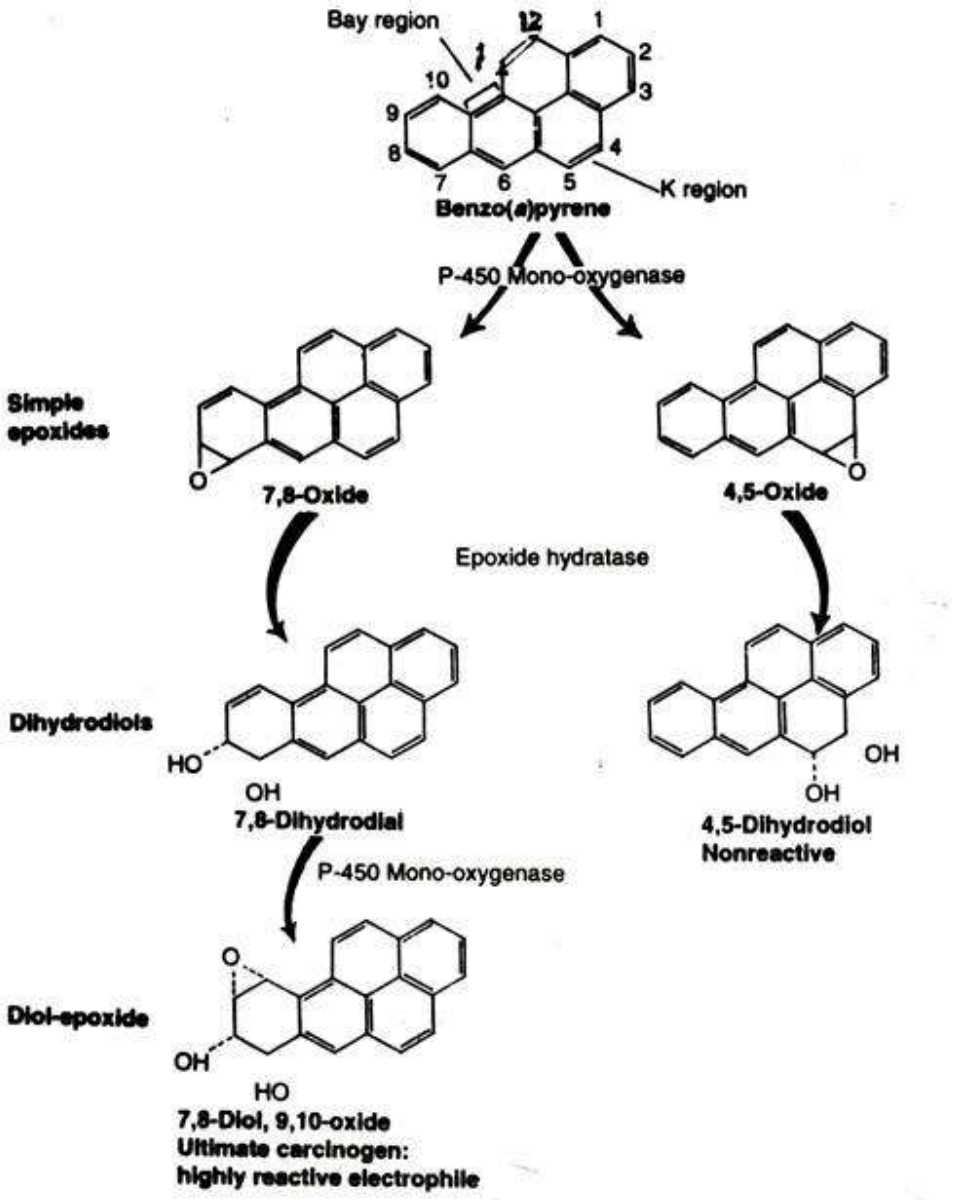


Fig. 23.3: Steps of metabolic activation of benzo(a)pyrene—a powerful carcinogen.

On the other hand, promotion is a gradual, partially reversible process that needs prolonged exposure to promoting agents. If a cell that has already undergone initiation is exposed to a promoting agent, the cell starts to divide and the number of genetically damaged cells goes up.

As the damaged cells continue to divide, a gradual selection for cells showing higher growth rate and invasive properties occurs—leading to the formation of malignant tumour. The promotion phase continues for longer period. That is why cancer does not develop just after exposure to a carcinogenic agent.The mechanism of action of promoting agents have come from the studies of phorbol esters which are present in castor oil and act as tumour promoters. Phorbol esters bind to the plasma membrane and activate protein

kinase C. Protein kinase C is a component of the phosphoinositide signalling pathway whose activity is normally controlled by the second messenger, diacylglycerol.

The activation of protein kinase C leads to phosphorylation of many target proteins and, consequently, activates the transcription factor API which switches on the transcription of genes involved in stimulating cell proliferation. Therefore, the mode of action of phorbol esters gives an insight into the possible mechanism of action of a promoting agent.

Energy that travel through space is known as radiation. Natural source of radiation to which humans are generally exposed are ultraviolet rays, cosmic rays and emission from radioactive elements. We are also exposed to another high- energy radiation like X-ray. Medical, industrial and military activities generally create the high-energy radiation.Sunlight has the ability to cause skin cancer in people who spend long hours in the sunlight. Sunlight contains ultraviolet rays which are also absorbed by normal skin pigmentation. Hence, for this reason, dark-stained or black people usually have lower rates of skin cancer than fair-skinned individual.Because ultraviolet radiation is very weak to pass through the skin, it does not induce any other type of cancer except skin cancer. It is more or less restricted superficially on skin because skin cancer rarely metastasizes.

This type of cancer can be cured by easily removing the affected site surgically. Xeroderma pigmentosum is a type of inherited malignant disease. Individuals with this malignant disease develop extensive skin tumours after exposure to sunlight. Homozygotes for the autosomal recessive mutation responsible for xeroderma pigmentosum are less efficient in the repair of DNA damaged by exposure to ultraviolet light.X-rays are high energy radiation. They are strong enough to penetrate the skin and reach internal organs. X-rays thus make a serious cancer hazard because they are able to induce gene mutation or DNA damage. Many radioactive elements emit radiation. It also acts as carcinogen and causes cancer.Marie Curie, the co-discoverer of the radioactive elements polonium and radium, died of a form of leukemia that appeared to be caused by her extensive exposure to radioactivity. Another example of radiation-induced cancer occurred in New Jersey in 1920. A group of women was employed by a factory that produced watch which glow in the dark. The luminescent point used to point the watch dial contained radium.The paint was applied with a fine-tipped brush that the employee frequently wetted with their tongue. During this process, minute quantities of radium were ingested through saliva in the digestive system from where they were readily abs, -bed and distributed in the different cells ana tissues thorough circulatory system.Several years later these women suffered from bone cancer caused by radioactive radium that had gradually become concentrated in their bone.

The most well-known horrifying examples of radiation-induced cancer occurred in Japan and in Nevada of United States. In 1945 atomic bombs were exploded over Hiroshima and Nagasaki. The massive fallout of radioactive elements increased the incidence of leukaemia, lymphomas and cancers of the thyroid, breast,' uterus and gastrointestinal tract.Similarly, in Nevada, people suffered from cancer due to the radioactive fallout during nuclear bomb testing. It is suggested that radioactive carcinogen is thought to initiate malignant transformation by causing DNA damage. Alternatively, it is also explained that subsequent exposure of radiation damaged cells to promoting agents stimulates the cell to divide abnormally and form tumour.

Role of Virus in Cancer formation:

There are many viruses which are capable of causing tumour in animals, human as well as plants (Table 23.2). These viruses are called tumour viruses or oncovirus. Some tumour viruses have RNA genome and are known as DNA tumour viruses.

Some tumour viruses have DNA genome and are known as retroviruses. Retrovirus replicates via synthesis of a DNA provirus in the infected cells. In addition, HIV is indirectly responsible for the cancer that develops in AIDS patient as a result of immunodeficiency.

Table 23.2: List of Chemical Carcinogens and Type of Cancer induced by such chemicals

Carcinogen	Type of cancer induced
Acrylonitrile	Colon, lung
4-Aminodiphenyl	Bladder
Aniline derivatives	Bladder
Arsenic compounds	Lung, skin
Asbestos	Lung, mesothelium
Benzene	Leukemia
Cadmium salts	Prostate, lung
Carbon tetrachloride	Liver
Chromium and chromates	Lung, nasal sinuses
Diethylstibestrol (DES)	Uterus, vagina
Lead	Kidney
Mustard gas	Lung, larynx
α-Naphthylamine	Bladder
Nickel	Lung, nose
Organochloride pesticides	Liver
Polychlorinated biphenyls	Liver
Radon	Lung
Soot and tars	Skin, lung, bladder
Vinyl chloride	Liver, lung, brain
Wood and leather dust	Nasal sinuses
Tobacco smoke, which contains the following:	Lung, oral cavity, larynx, esophagus, stomach, pancreas, others
Aminostilbene, arsenic, benz[*a*]anthracene, benz[*a*]pyrene, benzene, benzo[*b*]fluoranthene, benzo[*c*]phenanthrene, benzo[*j*]fluoranthene, cadmium, chrysene, dibenz[*a,c*]anthracene, dibenzo[*a,e*]fluoranthene, dibenz[*a,b*]acridine, dibenz[*a,j*]acridine,dibenzo[*c,g*]carbazone, N-dibutylnitrosamine, 2,3-dimethylchrysene, indeno[*1,2,3-c,d*]pyrene, S-methylchrysene, S-methylfluoranthene, α-naphthylamine, nickel compounds, N-nitrosodimethylamine, N-nitrosomethylethylamine, polonium-210, N-nitrosodiethylamine, N-nitrosonornicotiné, N-nitrosoanabasine, N-nitrosopiperidine	

The herpes viruses are the most complex animal viruses. The genome length of these viruses is 100-200 Kb. Many herpes viruses cause tumour in many animals such as frogs, chickens, monkeys etc. Epstein-Barr virus, a member of herpes virus, can trigger the development of some human malignancies including Burkett's lymphoma in some region of Africa and nasopharyngeal carcinoma in China.It also causes B-cell lymphomas in AIDS patient and other immunosuppressed persons. Cell transformation by herpes viruses is not fully understood because of the complexity of their genome. But it is evident that some viral genes are required to induce transformation of lymphocytes.Of the DNA tumour viruses, the papoviruses are the best studied DNA tumour viruses from the standpoint of molecular biology and have received particular attention because they have been critically important as models for understanding the molecular basis of cell transformation.The genome size of papoviruses is small (approximately 5 Kb). Simian virus 40 (SV_{40}) and polyomavirus are the important and commonly known member of papoviruses. Both these viruses are similar in size and general structure.

A virus usually multiplies in specific cells derived from animals in which the virus normally grows. Such cells are called permissive cells. Cells which do not allow the viruses to grow are called non-permissive cells.SV_{40} and polyoma viruses, on entering their respective host cells, undergo one of the two types of behaviour—they enter the permissive cell of the host, undergo the lytic phase, and multiply within host cell, ultimately killing them.Since a permissive cell is killed as a consequence of virus replication, it cannot become transformed. Sometimes viruses enter non-permissive cells and are not able to multiply, i.e., virus replication is blocked. In this case, the viral genome sometimes integrates into cellular DNA and expression of specific viral genes results in transformation of the infected cells.The SV_{40} and polyoma virus genes that trigger cell transformation have been identified, isolated and sequenced by molecular analysis. The genome of SV_{40} and polyomavirus are divided into early and late regions. The early region is expressed immediately after infection and is needed for synthesis of viral DNA.

The late region is not expressed until after viral DNA replication has begun. The early region of SV_{40} codes for two proteins which are known as small (17 Kd) and large (94 Kd) T-antigens. In addition to small and large regions, the genome of polyomavirus contains a third early region which is called as middle T region. It codes for a protein of about 55 Kd. Experimentally, it has been shown that large T of SV_{40} is sufficient to induce transformation and the middle T region of polyoma virus is primarily responsible for transformation. During lytic cycle, the early region proteins are needed to initiate viral DNA replication as well as to stimulate host cell gene expression and DNA synthesis.Since the replication viral DNA is dependent on host cell enzymes, therefore stimulation of gene expression of the host cell is a critical event in the viral life cycle. Most of the cells of adult animal cells become non-dividing. So the enzymes required for cell division are not available within the cell.

Therefore they must be stimulated to divide in order to induce the enzymes needed for viral DNA replication. This stimulation of cell division by the early gene products of virus can lead to transformation if the viral DNA becomes stably integrated and expressed in a nonpermissive cells. The early region proteins of SV_{40} and polyoma virus induce transformation by interacting with host proteins that regulate cell division. The papilloma viruses are small DNA viruses. The genome length of such viruses is approximately 8 Kd. Some of these viruses induce only benign tumours such as warts. But some others cause malignant carcinomas— particularly cervical and anogenital cancers. Cell transformation by papilloma viruses occurs from the expression of two early region genes E_6 and E_7.The hepatitis B viruses are another group of DNA virus. They have the smallest genomes which is approximately 3 Kb. These viruses mainly infect the liver cells and cause liver damage. But how they induce cell-transformation is not clearly known.Possibly tumour results from expression of a viral gene. Alternatively, the chronic cell damage of liver simply induce the continuous cell division which, ultimately, causes the cell transformation.

The retroviruses, one family of RNA viruses, also cause human cancer. For example, human T-cell lymphotropic virus type-I (HTLV- I), a RNA virus, is the causative agent of T-cell leukemia. A related virus

(HTLV-II) cause a rare form of leukaemia called hairy T- cell leukaemia.HIV (Human immunodeficiency virus) is the causative agent of AIDS. These viruses, i.e., HTLV-I, HTLV-II, HIV, actually does not cause cancer by directly converting a normal cell into a tumour cell. The AIDS patients become susceptible to high incidence of some malignancies like lymphomas and Kaposi's sarcoma due to immunosuppression of the patient.RNA viruses have an RNA genome which is extended at either end by a long terminal repeat (LTR). The LTR contains many of the signals that allow retrovirus to function (Fig. 23.4). Retroviruses use their genomic RNA as a template to make DNA with the help of reverse transcriptase.

This DNA is then integrated into host's DNA as DNA the provirus. The DNA provirus is transcribed to yield genome length RNA provirus directed transcription involves a promoter—a sequence that directs the RNA polymerase to a specific initiation site and an enhancer—a sequence that facilitates transcription.

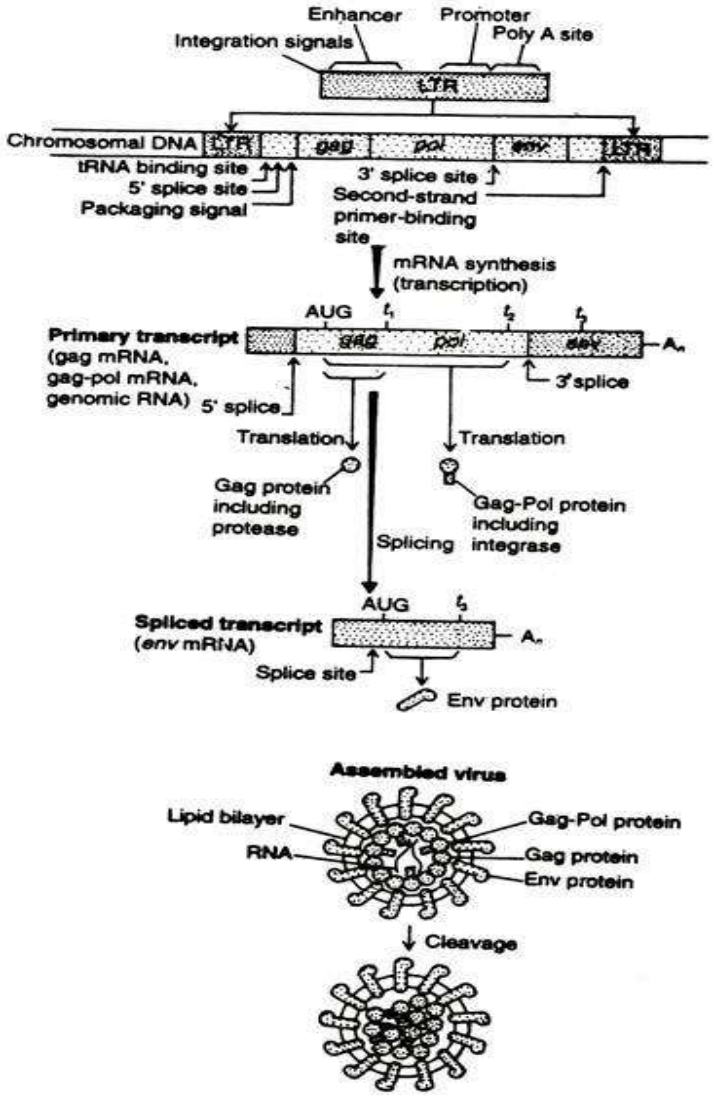


Fig. 23.4: Genetic elements of proviral DNA and the corresponding gene products.

The promoter and enhancer are located in the LTR. The primary transcript serves as the genomic RNA for progeny virus particles and as mRNA for the gag and pol genes. In addition the full length RNA is spliced to yield mRNA for env.The gag gene encodes the viral protease and structural proteins of the virus particle, pol encodes reverse transcriptase and integrase and env encodes envelope glycoproteins. These three genes are only required for viral replication but play no role in cell transformation.

This type of retrovirus causes tumour only when any mutation results at the time of integration of pro-viral DNA within or adjacent to host's genome. But there are some other retroviruses which contain specific genes which are responsible for the induction of cell transformation and acts as potent carcinogens.The first cancer causing gene is f6und in the retrovirus called Rous Sarcoma virus (Fig. 23.5) that produces sarcomas in chicken. It was later named src gene. Genes like src which are capable of inducing malignant transformation, are referred to as oncogenes. The identification of the first viral oncogene has provided a model for understanding many aspects of cancer development at the molecular level.

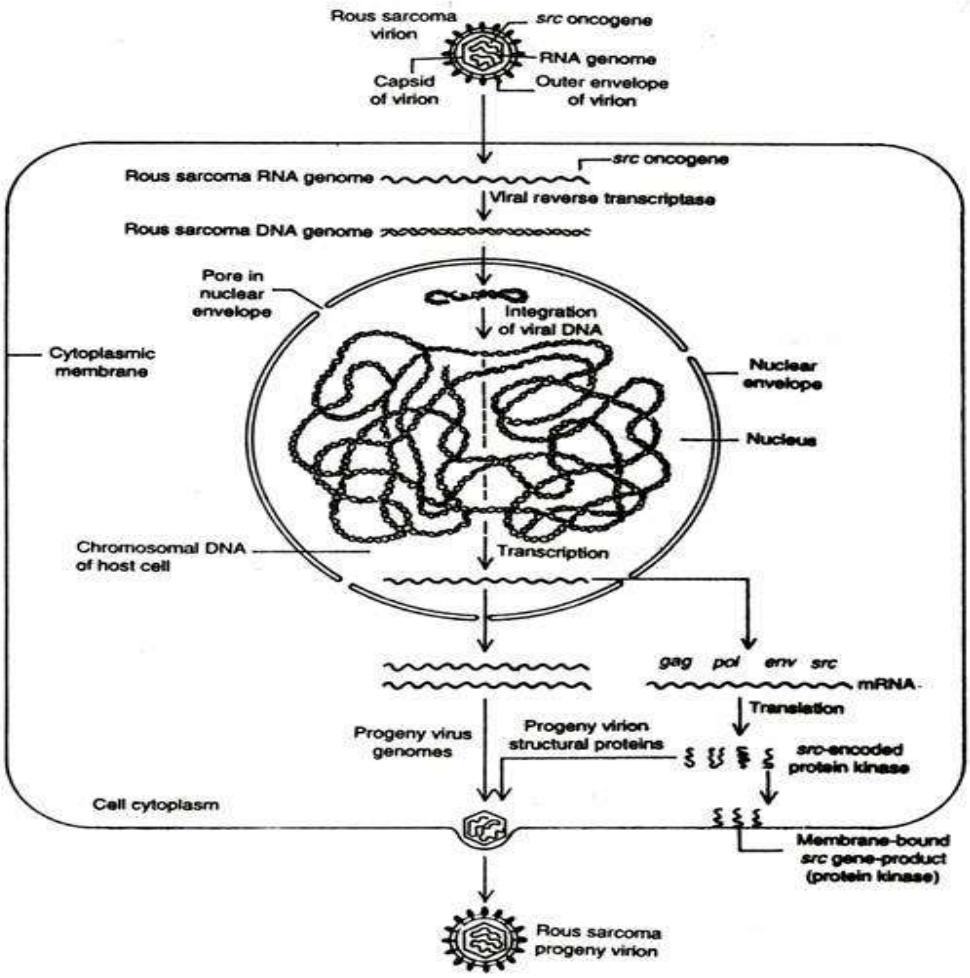


Fig. 23.5: Life cycle of Rous Sarcoma RNA tumour virus.

DNA Oncogenic Viruses:

Oncogenic viruses are distributed in several families of DNA viruses. These include Herpesviridae, Poxviridae, Papovaviridae and Hepadnaviridae. The Herpesviridae include the Epstein-Barr Virus (EBV) which has been found as the cause of two forms of human cancers — Burkitt's lymphoma and nasopharyngeal carcinoma. EBV has also been implicated with Hodgkin's disease, a cancer of lymphatic system. Other herpes-viruses have been associated with human cancers of lip and cervix.

The Papilloma viruses belonging to the Papovaviridae cause benign tumours as well as cancer in several species including human. In humans, papilloma viruses cause uterine (cervical) cancer. Another member of Papovaviridae, the Simian Virus 40 (SV40) is among the best studied DNA tumour viruses.Natural host of SV 40 is cultured fibroblast cells of monkey. Such a cell culture is called permissive, because it allows viral multiplication and release of progeny viruses by cell lysis. On the other hand, when SV 40 is inoculated into non-permissive cell cultures e.g. the fibroblast cells of mice, the virus cannot multiply, but in a small number cells the viral DNA is, integrated with the host DNA causing their transformation into cancer cells.

Due to integration into the host chromosome viral multiplication and cell lysis are absent. The phenomenon is comparable to lysogeny observed in temperate phage infection of bacteria. Integration of some DNA viruses is site-specific i.e. the viral DNA is inserted into a host chromosome at a specific site. But papova-viruses do not have such specificity and can be inserted at random.Hepatitis B virus (HBV) belonging to the Hepadnaviridae causes cancer of liver. Many animal experiments have yielded results which clearly indicate a connection of HBV and liver cancer. Although direct proof is lacking in case of human beings, a survey revealed that all people with liver cancer had a previous infection of HBV.

RNA Oncogenic Viruses:

Among the RNA viruses only some members of the family Retro-viridae can cause cancer. Other RNA viruses which replicate by RNA replicase are non-oncogenic. Retroviruses which have a single- stranded RNA genome replicate via a double-stranded DNA produced by an RNA-dependent DNA polymerase (reverse transcriptase) and they insert the DNA copy into the host chromosome as a provirus.

Rous Sarcoma Virus (RSV) is of historical importance, because it was the first tumour-inducing virus to be studied. RSV is a retrovirus with a single-stranded RNA genome and its DNA copy is integrated into a specific site of the host chromosome as a provirus.Research on RSV revealed identification of a cancer-inducing gene (an oncogene) in RSV genome. This gene, called src, is not essential for viral replication, as it does not code for any viral proteins. Later, it was discovered that a copy of the src gene is present in the host chromosome of normal cells and it was not oncogenic.

Thus, the viral src gene which is oncogenic is derived from the host. How the non-oncogenic chromosomal src gene is converted to an oncogene in RSV is not clearly understood. It may occur through a mutation. The entry of a chromosomal gene into the viral genome possibly occurs through a process similar to that which operates in restricted transduction in bacteria.It is thought that the RSV DNA produced through reverse transcription is inserted next to the chromosomal src gene and during transcription of the RSV RNA genome, src gene might be included. In this way, src gene might enter into the viral genome. RSV causes cancer in chicken.

Similar retroviruses are known to cause cancer in other animals including monkey. But definite evidence of retroviruses causing cancer in humans was not available until 1980. In that year Gallo isolated a virus that could transform normal T-lymphocytes into cancerous T-lymphocytes causing a disease, called T-cell leukaemia.The virus is known as Human T-cell Leukaemia Virus (HTLV). Later research during 1990s has

confirmed the role of HTLV in causing human leukaemia. Another HTLV was later discovered causing hairy cell leukaemia in man.The malignant leucocytes develop hairy outgrowths on their surface. The second virus has been designated as HTLV-II. These retroviruses have been shown to transform normal T-cells by a regulatory protein which stimulates uncontrolled cell division. Besides leukaemia, HTLV is also known to cause neurological disorders, like spastic para-paresis. T-cell leukaemia is more or less restricted in several countries, like Japan, West Indies and some parts of West Africa.

Probable Questions:

1. Write down the differences between normal cells and cancerous cells.
2. Write down the types of cancers.
3. How cancer is developed?
4. Write down characteristics of cancerous cells.
5. What are the causes responsible for onset of cancer?
6. Describe the role of DNA virus in onset of cancer.
7. Describe the role of RNA virus in onset of cancer.

Suggested readings:

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7th Edition.
6. Gene cloning and DNA Analysis by T. A. Brown, Sixth Edition.
7. Genetics . Verma and Agarwal.

Unit-II

Concept of oncogene and their role in cancer, tumour suppressor and apoptotic genes. Chromosomal basis of human cancer

Objective: In this unit you will know about Oncogene and tumour suppressor gene and their role in cancer. You will also learn about chromosomal basis of human cancer.

Oncogenes:

Oncogene is a type of specific viral gene that is capable of inducing cancer or cell transformation—either in the body of host or in the tissue in culture. After the discovery of src oncogene in RSV, more than 40 different highly oncogenic retroviruses have been isolated (Table 23.3) from a variety of animals like mice, rat, cat, chickens, turkeys, monkeys etc.

All these viruses contain at least one (in some cases two) oncogene like RSV. These oncogene are not needed for viral replication but is responsible for cell transformation. In some cases different viruses contain the same oncogenes. Many of these genes encode protein which, in turn, acts as the key components of signalling pathways that induces cell transformation.

Table 23.3: Examples of Tumour Viruses

Class	Examples	Tumours induced	Organism
DNA viruses:			
Herpesviruses	Lucke virus	Kidney adenocarcinoma	Frogs
	Epstein-Barr virus (EBV)	Burkitt's lymphoma, nasopharyngeal carcinoma	Humans
	Marek's disease virus	Lymphoma	Chickens
Papovaviruses	Shope papilloma virus	Papillomas	Rabbits
	SV-40	Subcutaneous, kidney and lung sarcomas	Hamsters
	Polyoma	Liver, kidney, lung, bone, blood vessels, nervous tissue, connective tissues	Mice
	Human papillomaviruses	Cervical cancer	Humans
Hepatitis B virus		Liver cancer	Duck, Woodchucks, squirrels, human
Adenoviruses	Human adenoviruses	Subcutaneous, intraperitoneal, intracranial	Hamsters
RNA viruses:			
B-type viruses	Bittner mammary tumor virus	Mammary carcinoma	Mice
C-type viruses	Rous sarcoma virus	Sarcomas	Birds, mammals
	Murine leukemia viruses (Gross, Moloney, Friend, Rauscher, and others)	Leukemia	Mice
	Feline leukemia virus	Leukemia	Cats
	Murine sarcoma virus	Sarcoma	Mice
	Feline sarcoma virus	Sarcoma	Cats
	Avian leukemia viruses (avian myeloblastosis and others)	Leukemia	Chickens
	Human T-cell leukemia virus	Leukemias/Lymphomas	Humans
Plant viruses	Wound tumor virus	Roots and stems	Plants

Oncogene in Human Cancer:

Direct evidence for the involvement of cellular oncogenes (the term cellular oncogene is generally used to distinguish this group of cancer- causing genes from viral oncogenes) in human tumour was first derived from gene transfer experiment carried out in the laboratories of Robert Weinberg and Geoffrey Cooper in the early 1980s.

In this process, a DNA segment isolated from tumour cells are artificially introduced into normal cells to see its subsequent changes. DNA isolated from a human bladder carcinoma was found to efficiently induce malignant transformation of recipient mouse cells in culture. This experiment reveals that the human tumour contains a cellular oncogene.The first human oncogene identified in gene transfer experiment was the ras oncogene. The ras oncogenes are not present in normal cells, but they are generated in tumour cells as a consequence of point mutation of the ras proto-oncogene. This results in the change of a single amino acid at critical position of the ras protein molecule encoded by ras gene.

The first such mutation was the substitution of valine for glycine at position 12. A single nucleotide, change which alters codon 12 from GGC (Gly) to GTC(Val) is responsible for the transforming activity. This is detected in bladder carcinoma DNA. The ras gene encodes membrane-bound guanine-nucleotide binding proteins (G- protein) that plays a central role in the transmission of singles from receptor-bound external growth factor to the cell interior.

During this process, GTP is hydrolysed into GDP. Therefore, Ras protein alternates between active (GTP bound) and inactive (GDP bound) states. But oncogenic ras proteins remain in the active GTP bound state and drive unregulated cell proliferation leading to the development of malignancy.In human tumour, point mutation is an important mechanism by which proto-oncogenes are converted into oncogenes. Besides this, the gene rearrangement—resulting mainly from chromosome translocation—sometimes lead to the conversion of proto-oncogene to oncogene.The classical example regarding the conversion of proto-oncogene to oncogene due to translocation of chromosome is the Burkitt's lymphoma. It produces the malignancy of the antibody producing B-lymphocytes.

In this case a piece of chromosome(s) 8 carrying c-myc proto-oncogene is trans-located to the immunoglobulin heavy chain locus on chromosome 14 (Fig. 23.6). Since the antibody genes are extremely active in lymphocytes, the transcriptional regulation of the adjacent myc proto-oncogene is disturbed, resulting in an abnormal pattern of synthesis of the myc protein product. Such abnormal pattern of expression of the c-myc gene—which encodes transcription factor normally induced in response to growth factor stimulation—is sufficient to drive cell proliferation and contribute to tumour development.

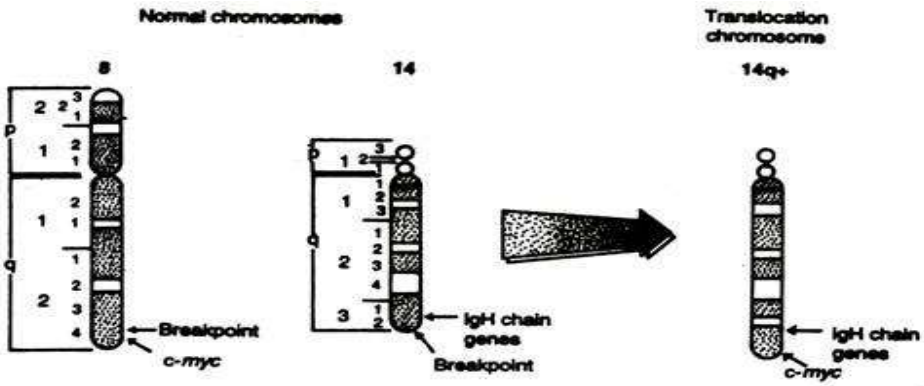


Fig. 23.6: Translocation of a c-myc protooncogene from chromosome 8 to 14.

Translocation of some proto-oncogene often causes the rearrangement of coding sequences which lead to the formation of abnormal gene products. In chronic myelegenousleukemia, the abl proto-oncogene is translocated from chromosome 9 to chromosome 22 forming Philadelphia chromosome (Fig. 23.7).The abl proto-oncogene which contains two alternative first exon (1A and IB) is joined to the middle to the bcr gene on chromosome 22. Exon IB is deleted as a result of the translocation. Transcription of the fused gene initiates at the bcr promotor and continues through abl. Splicing then generates a fused bcr/abl mRNA, in which abl exon 1A sequences are also deleted and bcr sequences are joined to abl Exon 2.

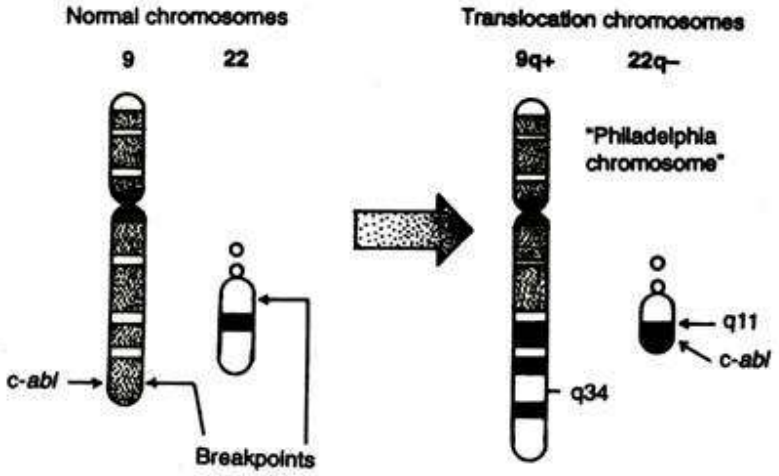


Fig. 23.7: Reciprocal translocation between chromosomes 9 and 22 that produce Philadelphia chromosome.

The bcr/abl mRNA is translated to yield a recombinant bcr/abl fusion protein in which the normal amino terminus of abl proto-oncogene has been replaced by bcr amino acid sequences. The fusion of bcr sequences results in aberrant activity and altered subcellular localisation of the abl protein tyrosine kinase, leading to cell transformation.Gene amplification occurring in the tumour cell is a common process by which proto-oncogenes are converted to oncogene. Gene amplification takes place due to an increase of the number of copies of a gene resulting from the repeated replication of a region of DNA.

Therefore, gene amplification leads to the overproduction of a particular protein or enzyme from the amplified gene. A prominent example of oncogene amplification is the involvement of the N-myc gene in neuroblastoma, a tumour of embryonal neuronal cells. Amplified copies of N-myc gene are frequently present in rapidly growing tumour. Hence it indicates that N-myc amplification is related with the

development of neuroblastomas. Amplification of erb B-2 which encodes a receptor protein kinase is similarly associated to the development of breast and ovarian carcinomas.

Table 23.4: Retroviral Oncogenes

Oncogene	Virus	Species
abl	Abelson leukemia	Mouse
akt	AKT8 virus	Mouse
cbl	Cas NS-1	Mouse
crk	CT10 sarcoma	Chicken
erbA	Avian erythroblastosis-ES4	Chicken
erbB	Avian erythroblastosis-ES4	Chicken
ets	Avian erythroblastosis-E26	Chicken
fes	Gardner-Arnstein feline sarcoma	Cat
fgr	Gardner-Rasheed feline sarcoma	Cat
fms	McDonough feline sarcome	Cat
fos	FBJ murine osteogenic sarcome	Mouse
fps	Fujinami sarcome	Chicken
jun	Avian sarcoma-17	Chicken
kit	Hardy-Zuckerman feline sarcome	Cat
maf	Avian sarcoma-AS42	Chicken
mos	Moloney sarcoma	Mouse
mpl	Myeloproliferative leukemia	Mouse
myb	Avian myeloblastosis	Chicken
myc	Avian myelocytomatosis	Chicken
qin	Avian sarcoma 31	Chicken
raf	3611 murine sarcoma	Mouse
rasH	Harvey sarcoma	Rat
rasK	Kirsten sarcoma	Rat
rel	Reticuloendotheliosis	Turkey
ros	UR2 sarcoma	Chicken
sea	Avian erythroblastosis-S13	Chicken
sis	Simian sarcoma	Monkey
ski	Avian SK	Chicken
src	Rous sarcoma	Chicken
yes	Y73 sarcome	Chicken

Subsequent studies have discovered a number of oncogenes (Table 23.4) which are associated with human tumour. Among them chromosomal location of some oncogenes are shown in Fig. 23.8.

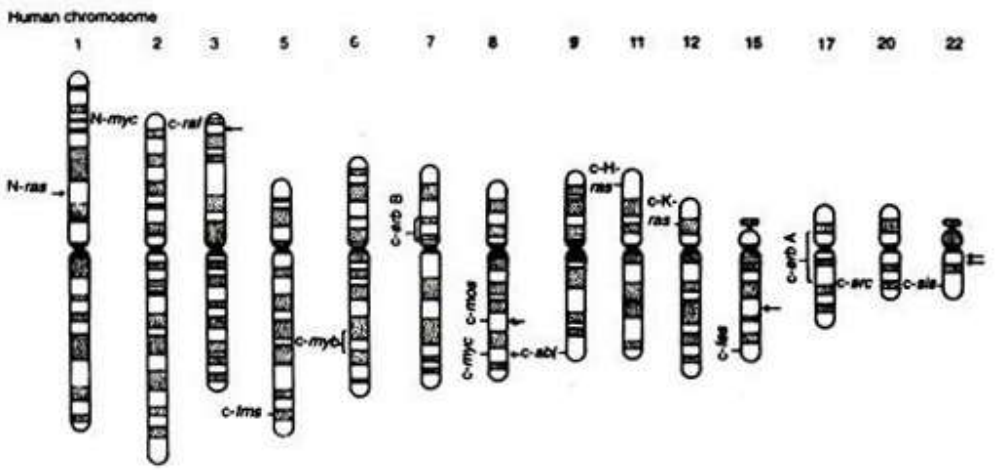


Fig. 23.8: Chromosomal location of some human protooncogenes.

Functions of Oncogene Products:

We have understood that alternation in normal genes, proto-oncogenes, can convert them into oncogenes that code for proteins that are abnormal in structure or are produced in inappropriate amounts. The proteins encoded by the normal genes regulate normal cell proliferation. But the protein encoded by the corresponding oncogene proteins drives the uncontrolled proliferation of the cancer cells.

In addition, some oncogene products involved in other aspects of the behaviour of cancer cells such as defective differentiation and failure to undergo programmed cell death. Besides this, majority of oncogene proteins function as elements of the signalling pathways that regulate cell proliferation in response to growth factor stimulation.These oncogene proteins include polypeptide growth factors, growth factor receptors, elements of intracellular signalling pathway and transcriptional factors (Table 23.5).

Table 23.5: Representative Oncogenes of Human Tumours

Oncogene	Type of cancer	Activation mechanism
abl	Chronic myelogenous leukemia, acute lymphocytic leukemia	Translocation
bcl-2	Follicular B-cell lymphoma	Translocation
*E2A/pbx*1	Acute lymphocytic leukemia	Translocation
erb B-2	Breast and overian carcinomas	Amplification
gip	Adrenal cortical and ovarian carcinomas	Point mutation
gli	Glioblastoma	Amplification
gsp	Pituitary and thyroid tumors	Point mutation
hox-11	Acute T-cell leukemia	Translocation
lyl	Acute T-cell leukemia	Translocation
c-*myc*	Burkitt's lymphoma	Translocation
c-*myc*	Breast and lung carcinomas	Amplification
L-*myc*	Lung carcinoma	Amplification
N-*myc*	Neuroblastoma, lung carcinoma	Amplification
PML/RA/Rα	Acute promyelocytic leukemia	Translocation
*PRAD*1	Parathyroid adenoma	Translocation
*PRAD*1	Breast carcinoma	Amplification
rasH	Thyroid carcinoma	Point mutation
rasK	Colon, lung, pancreatic, and thyroid carcinomas	Point mutation
rasN	Acute myelogenous and lymphocytic leukemias, thyroid carcinoma	Point mutation
ret	Thyroid carcinoma	DNA rearrangement

If the oncogenes induce uncontrolled cell growth that leads to cancer then it is obvious that the products of these genes would act by stimulating all division in some manner.For example, the product of the v-sis oncogene (the v stands for virus) of simian sarcoma virus is closely related to a polypeptide growth hormone called platelet-derived growth factor (PDGF). This factor produced by platelets promotes wound healing by stimulating growth of cells at wound site.

Simian sarcoma virus with v-sis gene in their genome when injected into the body of woolly monkey, induce sarcoma. They are also able to transform fibroblasts growing in culture to a tumorous state. This type of cellular transformation occurs by a mechanism which is possibly related to the effect of normal PDGF on cells at the wound site.Other oncogenes encode products that are identical to growth hormone as well as hormone receptors. For example, oncogene erb B and fms encode proteins that are closely related to the receptors for epidermal growth factor (EGF) and colony stimulating factor-1 (CSF-1).CSF-1 is a growth factor that stimulates growth and differentiation of macrophages. The receptor of this growth factor is a trans membrane-protein with growth factor domains on the outside of the cell and protein kinase domains on the inside of the cell (Fig. 23.9).

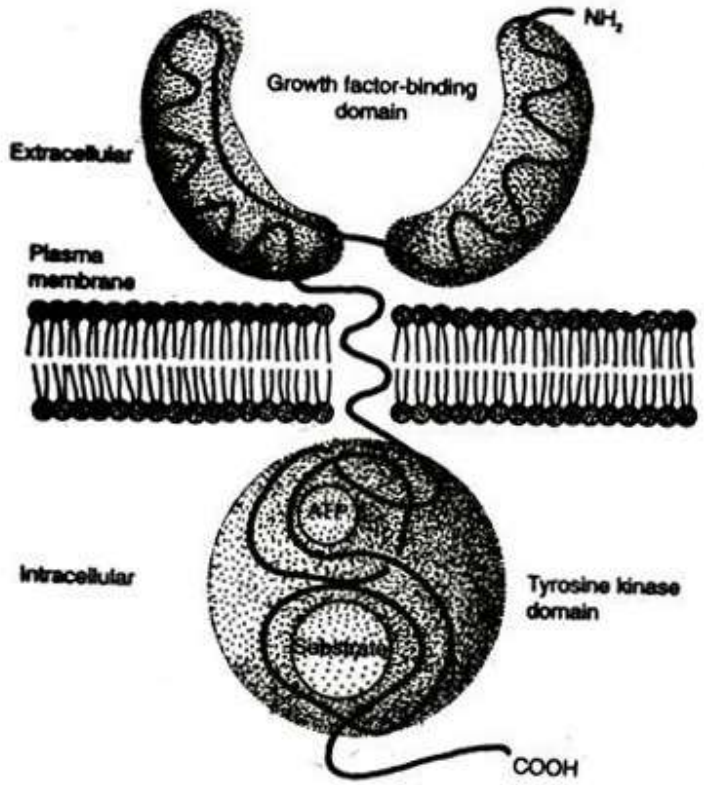


Fig. 23.9: Structure of transmembrane growth factor receptors.

These receptors are key components in trans-membrane signalling pathways. The erb gene product is an analog of the nuclear receptor for the thyroid hormone T_3. Therefore, all of the gene products are undoubtedly involved in intercellular communication circuit which control cell division during the growth and development of highly differentiated tissue.Protein tyrosine kinase is a trans-membrane receptor that is capable of transmitting a perfect signal instructing a cell to divide. Alternation in the structure and function of this enzyme will transmit a wrong signal instructing the cell to divide when it normally should not divide—the result will be tumour formation.Following the discovery that the src oncogene codes for a protein kinase, more than 20 other oncogenes have also been found to code for protein tyrosine kinases. These oncogene encoded tyrosine kinases can be subdivided into two main classes such as receptor protein tyrosine kinases and non-receptor protein tyrosine kinases.Receptor protein tyrosine kinases are trans--membrane proteins that contain a growth factor receptor domain which are exposed on the outer surface of the plasma membrane and a tyrosine kinase catalytic domain at the inner surface of the plasma membrane.In a normal receptor of this type, first appropriate growth such as PDGF, EGF, binds with receptors site and activates protein tyrosine kinase domain. Activation of protein tyrosine kinase stimulates cell proliferation through activation of the membrane associated G protein Ras (Fig. 23.10).

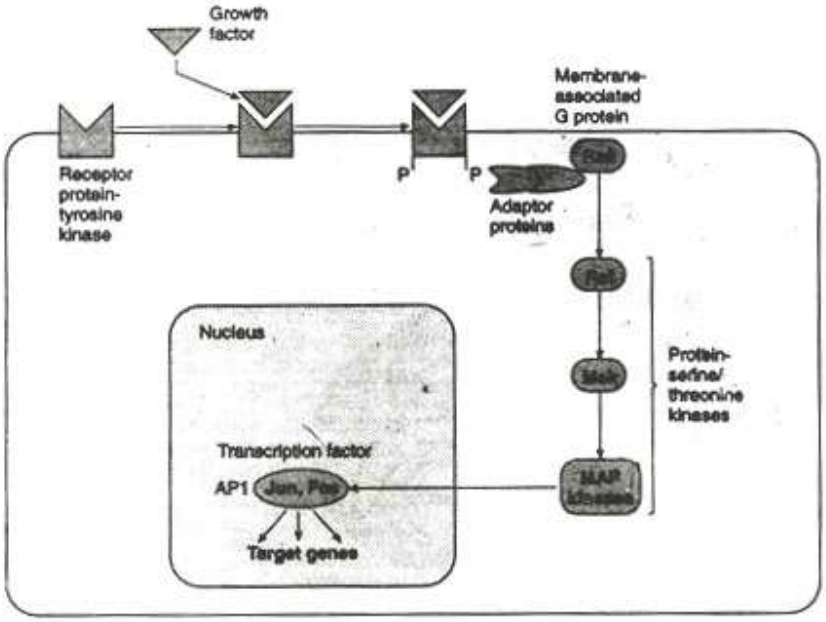


Fig. 23.10: Activation of protein tyrosine kinase that stimulates cell proliferation through activation of the membrane associated G protein Ras.

Activation of Ras triggers the phosphorylation of a series of cytoplasmic protein-serin/theronine kinase, thereby leading to phosphorylation of the nuclear API transcription factor which, in turn, activates genes involved in stimulating cell proliferation.Oncogenes can code for abnormal receptor protein- tyrosine kinases in which the growth factor binding site is disrupted leading to unregulated activity of the protein tyrosine kinase site.

Non-receptor protein tyrosine kinase are usually bound to the membrane's cytoplasm or free in the cytosol. The non-receptor protein tyrosine kinase is encoded by the src gene. Oncogene-encoded non-receptor kinases often show excessive unregulated protein-tyrosine kinase activity.Another group of oncogenes code for plasma membrane associated G proteins. In human cancer, ras oncogene shows almost resemblance with cellular ras gene of the host except that ras oncogene is the mutant form in contrast to cellular ras gene.

Hence mutant ras G proteins are produced. They retain bound GTP instead of hydrolyzing it to GDP. As a result mutant ras protein in its active form mislead the transmission of signal from external growth factors. Hence the host cells undergo abnormal cell division.Most of the protein kinase activity showed by mammalian cells catalyses the phosphorylation of the amino acids serine and theonin, not tyrosine. These protein-serine/threonine kinase like protein-tyrosine kinase can be encoded by oncogene.

The most important oncogene belonging to this group is the raf oncogene. It codes for a protein serine/threonine kinase that transmits signals from plasma membrane Ras protein to the cell interior.Some oncogenes code for proteins that function within the nucleus, particularly in the regulation of gene transcription. The examples of such oncogenes are the jun and fos oncogene which code for proteins that make up the AP! transcription factor.The AP_1 factor regulates the expression of a group of genes that are involved in stimulating cell proliferation. The myc oncogene, associated with several kinds of human cancer, also appears to code for a transcription factor.

Proto-Oncogene:

It is well-established that oncogenic virus contains a relatively small number of genes which has facilitated the identification of the viral genes that cause cell to become malignant. The first cancer-causing gene to be identified occurs in Rous sarcoma virus, a small retrovirus that produces sarcomas in chickens.An unexpected feature of retroviral oncogene is their lack of involvement in virus replication while other viral gene involves efficiently in the same process.

Again, the existence of viral oncogene is not an integral part of the virus life cycle. Therefore, the origin and existence of viral oncogene leads to a new line of investigation. Such investigations have led to the surprising discovery that the src gene is not present only in cancer cells.Using nucleic acid hybridisation techniques, it has been shown that DNA sequence that Eire homologous to— but not identical with—the Rous src gene can be detected in the genome of normal cells of a wide variety of organisms including salmon, mice, cows, birds and humans.

The unexpected discovery that cells contain DNA sequences that are closely related to viral oncogenes has been substantiated by studies on a variety of other tumour viruses and, in each case, they resemble genes present in the genome of normal cell.The term proto- oncogene has been introduced to refer to these normal cellular genes that closely resemble oncogenes. The resemblance of viral oncogenes to proto-oncogene suggests that viral oncogenes may have originally been derived from normal cellular genes.According to this concept, the first step in the creation of retro-viral oncogenes took place million years ago when the ancient virus infected cells and became integrated in the host chromosomal DNA adjacent to normal cellular proto-oncogenes.When the integrated pro-viral DNA was later transcribed to regenerate new viral RNA molecules, the adjacent proto-oncogene sequences might have been transcribed as well. In this way, a viral RNA molecule containing normal proto- oncogene sequences could have been created.

Since a proto-oncogene would initially serve no useful purpose for a virus, it would be free to mutate during subsequent cycles of viral infection. Such mutation would eventually convert proto-oncogene into an oncogene.Therefore, the realisation that oncogenic viruses contain genes that cause cell to become malignant raise the question of whether genetic alteration are also involved in non-virus induced cancers. The ability of many carcinogens to act as mutagens provides the reason to believe that genetic changes play a role in non-viral carcinogenesis.Besides this, recent research suggests that cellular oncogenes are derived from normal proto-oncogenes by at least five mechanisms:

(i) Point Mutation:

The simplest mechanism for converting a proto- oncogene into an oncogene, it involves a single base pair substitution or point mutation.

(ii) Local DNA Rearrangement:

The second mechanism for creating oncogenes is based on DNA rearrangements that cause either deletions or base sequence exchanges between proto-oncogene and surrounding genes.

(iii) Insertional Mutagenesis:

The evidence of third mechanism comes from the findings that some cancer-causing retrovirus lack oncogenes and these particular viruses cause cancer by integrating a DNA copy of their genetic information into a host chromosome in a region where a proto-oncogene is located and thus disrupt the structure of the host proto-oncogene and thereby convert it into an oncogene.

(iv) Gene Amplification:

The fourth mechanism for creating oncogenes uses gene amplification to increase the number of copies of a particular protogene. This overproduction of copies of a particular proto- oncogene leads to malignant transformation.

(v) Chromosomal Translocation:

The fifth mechanism for creating an oncogene involves chromosomal translocation. It is a process where a portion of one chromosome is physically broken and joined to another chromosome. As a result, the broken segment containing proto-oncogene is transferred from its normal location to a new location where it is converted as oncogene.

Tumour Suppressor Genes:

We have now seen how the presence of an oncogene can stimulate uncontrolled cell growth and division, thereby fostering the development of malignancy. Cancer can also be induced by the loss of tumour suppressor genes that normally inhibit cell proliferation.The term tumour suppressor gene implies that the normal function of gene of this type is to restrain cell growth and division. In other words, tumour suppressor genes act as brakes on the process of cell proliferation and inhibits tumour development.

In many tumours these genes are lost or inactivated, thereby removing negative regulators of cell proliferation and contributing to the abnormal proliferation of tumour cells. Normally, the function of tumour suppressor gene is just opposite to oncogene.The first evidence of the activity of tumour suppressor gene came from somatic cell function experiment done by Henry Harris etal in 1969. The fusion of tumour cells with normal cell yields hybrids that contain chromosomes from both parents.

Such hybrids are usually non-tumerogenic. Suppression of tumourogenicity by cell fusion indicates that genes derived from the normal cell definitely suppress the tumour development.The first suppressor gene to be identified is involvement in hereditary retinoblastoma, a rare type of eye cancer that develops in children who have a family history of the disease. Such children inherit a chromosomal deletion in a specific region of one copy of chromosome 13.

Although the deletion occurs in all cells, only a few in the retina actually become malignant because the initial deletion in chromosome 13 does not cause cancer by itself; for cancer to develop, a subsequent mutation must also occur in the same region of the homologous chromosome 13.It has, therefore, been concluded that chromosome 13 contains a gene on homologous chromosome of a normal diploid cell where such gene normally functions to inhibit retinoblastomas. In inherited retinoblastoma one defected copy of gene is genetically transmitted.The loss of this single copy of gene is compensated by the identical second copy of the gene present on the same region of the second copy of chromosome 13.Therefore loss of a single copy of gene is not by itself sufl5cient to trigger tumour development, but retinoblastoma almost always develops in these individuals as a result of a second somatic mutation leading to further loss of the function of the remaining second copy of normal gene.

The gene lost in hereditary retinoblastoma is called RBI. It is a tumour suppressor gene that codes for the nuclear protein p^{RB} that inhibits expression of a group of genes whose products are needed for uncontrolled cell proliferation. In hereditary retinoblastoma a defective or copy of the RBI gene is inherited from the affected person.Hence a lack of p^{RB} resulting from loss of both copies of RBI (one due to deletion and other due to a second somatic mutation) can lead to uncontrolled proliferation which ultimately causes the development of retinoblastoma. In nonhereditary cases, two normal RBI genes are inherited and retinoblastoma develops only if two somatic mutations in adult inactivate both copies of RBI in the same cell.

Table 23.6: Main classes of oncogenes categorised by nature of their Protein Products

Nature of Protein Product	Examples of Oncogenes	Comments
Growth factors	it sis	Platelet-derived growth factor (PDGF)
Protein-tyrosine kinases	*erb B*	Membrane receptor of epidermal growth factor (EGF)
	fms	Membrane receptor for colony-stimulating factor-1 (CSF-1)
	src, yes, fgr	Membrane nonreceptor proteintyrosine kinases
Membrane-associated G proteins	*ras*	Membrane-associated GTP-binding protein
	gsp	G_s(α sub-unit)
	gip	G_i(α sub-unit)
Protein-serine/threonine kinases	*raf, mos*	Cytoplasmic protein-serine/threonine kinases
Transcription factors	*jun, fos*	Components of AP1 transcription factor
	erb A	Thyroid hormone receptor

Following the discovery of the RBI gene several other tumour suppressor genes have been identified (Table 23.6). The second suppressor gene is p^{53} which is frequently inactivated in a wide variety of human cancer including leukemia's, lymphomas, sarcomas, bredn tumour and carcinomas of many tissues including breast, colon and lung.The p^{53} protein is a nuclear transcriptional factor that switches on the activity of genes that arrest cells in the G_1 phase of the cell cycle. Normally, the production of the p^{53} protein is stimulated when DNA is damaged due to exposure to ultraviolet ray or DNA damaging agents.

Hence p^{53} appears to act like a molecular policeman that checks the cell for DNA damage and prevents the cell from proliferation if damage is detected. The loss of p^{53} function allows the survival and reproduction of cells in which DNA damage has led to the production of oncogenes and/or the loss of other tumour suppressor genes.In addition to mediating cell cycle arrest P^{53} is required to apoptosis induced by DJNA damage. Unrepaired DNA damage normally induces apoptosis that eliminates cells which might develop into cancer. Cells lacking p fail to undergo apoptosis.

This failure contributes to the resistance of many tumours to chemotherapy. The failure of function of p is thought to account for the high frequency of p^{53} mutations that lead to inactivation of p^{53}.Like p^{53}, the INK4 is a tumour suppressor gene that prevents lung cancer. Similarly, two other tumour suppressor genes such as APC and DCC prevent colon cancer. When these genes are deleted or mutated, such cancers develop.The product of RBI and INK4 tumour suppressor genes regulate cell cycle progression at the same point. These

genes inhibit passage through the restriction point in G_1 by suppressing transcription of a number of genes involved in cell cycle progression and DNA synthesis.

A rare hereditary form of colon cancer, familial adenomatous polyposis, is produced due to inherited mutation of the APC gene. In this type of cancer hundreds of polyps or benign colon adenomas are produced within the colon of an individual. Some of these polyps are transformed into malignancy.Inactivated or mutated form of some additional tumour suppressor genes is also associated with the development of breast, ovarian and pancreatic carcinomas as well as in some rare inherited cancer syndromes such as Wilm's tumour (a childhood kidney tumour).The tumour suppressor gene of Wilm's tumour is WT1 which is frequently inactivated in Wilm s tumour. The product of WT1 gene appears to suppress transcription of a number of growth factor inducible genes.

Prevention and Treatment of Cancer:

There is a general belief among the common people that cancer cannot be cured. Although this is partially true, it depends on several aspects of the patient and the time of detection. In many cases, when it is clinically detected then it is already late and it goes beyond the treatment.Actually, cancer is a disease that ultimately has to be understood at the molecular and cellular level. In fact many cancers can be cured if they are detected at the early stage of its development. In case of hereditary cancer, regular testing may allow early detection.

Therefore, whether cancer is curable or not is a debatable question. With the help of modern and sophisticated technology, cell biologists are always trying to improve the methods for prevention and treatment of cancer.

The first step in preventing cancer is to identify the agents that cause cancer. For example, it is already known that tobacco smoke causes cancer. So just to prevent the possibility of this type of lung cancer, it is advisable simply to avoid tobacco smoke.Similarly the discovery of carcinogenic properties of X-ray and sunlight suggests that individuals should avoid unnecessary medical X-ray and use protective lotions during long time exposure to sunlight.

Epidemiological data also allow potential carcinogens to be identified in exposed human population. The epidemiological approach is based on comparison of cancer rates among various groups of people exposed to different environmental conditions.For example, when Japanese individuals move to the United States heir susceptibility to developing stomach and lung cancer changes to reflect the rates for such cancers in the United States.Therefore, the comparison of the frequency of stomach and lung cancer in Japan, in the United States and in Japanese immigrants to the United States suggests that environmental factors play a prominent role in causing cancer. Epidemiological data have played an important role in identifying some of the environmental factors that may cause cancer.The Ames test is a rapid screening method tor identifying potential carcinogens This method is based on the rationale that most carcinogens act as mutagens, it measures the ability of potential carcinogens to induce mutations in a strain of bacteria that lack the ability to synthesize the amino acids histidine.

Each bacterial cell that has mutated to a form in which it no longer needs histidine will grow into a colony that can be counted. The number of colonies indicates the mutagenic potency of the substance to be tested.Chemicals to be tested m the Ames test are first incubated with a liver homogenate because many of the chemicals to which humans are exposed only become carcinogenic after they have undergone bio-chemical modification in the liver (Fig. 23.11) Cancer can be prevented in several other ways A person can modify his life style in order to reduce the risk of developing cancer.Change of life style sometimes requires minimizing the exposure to carcinogens. Tobacco smoking and extensive meat consumption are the probable causative factors of cancer. If any person method of treatment is most effective when the cancer is detected

at the early stage of development and when metastasis has not occurred.This method is not effective when the cancer has already been disseminated throughout the body by the process of metastasis. Therefore, early detection of cancer is very important for its treatment.

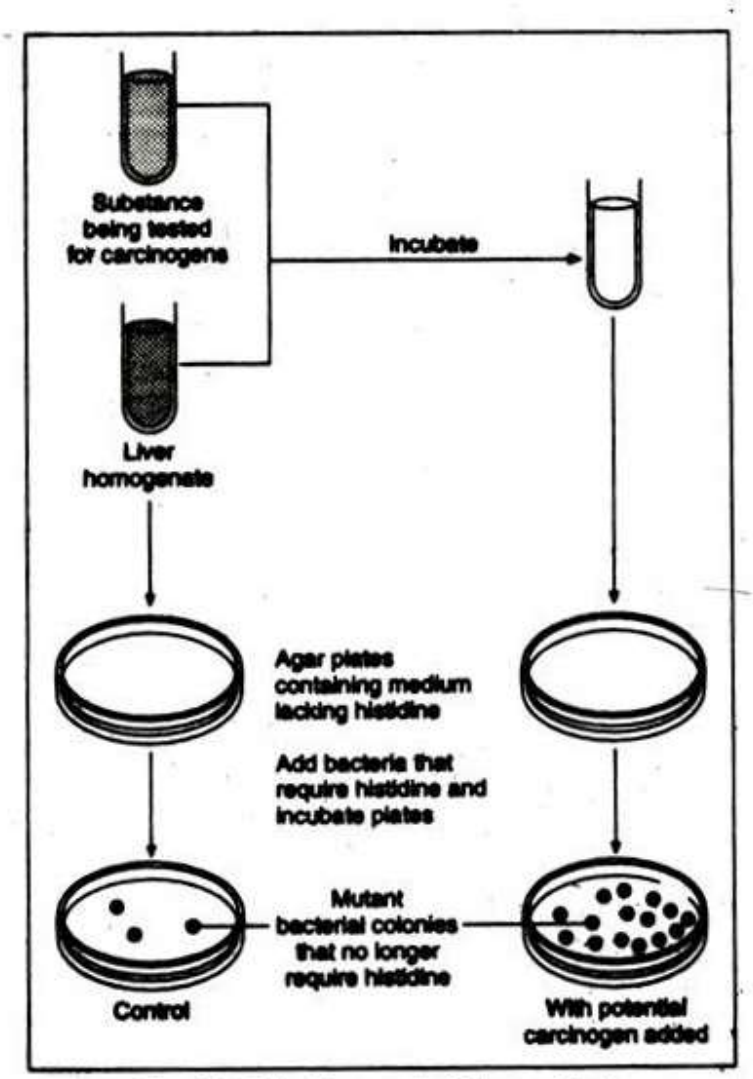


Fig. 23.11: Protocol of Ames Test.

Treatment of cancer-affected part of body with the help of X-radiation is another alternative method of curing cancer. X-ray is very effective for killing the cancer cells that are actively proliferating. The cells that are engaged in DNA synthesis prior to cell division, or are on the way of mitosis, are very sensitive to X-ray.But the main problem of using X- ray for the treatment of cancer is that normal and healthy dividing cells of the body-such as blood-forming cells in the bone marrow are also destroyed along with the cancer cells.

Moreover, X-radiation itself is carcinogenic. Hence there is always a chance of developing cancer after X-ray treatment. In spite of such risk posed by X-ray treatment, it is effective for the treatment of certain types of cancers like skin cancer, Hodgkin's disease and specific forms of testicular and bone cancer.Chemotherapy is another approach for treating cancer. This method is based on the use of certain drugs that are designed to kill the proliferating cells as in radiation treatment. This method is also effective

when the cancerous cells have already metastasized.The drugs are generally injected in the body and the circulatory system helps the drug to spread throughout the body. Some drugs used m cancer chemotherapy are given in the Table 23.7.

Table 23.7: Tumour Suppressor Genes

Gene	Type of cancer
APC	Colon/rectum carcinoma
BRCA1	Breast and ovarian carcinomas
BRCA2	Breast carcinoma
DCC	Colon/rectum carcinoma
DPC4	Pancreatic carcinoma
INK4	Melanoma, lung carcinoma, brain tumours, leukemias, lymphomas
NF1	Neurofibrosarcoma
NF2	Meningioma
P^{53}	Brain tumours, breast, colon/rectum, esophageal, liver, and lung carcinomas; leukemias and lymphomas
Rb	Retinoblastoma, sarcomas; bladder, breast, and lung carcinomas
VHL	Renal cell carcinoma
WT1	Wilm's tumour

Like radiation, chemotherapeutic drugs also kill the normal and healthy cells along with cancer cells. This type of treatment has also some toxic side-effect-like loss of hair (caused by destruction of hair follicle cells), diarrhoea (caused by destruction of cells of the intestinal lining) and susceptibility to infections (caused by destruction of blood cells).Sometimes two or more combination of drugs are also used for the treatment of cancer.Besides its side- effects and other disadvantages, it is true that, for certain types of cancer, chemotherapy is very successful for curing cancer like Burkitt s lymphoma, chorio carcinoma, acute lymphocytic leukaemia, Hodgkin's disease, lymphomas, mycosis fungoides, Wilm's tumour, Ewing s sarcoma, thabdomyosarcoma, retinoblastoma, and embryonal testicular tumours etc.

Table 23.8: Some drugs used in Cancer Chemotherapy

Class	Examples	Mechanism of Action
1. Antimetabolites	Methotrexate 5-Fluorouracil 6-Mercaptopurine	Inhibit enzymatic pathways for biosynthesis of nucleic acids by substituting for normal substrates
2. Antibiotics (sybstances produced by microorganisms)	Actinomycin D Adriamycin Daunorubicin	Bind to DNA
3. Alkylating agents	Nitrogen mustard Chlorambucil Cyclophosphamide Imidazole carboximides	Crosslink DNA
4. Mitotic inhibitors	Vincristine Vinblastine Taxol	Interfere with mitotic spindle
5. Hormones	Estrogen (for prostate cancer) Cortisone Progesterone Androgens	Inhibit growth of hormone-sensitive cells by interacting with hormone receptors
6. Miscellaneous agents	L-Asparaginase	Hydrolyzes asparagine

Although the use of surgery, radiation and chemotherapy has led to increased survival rates for certain kinds of cancer, many malignancies do not respond well to such treatment. Recent experimentation is attempting to exploit the ability of the immune system to recognize and kill tumour cells. This type of treatment is known as immunotherapy.

The basic principle of immunotherapy is to exploit the ability of the immune system to recognise and kill tumour cells. Tumour cells tend to show cell surface antigens which make them recognisable by the immune system. Initially, some scientists attempted to utilise a person's own lymphocytes to kill cancer cells.For this experiment, lymphocytes were isolated from the blood of cancer patients and grown in culture in presence of Interlaken 2 to stimulate the cancers destroying properties of the cell. The result was the isolation of a population of killer T-cells that were specifically targeted against the patient's tumour.

These cells, called tumour-infiltrating lymphocytes (TILs), were injected back into the patients from whom the blood was drawn. TILs axe more effective in inducing tumour regression. Recently TILs are made even more effective by using recombining DNA technique to insert some genes whose product enhances the additional potency of the TILs.A protein produced by march-panes called tumour necrosis factor (TNF) is effective in promoting the destruction of cancer cells if the TNF gene were inserted into the TILs.Obviously, the genetically engineered TILs would be more effective than normal TILs and would be more powerful to killing the tumour cells. Currently this technique is being tested in the hope of finding ways to promote immune destruction of cancer cells.

Chromosomal basis of human cancer:

Cancer is also regarded as a chromosomal disease. Accordingly carcinogenesis is initiated by random aneuploidies, which are induced by carcinogens or spontaneously. Since aneuploidy unbalances 1000s of genes, it corrupts teams of proteins that segregate, synthesize and repair chromosomes. Aneuploidy is therefore a steady source of chromosomal variations from which, in classical Darwinian terms, selection encourages the evolution and malignant progression of cancer cells. The rates of specific chromosomal variations can exceed conventional mutations by 4-11 orders of magnitude, depending on the degrees of aneuploidy. Based on their chromosomal constitution cancer cells are new cell "species" with specific aneusomies, but unstable karyotypes. The cancer-specific aneusomies generate complex, malignant phenotypes through the abnormal dosages of 1000s of genes, just as trisomy 21 generates Downsyndrome.
Although chromosomal changes are highly variable, they can be grouped into two general categories. In balanced structural changes, the genetic material is exchanged evenly. An example of a balanced structural change is the Philadelphia chromosome translocation . In that case, although genetic information was

rearranged into an abnormal gene, it resulted from an evenexchange of DNA. Conversely, in nonreciprocal or unbalanced structural changes, the exchange is not equal, and genetic material is added or lost. This can range from the loss or gain of a single base pair to the loss or gain of entire chromosomes. Scientists have hypothesized that the primary pathogenetic changes in cancer result from balanced rearrangements, while the secondary changes that occur during cancer progression are from unbalanced changes. Cancer is a multistep, progressive disease, and early chromosomal changes provide the cell with a proliferative advantage. Often, these changes hijack or interfere with the normal cellular control mechanisms by disrupting proto-oncogenes and tumor suppressor genes and allowing additional changes to occur in the genome. Cancer cells generally gain multiple types of chromosomal aberrations during tumor progression, including rearrangements, deletions, and duplications. As a result, the genome becomes progressively moreunstable.

Chromosomal rearrangements :

Chromosomal rearrangements can lead to cancer either by forming a hybrid gene or by causing disregulation of a gene. Since the discovery of a particular chromosomal rearrangement, thousands of other chromosomal aberrations have been determined to be associated with cancer. These chromosomal changes are the signature of gene deregulation in cancer and lead to instability of the genome. Chromosomal changes are highly variable in different cancers, and the resultant phenotypic effects are equally variable.

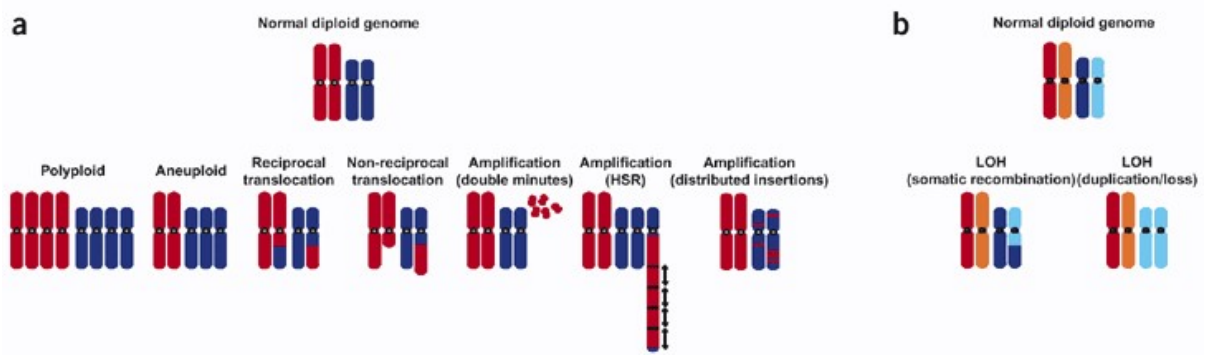


Fig 6: Different types of Chromosomal aberrations. (A) Chromosomal aberrations with altered gene number or sequence (B) Chromosomal aberrations with intact gene number or sequence The Philadelphia (Ph^1) chromosome, a small acrocentric chromosome seen in 90% of patients with chronic myeloid leukemia. The Ph^1 chromosome is one product of a balanced reciprocal 9;22 translocation. The breakpoint on chromosome 9 is within an intron of the *ABL1* oncogene. The translocation joins the 3' part of the *ABL1* genomic sequence onto the 5' part of the *BCR* (breakpoint cluster region) gene on chromosome 22, creating a novel fusion gene. This chimericgene is expressed to produce a tyrosine kinase related to the *ABL* product but with abnormal transforming properties.

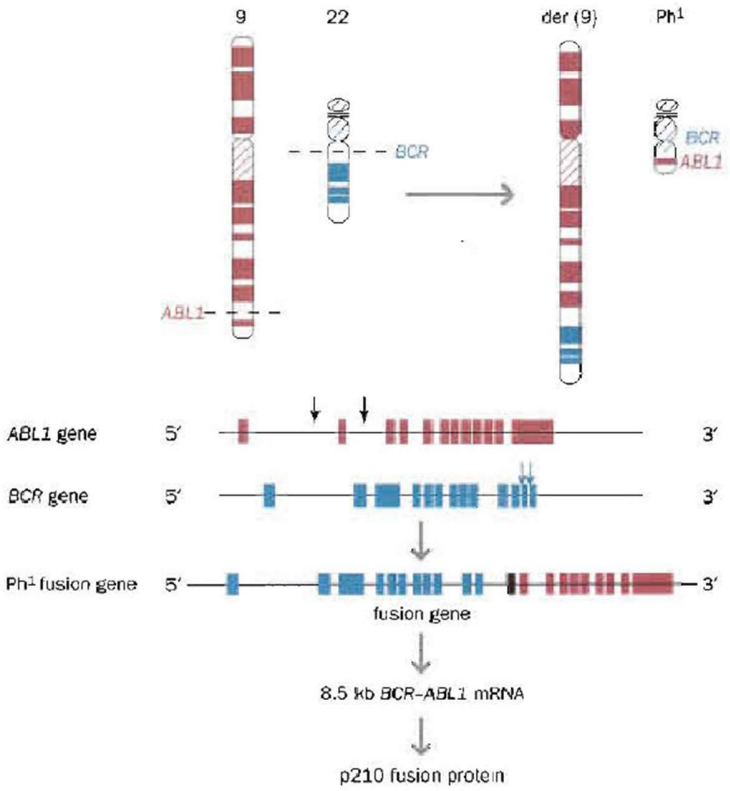


Fig 7: Chromosomal arrangement in Philadelphia chromosome.

Many other tumor-specific recurrent rearrangements that produce chimeric oncogenes have now been recognized**.** This mechanism is seen in 15-25% of leukemias, lymphomas, and sarcomas, but has been reported in only 1 % or less of the common solid epithelialtumors.Burkitt lymphoma is known to be associated with activation of the MYC oncogene A characteristic chromosomal translocation, t(8;14)(q24;q32), is seen in 75-85% of patients**.** The remainder have t(2;8)(p12;q24) or t(8;22)(q24;qll). Each of these translocations juxtaposes the MYC oncogene (normally located at 8q24) close to an immunoglobulin (IG) locus. This may be *IGH* at 14q32, *IGK* at 2p12, or *IGL* at 22qll. The translocation bring the oncogene under the influence of regulatory elements that normally ensure high expression of the immunoglobulin genes in antibody-producing B cells. In the 8; 14 translocation, the MYC and *IGH* genes are in opposite transcriptional orientations, head to head. Often, depending on the precise breakpoint, exon 1 of the MYC gene (which is noncoding) is not included in the translocated material. Deprived of its normal upstream controls and placed in an active chromatin domain, MYC isexpressed at an inappropriately high level. Between 25% and 65% of all B-cell malignancies involve the activation of one or another oncogene by an immunoglobulin enhancer, and many T- cell malignancies involve a similar activation by an enhancer at a T-cell receptor locus.

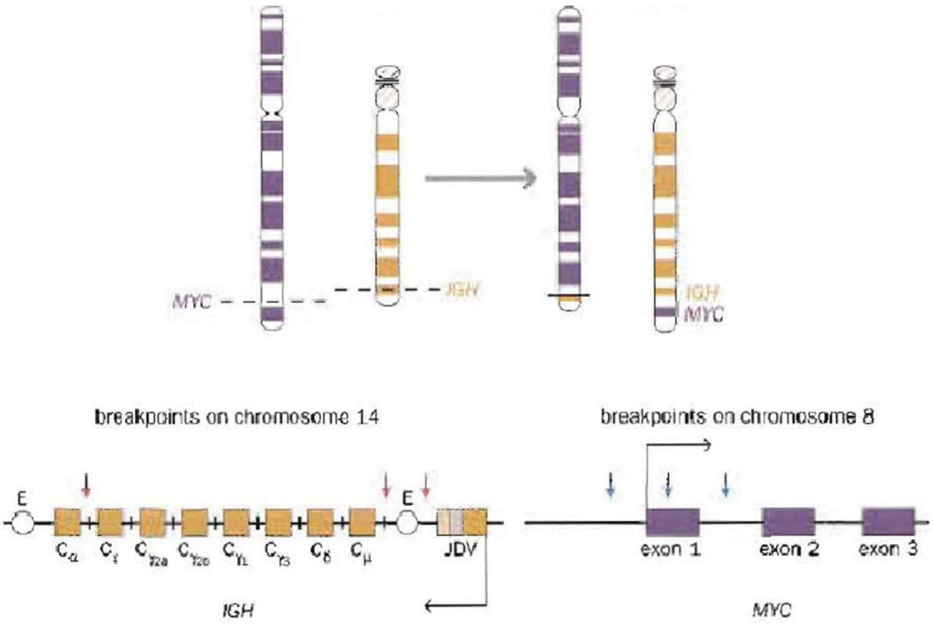


Fig 8: Chromosomal arrangement in Burkitt's lymphoma

Deletions and Duplications:

In cancers such as Wilms' tumor and retinoblastoma, gene deletions or inactivation are responsible for initiating cancer progression. In fact, inactivation of tumor suppressor genes is associated with many types of cancer, as chromosomal regions associated with tumor suppressors are commonly deleted or mutated. For example, deletions, inversions, and translocations are commonly detected in chromosome region 9p21 in gliomas, non-small-cell lung cancers, leukemias, and melanomas. These chromosomal changes inactivate a tumor suppressor called cyclin-dependent kinase inhibitor 2A. Along with these deletions of specific genes, large portions of chromosomes can also be lost. For instance, chromosomes 1p and 16q are commonly lost in solid tumorcells.Gene duplications and increases in gene copy numbers can also contribute to cancer and can be detected with transcriptional analysis or copy number variation arrays. For example, the chromosomal region 12q13-q14 is strikingly amplified in many sarcomas. This chromosomal region encodes a binding protein called MDM2, which is known to bind to a tumor suppressor called p53. When MDM2 is amplified, it prevents p53 from regulating cell growth, which can result in tumor formation.

Additionally, certain breast cancers are associated with overexpression and increases in copy number of the *ERBB2* gene, which codes for human epidermal growth factor receptor 2. Indeed, the presence of a high number of *ERBB2* copies has been found to be associated with aggressive forms of breast cancer. Therefore, measuring the *ERBB2* copy number can provide a diagnostic tool for breast cancer and other cancers. Along with these amplifications of specific genes, gains in chromosomal number, such as chromosomes 1q and 3q, are also associated with increased cancer risk.

Mutations in the genes necessary for DNA repair can additionally lead to rearrangements and duplications. For example, if a gene involved with chromosomal segregation is mutated, duplications and deletions are more likely. Furthermore, the accurate sorting and segregation of chromosomes during mitosis requires the activity of many gene products (proteins). Defects in the genes controlling the mitotic surveillance mechanisms necessary for chromosomal sorting can lead to chromosome instability and abnormalities in the number of chromosomes (polyploidy and aneuploidy), which can, in turn, lead to tumorigenesis. Another cause of genome instability and chromosomal aberrations in tumors is the presence of abnormal centromeres, which can lead to abnormal mitotic events with multiple spindles and result in the abnormal

loss ofchromosomes.

Probable Questions:

1. What is the difference between oncogene and protooncogene?
2. Write down the mechanism of oncogenic conversion of a protooncogene.
3. How oncogene causes cancer ?
4. Write down the oncogenic products which causes cancer?
5. What are tumour suppressor genes?
6. How tumour suppressor genes prevents cancer progression?
7. Write down the preventive measures of cancer.
8. How chromosomal rearrangement causes cancer?
9. How deletions in chromosomes causes cancer? Give suitable examples.
10. How duplications in chromosomes causes cancer? Give suitable examples.

Suggested readings:

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7th Edition.
6. Gene cloning and DNA Analysis by T. A. Brown, Sixth Edition.
7. Genetics . Verma and Agarwal.

Unit-III

Mutations and mutagenesis types of mutation; biochemical basis of mutations; mutagenesis; spontaneous and induced mutation; reversion as a means of detecting mutagens and carcinogens

Objective: In thisunit you will learn about types of mutation, biochemical basis of mutations, mutagenesis, spontaneous and induced mutation, reversion as a means of detecting mutagens and carcinogens

Definition of Mutations:

Mutation refers to sudden heritable change in the phenotype of an individual. In the molecular term, mutation is defined as the permanent and relatively rare change in the number or sequence of nucleotides. Mutation was first discovered by Wright in 1791 in male lamb which had short legs.Later on mutation was reported by Hugo de Vries in 1900 in Oenothera, Morgan (1910) in Drosophila (white eye mutant) and several others in various organisms. The term mutation was coined by de Vries.

Characteristics of Mutations:

Mutations have several characteristic features.

Some of the important characteristics of mutations are briefly presented below:

i. Nature of Change:

Mutations are more or less permanent and heritable changes in the phenotype of an individual. Such changes occur due to alteration in number, kind or sequence of nucleotides of genetic material, i.e., DNA in most of the cases.

ii. Frequency:

Spontaneous mutations occur at a very low frequency. However, the mutation rate can be enhanced many fold by the use of physical and chemical mutagens.

The frequency of mutation for a gene is calculated as follows:
Frequency of gene mutation = M / M + N

where, M = number of individuals expressing mutation for a gene, and

N = number of normal individuals in a population.

iii. Mutation Rate:
Mutation rate varies from gene to gene. Some genes exhibit high mutation rate than others. Such genes are known as mutable genes, e.g., white eye in Drosophila. In some genomes, some genes enhance the natural mutation rate of other genes. Such genes are termed as mutator genes.

The example of mutator gene is dotted gene in maize. In some cases, some genes decrease the frequency of spontaneous mutations of other genes in the same genome, which are referred to as anti-mutator genes. Such gene has been reported in bacteria and bacteriophages.

iv. Direction of Change:
Mutations usually occur from dominant to recessive allele or wild type to mutant allele. However, reverse mutations are also known, e.g., notch wing and bar eye in Drosophila.

v. Effects:
Mutations are generally harmful to the organism. In other words, most of the mutations have deleterious effects. Only about 0.1% of the induced mutations are useful in crop improvement. In majority of cases, mutant alleles have pleiotropic effects. Mutations give rise to multiple alleles of a gene.

vi. Site of Mutation:
Muton which is a sub-division of gene is the site of mutation. An average gene contains 500 to 1000 mutational sites. Within a gene some sites are highly mutable than others. These are generally referred to as hot spots. Mutations may occur in any tissue of an organism, i.e., somatic or gametic.

vii. Type of Event:
Mutations are random events. They may occur in any gene (nuclear or cytoplasmic), in any cell (somatic or reproductive) and at any stage of development of an individual.

viii. Recurrence:
The same type of mutation may occur repeatedly or again and again in different individuals of the same population. Thus, mutations are of recurrent nature.

Classification of Mutations:

Mutations can be classified in various ways. A brief classification of mutations on the basis of:

(1) Source,(2) Direction,(3) Tissue,(4) Effects,(5) Site,(6) Character, and(7) Visibility

TABLE 14.1. Classification and brief description of mutations

Basis of classification and type of mutation	*Brief Description*
1. Based on Source	
Spontaneous	Mutations that occur in nature
Induced	Mutations which are produced by the use of mutagenic agents.
2. Based on Direction	
Forward mutation	Any change from wild type allele.
Reverse mutation	A change from mutant allele to wild type.
3. Based on Tissue	
Somatic mutation	A mutation in somatic tissue.
Germinal mutation	A mutation in germ line cell.
4. Based on Survival	
Lethal	A mutation which kills the individual that carries it.
Sub-lethal	When mortality is more than 50% of individuals that carry mutation.
Sub-vital	When mortality is less than 50% of individuals that carry mutation.
Vital	When all mutant individuals survive.
5. Based on Site	
Nuclear mutation	A mutation in nuclear gene.
Cytoplasmic mutation	A mutation in cytoplasmic gene.
6. Based on Character	
Morphological	A mutation that alters morphological character of an individual.
Biochemical	A mutation that alters biochemical function of an individual.
7. Based on Visibility	
Macro-mutations	Mutations with distinct morphological changes in phenotype. Generally found in qualitative characters.
Micro-mutations	Mutations with invisible phenotypic changes. Generally observed in quantitative characters.

Types of Mutants:

The product of a mutation is known as mutant. It may be a genotype or an individual or a cell or a polypeptide.

There are four main classes of identifiable mutants, viz:

(i) Morphological,

(ii) Lethal,

(iii) Conditional

(iv) Biochemical.

These are briefly described below:

i. Morphological:

Morphological mutants refer to change in form, i.e., shape, size and colour. Albino spores in Neurospora, curly wings in Drosophila, dwarf peas, short legged sheep are some examples of morphological mutants.

ii. Lethal:

In this class, the new allele is recognized by its mortal or lethal effect on the organism. When the mutant allele is lethal all individuals carrying such allele will die; but when it is semi-lethal or sub-vital some of the individuals will survive.

iii. Conditional Lethal:

Some alleles produce a mutant phenotype under specific environmental conditions. Such mutants are called restrictive mutants. Under other conditions they produce normal phenotype and are called permissive. Such mutants can be grown under permissive conditions and then be shifted to restrictive conditions for evaluation.

iv. Biochemical Mutant:

Some mutants are identified by the loss of a biochemical function of the cell. The cell can assume normal function, if the medium is supplemented with appropriate nutrients. For example, adenine auxotroph's can be grown only if adenine is supplied, whereas wild type does not require adenine supplement.

Types of Mutation:

Mutations can be classified in various ways depending on the cause of the mutation, its effect on the function of the gene product or the kind of changes to the structure of the gene itself. Mutagenic agents such as carcinogens or high-energy radiation lead to changes to the genomic material. Some mutations occur as a natural byproduct of the error rate in DNA or RNA replication mechanisms. A mutation could be a loss-of-function or gain-of-function mutation, depending on whether the gene product is inactivated or has enhanced activity. In heterozygotes with two copies of every allele, some mutated gene products can suppress the effect of the wild- type allele. These are called dominant negative mutations. All these effects arise from a change to the structure of a gene or allied chromosomal material. These structural changes can be classified as substitutions, deletions, insertions, amplifications, ortranslocations.The term point mutation typically refers to the alteration of a single base pair of DNA or of a small number of adjacent base pairs. In this section, we will consider the effects of such changes at the phenotypic level. Point mutations are classified in molecular terms, which shows the main types of DNA changes and their effects on protein function when they occur within the protein- coding region of a gene

Substitution Mutations:

Substitution mutations are situations where a single nucleotide is changed into another. In organisms having double-stranded DNA or RNA, this usually means that the corresponding base pair is also altered. For example, an A:T base pair could be mutated into a G:C base pair or even a T:A base pair. Depending on the position of this change, it could have a variety of effects.

In highly conserved regions, both in the coding and regulatory stretches of DNA, mutations often lead to deleterious effects. Other, more variable stretches are more accommodating. In the promoter region or in other regulatory parts of the genome, a substitution mutation may change gene expression or the response of the gene to stimulus. Within the coding region, a substitution in the third or wobble position of a codon is called a silent mutation since there is no change to the amino acid sequence. When a substitution mutation results in a new amino acid but with similar properties-it is a neutral or a conserved mutation. For

instance, if aspartic acid is substituted with glutamic acid, there is a reasonable chance that there would be very few changes to the biochemistry of the protein.

Insertions and Deletions:

Insertions and deletions refer to the addition or removal of short stretches of nucleotide sequences. These types of mutations are usually more deleterious than substitutions since they can cause frame shift mutations, altering the entire amino acid sequence downstream of the mutation site. They can lead to a change in polypeptide length, either creating abnormally long proteins that cause aggregates or truncated polypeptides that are non-functional and can clog the translation machinery of the cell.

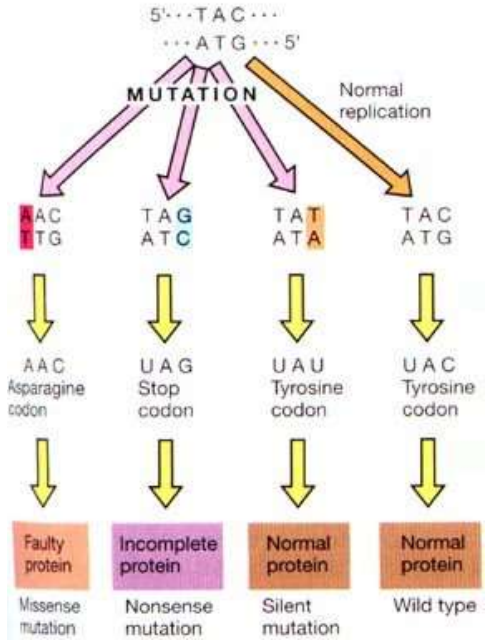


Fig 1: Types of Point mutation

Large-scale mutations:

Changes to the nucleotide sequence in genetic material can also occur on a large scale, sometimes involving thousands of base pairs and nucleotides. These kinds of mutations include amplifications, where segments of genetic material are present in multiple copies, and deletions, where a large chunk of genetic material is removed. Occasionally, some parts of the genome are translocated to a different chromosome, or reinserted into the same position, but in an inverted orientation. Translocations and deletions can bring together genes that are normally placed far apart from each other, either leading to the formation of mosaic polypeptides, or to the differential regulation of the genes within the segment.

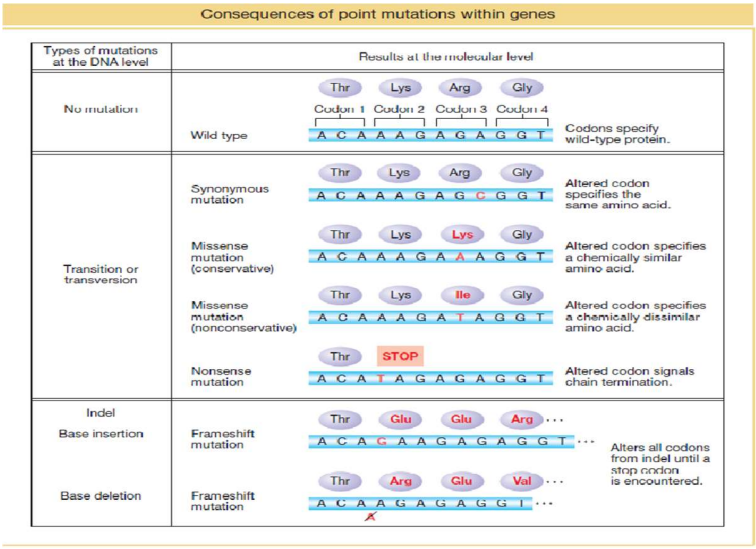


Table 1: Types of point mutation

Other Types of Mutation:

Based on change in genotype and phenotype, mutation are of two types : Pointmutation and Frameshiftmutation

1. Pointmutation

It occurs as a result of replacement of one nucleotide by other in specific nucleotide sequence of gene. Point mutation brings little phenotypic change as compared to frameshift mutation.
Point mutation are two types based on the base pair substitution.

i) Transition:

It is the point mutation occur by substitution of one purine by another purine or one pyrimidine by another pyrimidine.

ii) Transversion:

It is the point mutation occur by substitution of purine by pyrimidine and vice versa. Based on transcriptional property point mutation are of **three types.**

a) Silentmutation

b) Missensemutation

c) Non-sensemutation

a) Silentmutation:

It is also known as neutral mutation.

It is the mutation in which mutated codon codes same amino acids as the original codon. Since the amino acid is same as original one, it does not effects the structure and composition of protein.Silent mutation causes phenotype of bacteria remain similar to that of wild type.

b) Missense mutation:

In this mutation mutated codon codes different amino acid (other than original). Since new aminoacid coded by mutated codon is altered, the protein formed from it is also altered. Such protein can be less active or completely inactive.If altered aminoacids lie on active site of protein then such protein become completely non- functional. The missense mutation causes phenotypic change in organism.

c) Non sensemutation:

Mutation in which altered codon is stop codon or chain terminating codon, such mutation is called non-sense mutation. Non sense mutation causes incomplete synthesis. Such incomplete protein is always non-functional. Non-sense mutation bring greatest change in phenotype of an organism.

2. Frameshiftmutation

It occurs as a result of addition or deletion of nucleotide in the sequence of DNA. Addition or deletion of nucleotide causes shift of the reading frame of mRNA. In a mRNA each codon is represented by three bases without punctuation and insertion or deletion of a nucleotide changes the entire frame. So frame shift mutation bring greater phenotypic change than point mutation.
Insertion or deletion of one or two base pair of nucleotide causes shift in frame. However, insertion or deletion of three base pair adds or remove a whole codon, this results in addition of removal of single amino acid from polypeptide chain.

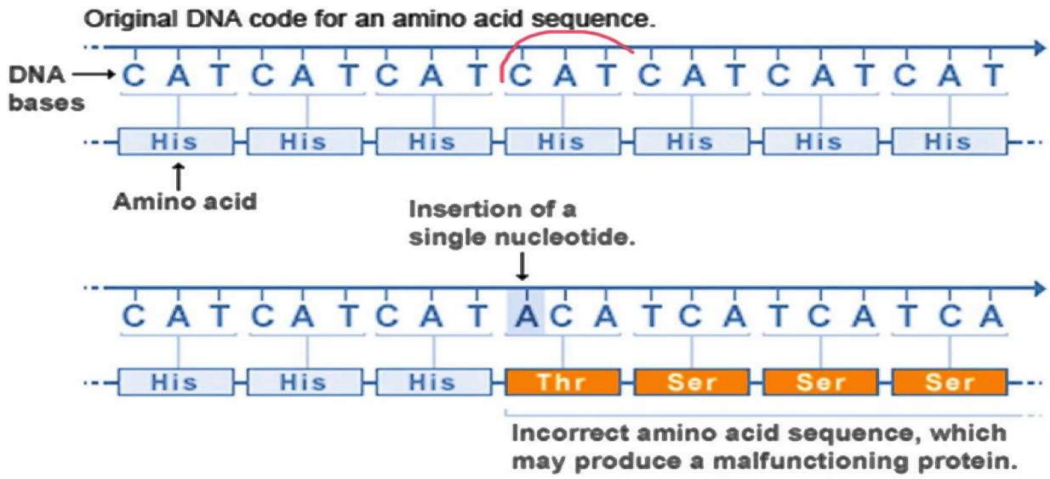


Fig 2: Frameshift Mutation

Agents of Mutations:

Mutagens:

Mutagens refer to physical or chemical agents which greatly enhance the frequency of mutations. Various radiations and chemicals are used as mutagens. Radiations come under physical mutagens. A brief description of various physical and chemical mutagens is presented below:

Physical Mutagens:

Physical mutagens include various types of radiations, viz. X-rays, gamma rays, alpha particles, beta particles, fast and thermal (slow) neutrons and ultra violet rays (Table 14.2)

A brief description of these mutagens is presented below:

TABLE 14.2. Commonly used physical mutagens (radiation), their properties and mode of action

Type of Radiation	*Main properties*	*Mode of action or changes caused*
1. X-rays	S.I., penetrating and non-particulate	Induce mutations by forming free radicals and ions. Cause addition, deletion, transitions and transversions.
2. Gamma rays	S.I., very penetrating and non-particulate	Induce mutations by ejecting atoms from the tissues. Cause all types of changes as above.
3. Alpha Particles	D.I., particulate, less penetrating and positively charged.	Act by ionization and excitation. Cause chromosomal and gene mutations.
4. Beta Rays Particles	S.I., particulate, more penetrating than alpha particles and negatively charged.	Act by ionization and excitation. Cause chromosomal and gene mutations.
5. Fast and Thermal Neutrons	D.I., particulate, neutral particles, highly penetrating	Cause chromosomal breakage and gene mutations.
6. Ultra Violet Rays	Non-ionizing, low penetrating	Cause chromosomal breakage and gene mutations.

i. X-Rays:

X-rays were first discovered by Roentgen in 1895. The wavelengths of X-rays vary from 10^{-11} to 10^{-7}. They are sparsely ionizing and highly penetrating. They are generated in X-rays machines. X-rays can break chromosomes and produce all types of mutations in nucleotides, viz., addition, deletion, inversion, transposition, transitions and trans-versions.
These changes are brought out by adding oxygen to deoxyribose, removing amino or hydroxyl group and forming peroxides. X-rays were first used by Muller in 1927 for induction of mutations in Drosophila.

ii. Gamma Rays:

Gamma rays are identical to X-rays in most of the physical properties and biological effects. But gamma rays have shorter wave length than X-rays and are more penetrating than X-rays. They are generated from radioactive decay of some elements like 14C, 60C, radium etc.

Of these, cobalt 60 is commonly used for the production of Gamma rays. Gamma rays cause chromosomal and gene mutations like X-rays by ejecting electrons from the atoms of tissues through which they pass. Now a days, gamma rays are also widely used for induction of mutations in various crop plants.

iii. Alpha Particles:

Alpha rays are composed of alpha particles. They are made of two protons and two neutrons and thus have double positive charge. They are densely ionizing, but lesser penetrating than beta rays and neutrons. Alpha particles are emitted by the isotopes of heavier elements.They have positive charge and hence they are slowed down by negative charge of tissues resulting in low penetrating power. Alpha particles lead to both ionization and excitation resulting in chromosomal mutations.

iv. Beta Particles:

Beta rays are composed of beta particles. They are sparsely ionizing but more penetrating than alpha rays. Beta particles are generated from radioactive decay of heavier elements such as 3H, 32P, 35S etc. They are negatively charged, therefore, their action is reduced by positive charge of tissues. Beta particles also act by way of ionization and excitation like alpha particles and result in both chromosomal and gene mutations.

v. Fast and Thermal Neutrons:

These are densely ionizing and highly penetrating particles. Since they are electrically neutral particles, their action is not slowed down by charged (negative or positive) particles of tissues. They are generated from radioactive decay of heavier elements in atomic reactors or cyclotrons. Because of high velocity, these particles are called as fast neutrons.

Their velocity can be reduced by the use of graphite or heavy water to produce slow neutrons or thermal neutrons. Fast and thermal neutrons result in both chromosomal breakage and gene mutation. Since they are heavy particles, they move in straight line. Fast and thermal neutrons are effectively used for induction of mutations especially in asexually reproducing crop species.

vi. Ultraviolet Rays:

UV rays are non-ionizing radiations, which are produced from mercury vapour lamps or tubes. They are also present in solar radiation. UV rays can penetrate one or two cell layers. Because of low penetrating capacity, they are commonly used for radiation of micro-organisms like bacteria and viruses.

In higher organisms, their use is generally limited to irradiation of pollen in plants and eggs in Drosophila UV rays can also break chromosomes. They have two main chemical effects on pyrimidine's.

The first effect is the addition of a water molecule which weakens the H bonding with its purine complement and permits localized separation of DNA strands. The second effect is to join pyrimidines to make a pyrimidine dimer.This dimerization can produce TT, CC, UU and mixed pyrimidine dimers like CT. Dimerization interferes with DNA and RNA synthesis. Inter-strand dimers cross link nucleic acid chains, inhibiting strand separation and distribution.

Chemical Mutagens:

There is a long list of chemicals which are used as mutagens. Detailed treatment of such chemicals is beyond the scope of this discussion.

The chemical mutagens can be divided into four groups, viz:

(a) Alkylating agents,

(b) Base analogues,

(c) Acridine dyes, and

(d) Others (Table 14.3).

A brief description of some commonly used chemicals of these groups is presented below.

TABLE 14.3. Some commonly used chemical mutagens and their mode of action

Group of mutagen	*Name of chemical*	*Mode of action*
1. Alkylating Agents	Ethyl methane Sulphonate	*AT* ↔ *GC* Transitions
	Methyl Methane Sulphonate	Transitions
	Ethyl Ethane Sulphonate	*GC* ↔ *AT* Transitions
	Ethylene Imines	Transitions.
2. Base Analogues	5 Bromo Uracil	*AT* ↔ *GC* Transitions
	2 Amino Purine	*AT* ↔ *GC* Transitions
3. Acridine Dyes	Acriflavin, Proflavin	Deletion, addition and frameshifts.
4. Others	Nitrous Acid	*AT* ↔ *GC* Transitions
	Hydroxylamine	*GC* ↔ *AT* Transitions
	Sodium Azide	Transitions

a. Alkylating Agents:

This is the most powerful group of mutagens. They induce mutations especially transitions and transversions by adding an alkyl group (either ethyl or methyl) at various positions in DNA. Alkylation produces mutation by changing hydrogen bonding in various ways.

The alkylating agents include ethyl methane sulphonate (EMS), methyl methane sulphonate (MMS), ethylene imines (EI), sulphur mustard, nitrogen mustard, etc.Out of these, the first three are in common use. Since the effect of alkylating agents resembles those of ionizing radiations, they are also known as radiomimetic chemicals. Alkylating agents can cause various large and small deformations of base structure resulting in base pair transitions and transversions.Transversions can occur either because a purine has been so reduced in size that it can accept another purine for its complement, or because a pyrimidine has been so increased in size that it can accept another pyrimidine for its complement. In both cases, diameter of the mutant base pair is close to that of a normal base pair.

b. Base Analogues:

Base analogues refer to chemical compounds which are very similar to DNA bases. Such chemicals sometimes are incorporated in DNA in place of normal base during replication. Thus, they can cause mutation by wrong base pairing. An incorrect base pairing results in transitions or transversions after DNA replication. The most commonly used base analogues are 5 bromo uracil (5BU) and 2 amino purine (2AP).

5 bromo uracil is similar to thymine, but it has bromine at the C5 position, whereas thymine has CH_3 group at C5 position. The presence of bromine in 5BU enhances its tautomeric shift from keto form to the enol form. The keto form is a usual and more stable form, while enol form is a rare and less stable or short lived form. Tautomeric change takes place in all the four DNA bases, but at a very low frequency.The change or shift of hydrogen atoms from one position to another either in a purine or in a pyrimidine base is known as tautomeric shift and such process is known as tautomerization.
The base which is produced as a result of tautomerization is known as tautomeric form or tautomer. As a result of tautomerization, the amino group ($-NH_2$) of cytosine and adenine is converted into imino group (-NH). Similarly keto group (C = 0) of thymine and guanine is changed to enol group (-OH).

5BU is similar to thymine, therefore, it pairs with adenine (in place of thymine). A tautomer of 5BU will pair with guanine rather than with adenine. Since the tautomeric form is short-lived, it will change to keto form at the time of DNA replication which will pair with adenine in place of guanine.

In this way it results in AT GC and GC —> AT transitions. The mutagen 2AP acts in a similar way and causes AT <-> GC transitions. This is an analogue of adenine.

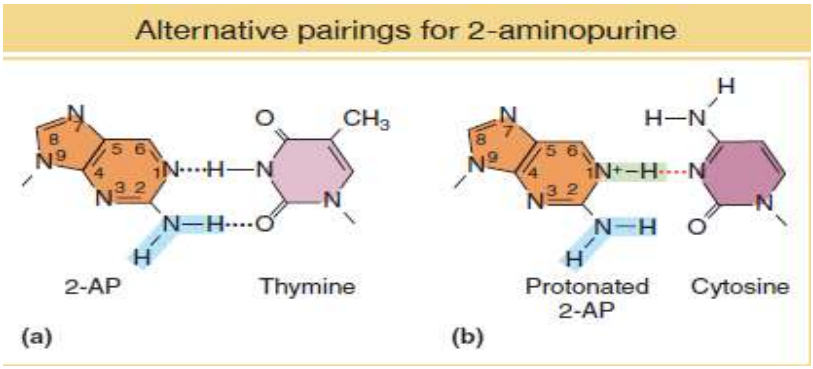


Figure: Base analogs

c. Acridine Dyes:

Acridine dyes are very effective mutagens. Acridine dyes include, pro-flavin, acridine orange, acridine yellow, acriflavin and ethidium bromide. Out of these, pro-flavin and acriflavin are in common use for induction of mutation. Acridine dyes get inserted between two base pairs of DNA and lead to addition or deletion of single or few base pairs when DNA replicates (Fig. 14.1).Thus, they cause frameshift mutations and for this reason acridine dyes are also known as frameshift mutagens. Proflavin is generally used for induction of mutation in bacteriophages and acriflavin in bacteria and higher organisms.

d. Other Mutagens:

Other important chemical mutagens are nitrous acid and hydroxy amine. Their role in induction of mutation is briefly described here. Nitrous acid is a powerful mutagen which reacts with C6 amino groups of cytosine and adenine. It replaces the amino group with oxygen (+ to – H bond). As a result, cytosine acts like thymine and adenine like guanine.

Thus, transversions from GC —> AT and AT —> GC are induced. Hydroxylamine is a very useful mutagen because it appears to be very specific and produces only one kind of change, namely, the GC —> AT transition. All the chemical mutagens except base analogues are known as DNA modifiers.

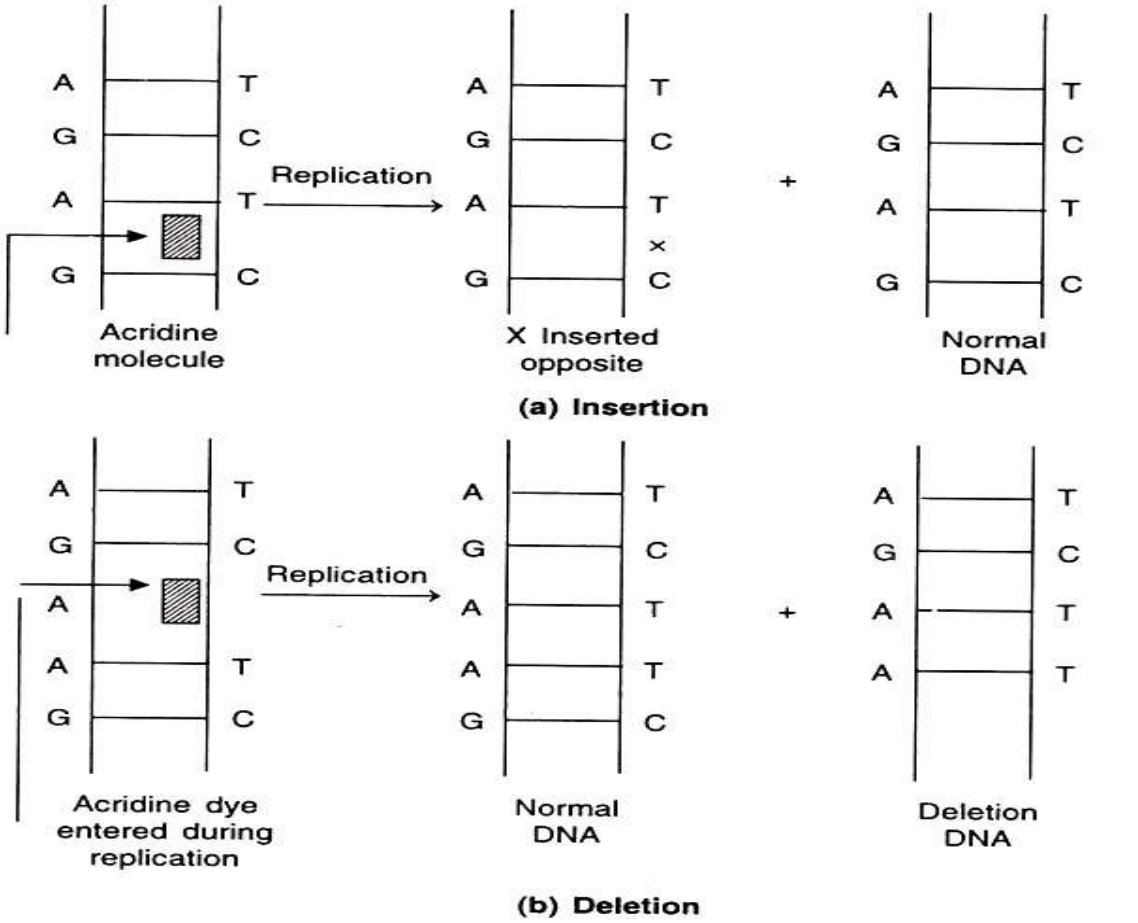


Fig. 14.1. Mode of action of acridine dyes : (*a*) insertion, (*b*) deletion.

Detection of Mutation:

Detection of mutations depends on their types. Morphological mutations are detected either by change in the phenotype of an individual or by change in the segregation ratio in a cross between normal (with marker) and irradiated individuals. The molecular mutations are detected by a change in the nucleotide, and a biochemical mutation can be detected by alteration in a biochemical reaction.The methods of detection of morphological mutants have been developed mainly with Drosophila. Four methods, viz., (1) CIB method, (2) Muller's 5 method, (3) attached X-chromosome method, and (4) curly lobe plum method are in common use for detection of mutations in Drosophila.

A brief description of each method is presented below:

i. CIB Method:

This method was developed by Muller for detection of induced sex linked recessive lethal mutations in Drosophila male. In this technique, C represents a paracentric inversion in large part of X-chromosome which suppresses crossing over in the inverted portion. The I is a recessive lethal. Females with lethal gene can survive only in heterozygous condition.The B stands for bar eye which acts as a marker and helps in identification of flies. The I and B are inherited together because C does not allow crossing over to occur between them. The males with CIB chromosome do not survive because of lethal effect.

The important steps of this method are as follows:

(a) A cross is made between CIB female and mutagen treated male. In F_1 half of the males having normal X-chromosome will survive and those carrying CIB chromosome will die. Among the females, half have CIB chromosome and half normal chromosome (Fig. 14.2). From F_1, females with CIB chromosome and male with normal chromosome are selected for further crossing.

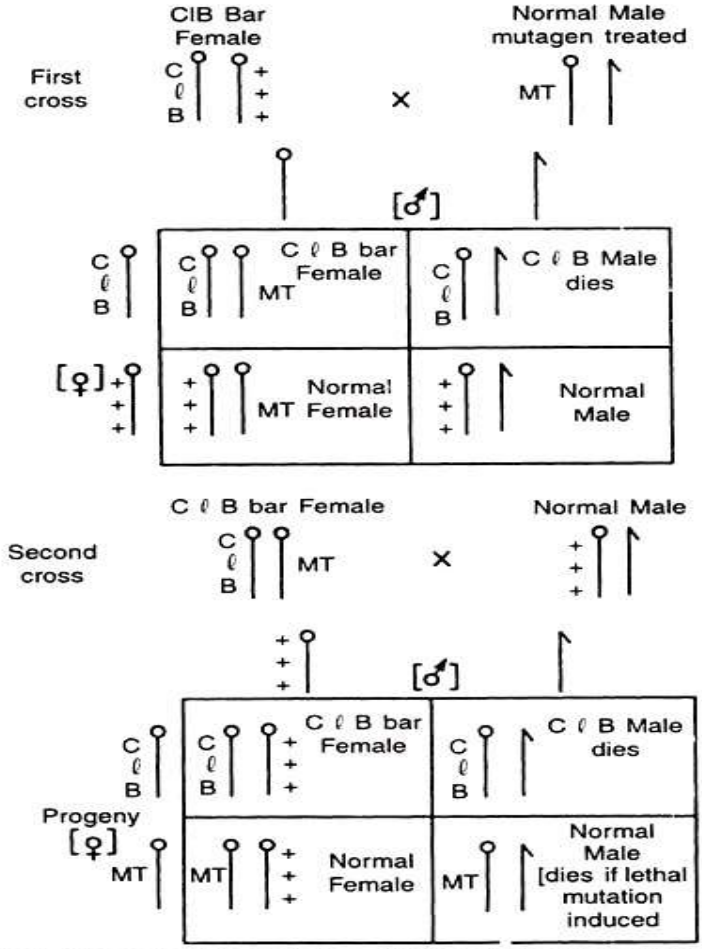


Fig. 14.2. Muller's CIB method for detection of lethal induced mutations in X-chromosome of **Drosophila**. Mt denotes mutagen treated X-chromsome.

(b) Now a cross is made between CIB female and normal male. This time the CIB female has one CIB chromosome and one mutagen treated chromosome received from the male in earlier cross.

This will produce two types of females, viz., half with CIB chromosome and half with mutagen treated chromosome (with normal phenotype). Both the progeny will survive. In case of males, half with CIB will die and other half have mutagen treated chromosome.

If a lethal mutation was induced in mutagen treated X-chromosome, the remaining half males will also die, resulting in absence of male progeny in the above cross. Absence of male progeny in F_2 confirms the induction of sex linked recessive lethal mutation in the mutagen treated Drosophila male.

ii. Muller's Method:

This method was also developed by Muller to detect sex linked mutation in Drosophila. This method is an improved version of CIB method. This method differs from CIB method in two important aspects. First, this method utilizes apricot recessive gene in place of recessive lethal in CIB method. Second, the female is homozygous for bar apricot genes, whereas it is heterozygous for IB genes in CIB method.In this method, the mutation is detected by the absence of wild males in F_2 progeny. This method consists of following important steps (Fig. 14.3).

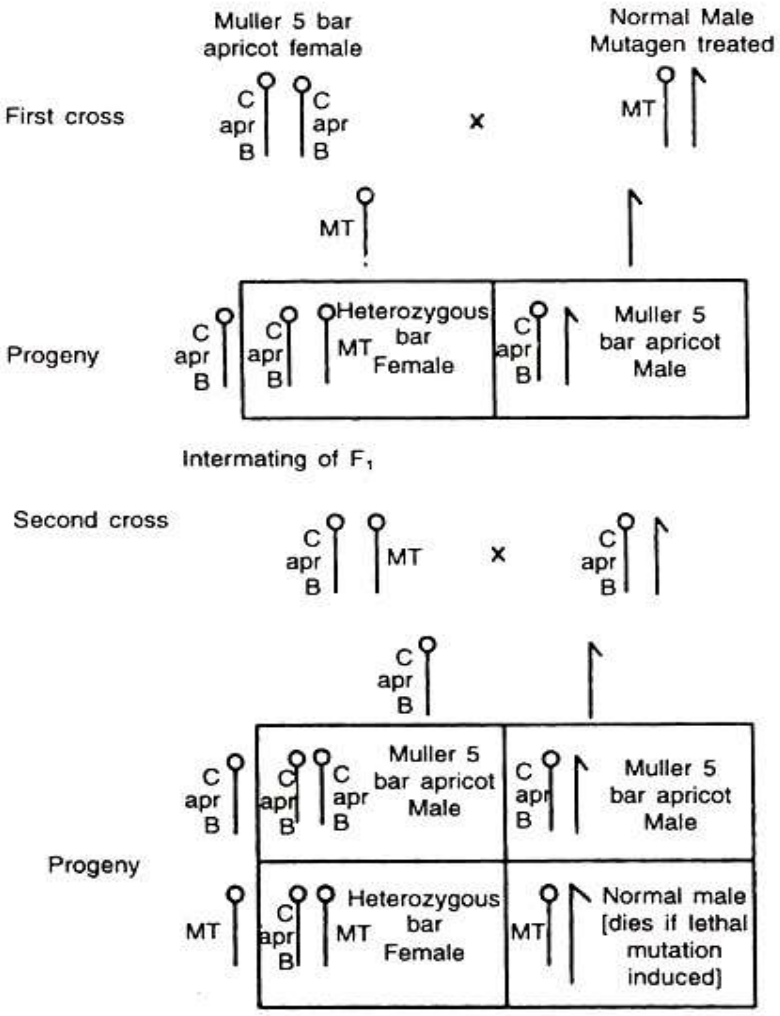


Fig. 14.3. Muller's 5 method of for detection of induced mutation in X-chromosome of Drosophila. Mt denotes mutagen treated X-chromosome.

a. A homozygous bar apricot female is crossed with mutagen treated male. In F_1 we get two types of progeny, viz., heterozygous bar females and bar apricot (Muller) males.

b. These F_1 are inter-mated. This produces four types of individuals. Half of the females are homozygous bar apricot, and half are bar heterozygous. Among the males, half are bar apricot (Muller 5) and half should be normal. If a lethal mutation is induced, the normal male will be absent in the progeny.

iii. Attached X-Method:

This method is used to detect sex linked visible mutations in Drosophila. In this method a female in which two X-chromosomes are united or attached together is used to study the mutation (Fig. 14.4). Therefore, this method is known as attached X-method. The attached X females (XXY) are crossed to mutagen treated male. This cross gives rise to super females (XX-X), attached female (XXY), mutant male (XY) and YY.The YY individuals die and super female also usually dies. The surviving male has received X-chromosome from mutagen treated male and Y chromosome from attached X-female. Since Y chromosome does not have corresponding allele of X-chromosome, even recessive mutation will express in such male which can be easily detected.

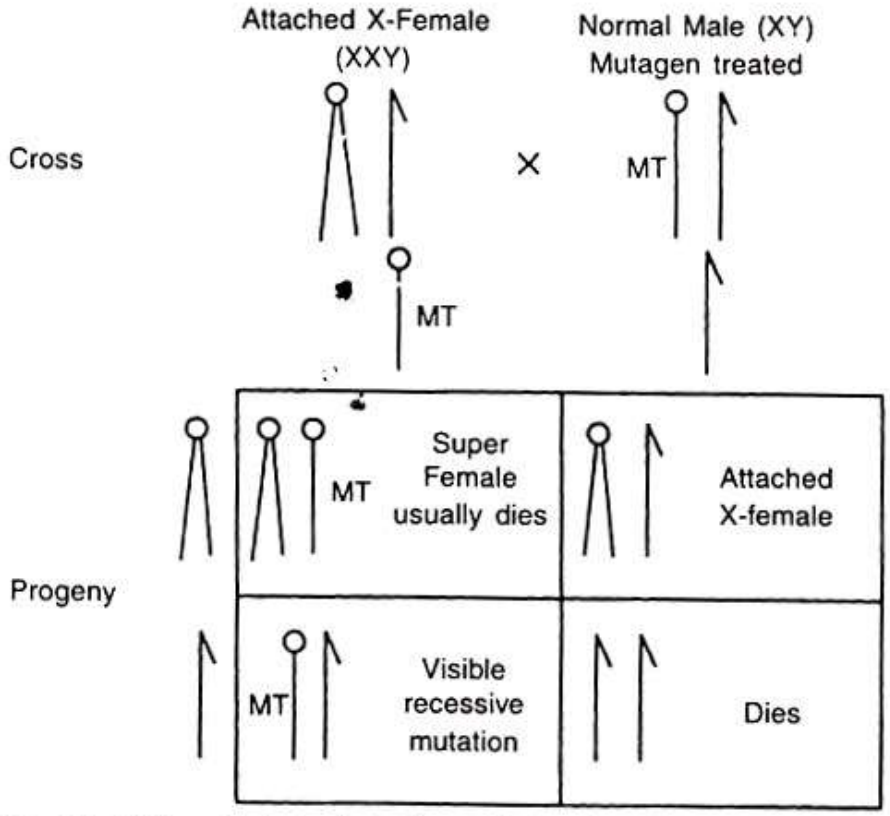


Fig. 14.4. Attach X-method for detection of induced visible mutation in chromosome of **Drosophila.** Mt. denotes mutagen treated X-chromosome.

iv. Curly Lobe-Plum Method:

This method is used for detection of mutation in autosomes. In this method curly refers to curly wings, lobe to lobed eye and plum to plum or brownish eye. All these three genes are recessive lethal. Curly (CY) and lobed (L) genes are located in one chromosome and plum (Pm) in another but homologous chromosome.Crossing over between these chromosomes cannot occur due to presence of inversion. Moreover, homozygous individuals for CYL or Pm cannot survive because of lethal effect. Only heterozygotes survive. Thus, this system is also known as balanced lethal system. This method consists of following steps (Fig. 14.5).

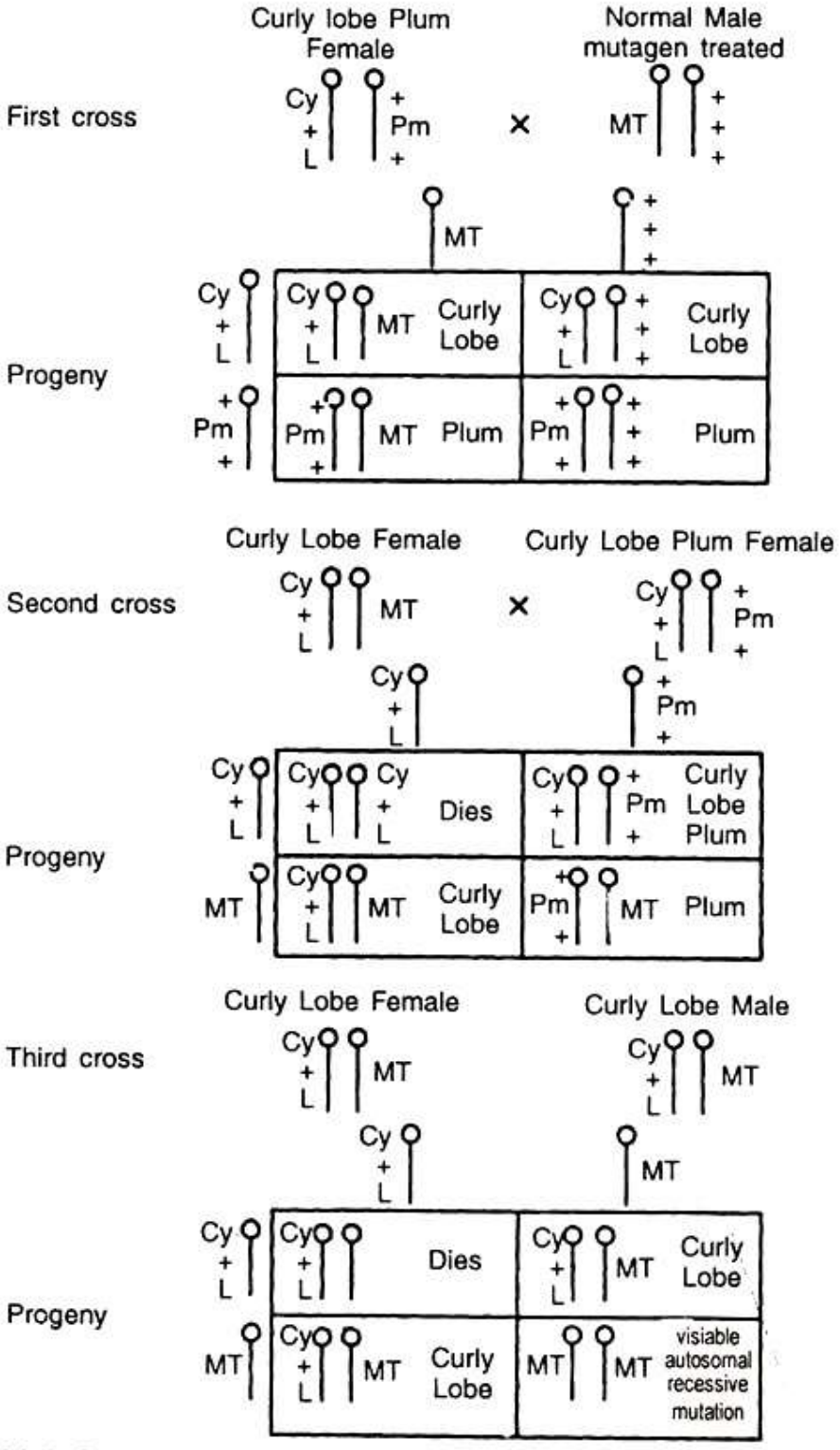


Fig. 14.5. Detection of induced autosomal recessive mutation in **Drosophila.**

a. A cross is made between curly lobe plum (CYL/Pm) female and mutagen treated male. This produces 50% progeny as curly lobe and 50% as plum.

TABLE 14.4. Comparison of Different Methods of Detection of Mutation in *Drosophila*

CIB Method	*Muller 5 Method*	*Attachad X Method*	*Curly lobe plum Method*
1. Used to detect sex linked lethal mutations.	Used to detect sex linked recessive lethal mutations.	Used to detect sex linked visible mutations.	Used to detect visible autosomes mutations.
2. Utilises heterozygous CIB females.	Uses homozygous bar apricot females.	Uses attached X female.	Used heterozygous curly lobe plum female.
3. CIB males die due to lethal effect.	No lethal effect in the parents.	YY individuals die to due to lethal effect.	Homozygous individuals for CYL/PM gene die.
4. Crossing over does not occur.	Crossing over does not occur.	Crossing over does not occur.	Crossing over does not occur.
5. Involves two crosses	Involves two crosses.	Involves one cross only.	Involves three crosses.
6. Mutation is detected by the absence of males in the progeny of second cross.	Mutation is detected by the absence of normal males in the progeny of second cross.	Males with visible mutations are found.	Individuals with treated autosomes show visible mutations.

b. In the second generation cross is made between curly lobe female and curly lobe plum male. This will give rise to curly lobe plum, curly lobe and plum individuals in 1 : 1 : 1 ratio and homozygous curly will die due to lethal effect. From this progeny, curly lobe females and males are selected for further mating.

c. In third generation, a cross is made between curly lobe female carrying one mutagen treated autosome and curly lobe male also carrying treated autosome. This results in production of 50% progeny as curly lobe, 25% homozygous curly lobe which die and 25% progeny homozygous for treated autosomes.

This will express as autosomal recessive mutation and constitute one third of the surviving progeny. A comparison of different methods of detection of mutation in Drosophila is given in Table 14.4.

Nutritional Deficiency Method of Mutations:

This method of detection of induced mutations is used in micro-organisms like Neurospora. The normal strain is treated with a mutagen and then cultured on minimal medium. A minimal medium contains sugar, salt, inorganic acids, nitrogen and vitamin biotin. The normal strain of Neurospora grows well on the minimal medium, but a biochemical mutant fails to grow on such medium.

This confirms induction of mutation. Then minimal medium is supplemented with certain vitamins or amino acids, one by one and the growth is observed. The medium which results in normal growth of mutagen treated mould indicates that the mutant lacks synthesis of that particular vitamin or amino acid, addition of which to the minimal culture medium has resulted in normal growth of treated strain.

Spontaneous Mutations:

Naturally occurring mutations are known as spontaneous mutations. Such mutations are induced by chemical mutagens or radiations which are present in the external environment to which an organism is exposed. Temperature also affects the frequency of spontaneous mutations. A rise of 10°C in the temperature leads to fivefold increase in mutation rate in an organism exposed to such variation in temperature.

Drastic change of temperature in any direction produces still greater effect on mutation frequency. External environmental conditions of any type, i.e., either extremely high or low leads to increase in the mutation frequency.

Internal environment of an organism also plays an important role in the induction of spontaneous mutations. For example, spontaneous rearrangements of DNA bases result in base pair transitions. Similarly, errors in DNA repair or replication can cause spontaneous mutations.

Selection of Different Types of Mutants

1. Resistant Mutants:

From a wild-type bacterial population which is susceptible to agents like bacteriophage, various drugs, ultra-violet light etc., mutants which are resistant to any of these agents can be selected directly by using selective media. After induction of mutation with a suitable mutagen, the treated population is allowed to grow under permissive conditions for some time to allow the expression of the mutant gene.

The culture is then dilution-plated in a suitable selective medium which would be expected to completely suppress the growth of the wild type bacteria and would allow only the mutants to grow and form colonies. The mutant colonies can be picked up and purified by dilution plating to obtain pure clones of mutants.

For example, for isolation of resistant mutants against a specific lytic bacteriophage, the selective medium would be one containing the particular phage, so that the wild-type which is susceptible would be eliminated by the lytic phage and only the phage-resistant mutants would form colonies.

Purification is necessary to eliminate any wild-type cells that might be present associated with the resistant cells. Similar procedure can be adopted for selection of drug-resistant mutants, in which case the selective medium would be a growth-supporting one containing the drug at a concentration which is normally inhibitory for the wild-type cells.

Similar procedure can also be used for isolation of several other types of mutants. For example, mutations resulting in reversion of auxotroph's to prototroph can be detected. If it is desired to obtain a prototroph (his^+) from a population of histidine auxotroph (his^-), a mutagen-treated population of the auxotroph may be plated on a medium without histidine. Such a medium will allow growth of the prototroph only.Another type of mutants that can be selected by a similar procedure is those having a wider substrate range in comparison to that of the wild type. This means that a mutant is capable of utilizing a particular substrate which the wild-type is unable to use. The mutagen treated population of the wild- type may be initially grown in a liquid medium containing that substrate.

As this medium would allow only the mutants to grow, an increase in the number of mutants would be expected (enrichment). Plating on the same selective medium would allow the growth of mutants which are capable of utilizing the substrate, while the wild-type cells will fail to grow, because of their inability to utilize the substrate.

2. Regulation-Defective Mutants:

The metabolic pathways are controlled by regulatory mechanisms. One of the important mechanisms is feed-back control of biosynthetic pathways in which the end-product inhibits the first enzyme of the sequence. The first enzyme in such case is an allosteric protein which can bind both the substrate and the end-product at different sites of the molecule.

Mutation may result in the formation of a defective enzyme protein with loss of regulatory function. Such regulation-defective mutants may arise spontaneously, or may be induced by treatment with mutagens. A well-known example is a naturally occurring defective mutant of Corynebacterium glutamicum which produces large quantity of glutamic acid and has been used for commercial production of this amino acid.

Regulation-defective mutants of bacteria can be detected by using specific antimetabolites in selection medium. An antimetabolite is generally a non-biological chemical compound which is a structural analogue of a metabolite. They are able to inhibit growth by stopping the synthesis of the metabolite by reacting with the first enzyme of the biosynthetic pathway of the particular metabolite. This property of an antimetabolite can be made use of in detection of defective mutants which have the ability to escape the regulatory mechanism.For detection of such regulation defective mutants, a mutagen-treated bacterial suspension containing 10^8 to 10^{10} cells/ml is spread over a medium containing an inhibitory concentration of the antimetabolite (specific for the metabolite). After incubation, only a few colonies develop in the plates, because most of the bacteria fail to grow in presence of the antimetabolite. The colonies develop only from the small number of antimetabolite-resistant mutants.However, all such resistant mutants may not be regulation-defective, because resistance may develop also by other means. The regulation-defective mutants can be identified in the plates by the presence of a halo of satellite colonies around the larger colonies of the mutants.The satellite colonies develop because the mutant colony secretes the metabolite into the medium which feeds the wild-type bacteria to make them grow. The satellite colonies are smaller in size, because they start developing later when the mutants secrete enough metabolite to overcome the effect of the antimetabolite (Fig. 9.67).

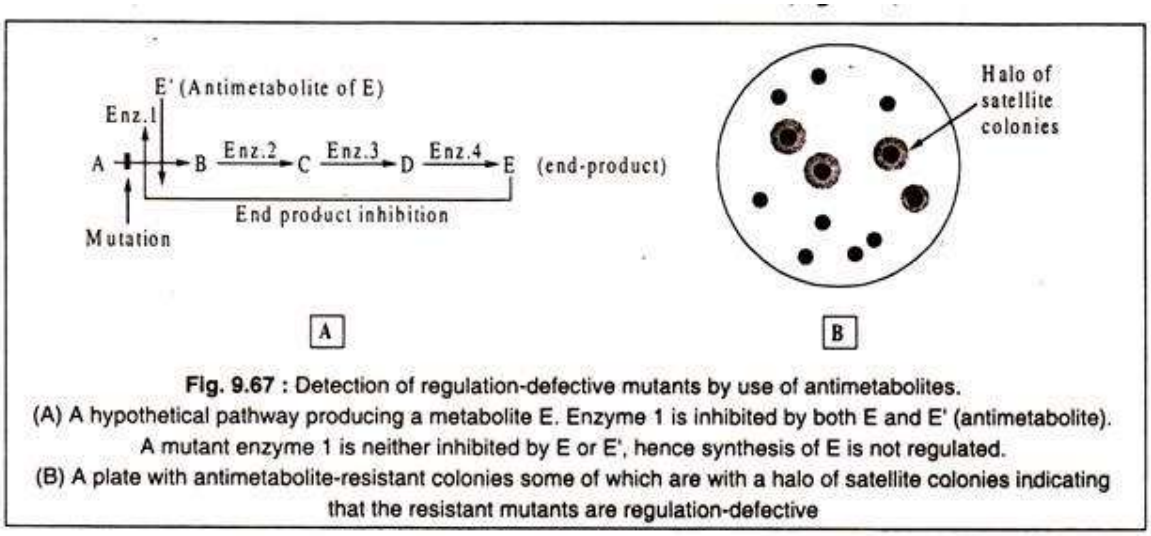


Fig. 9.67 : Detection of regulation-defective mutants by use of antimetabolites.
(A) A hypothetical pathway producing a metabolite E. Enzyme 1 is inhibited by both E and E' (antimetabolite). A mutant enzyme 1 is neither inhibited by E or E', hence synthesis of E is not regulated.
(B) A plate with antimetabolite-resistant colonies some of which are with a halo of satellite colonies indicating that the resistant mutants are regulation-defective

3. Auxotrophic Mutants:

An auxotrophic mutation differs from others in producing a loss of ability to synthesise an essential metabolite. It may be one of the protein amino acids, a nucleic acid base, a vitamin etc. As a result, the mutant loses ability to grow in a minimal medium which sustains growth of the wild-type, and it obligately requires supplementation of the metabolite which it no longer can synthesise.

Obviously, a straight-forward detection of auxotroph's is not possible in a selective medium. A laborious method would be to grow the mutagen-treated population in a medium which allows growth of both the wild-type and auxotroph's (complete medium) and then test each and every colony for its ability to grow in a complete medium and in a minimal medium plate.

Considering an average mutation rate of 10^{-6} to 10^{-7}, it can be realized that one million to 10 million colonies must be tested to find out a single auxotroph. However, treatment with mutagens can considerably increase the mutation rate. Even then, detection of auxotroph's remains a laborious task.

Two techniques which are used in conjunction to minimize this labour are the enrichment of the mutants in the total population by treatment with penicillin and the replica plating:

(a) Penicillin Enrichment Technique:

This method makes use of the bactericidal property of penicillin which is restricted to the actively growing organisms. The antibiotic cannot destroy the non-growing or resting bacteria. Thus, under conditions which support growth of the wild-type bacteria only, penicillin kills them preferentially and the mutants which do not grow survive.

As a result, the proportion of mutants in the total population increases substantially making their detection and isolation easier. For this purpose, the mutagen-treated population is grown in a minimal medium containing a lethal dose of penicillin for a period during which the wild-type bacteria grow and are killed by the antibiotic.The surviving bacteria having a higher proportion of mutants are centrifuged and washed to remove penicillin. They are suspended and plated on complete medium for detection of mutants by the replica plating technique. Penicillin treatment may have to be repeated to obtain the desired enrichment of the mutants.

(b) Replica-Plating Technique:

It is a simple but a very efficient method for detecting auxotrophic mutants. The technique developed by Lederberg makes it possible to test a reasonably large number of discrete colonies growing in a plate for the occurrence of mutants. Generally, 100 to 200 colonies growing in a single plate can be tested at a time, so that the labour and tediousness of examining each and every colony individually for detection of mutants are greatly minimized.The basic outfit (Fig. 9.68) consists of a wooden or metallic cylindrical block with a diameter slightly less than that of a standard Petri dish, a metallic collar and several pieces of velveteen cloth. The bristles of the cloth act as individual inoculating needles. The components are sterilized before use. The velveteen is spread tightly over the block and kept in place by the collar.

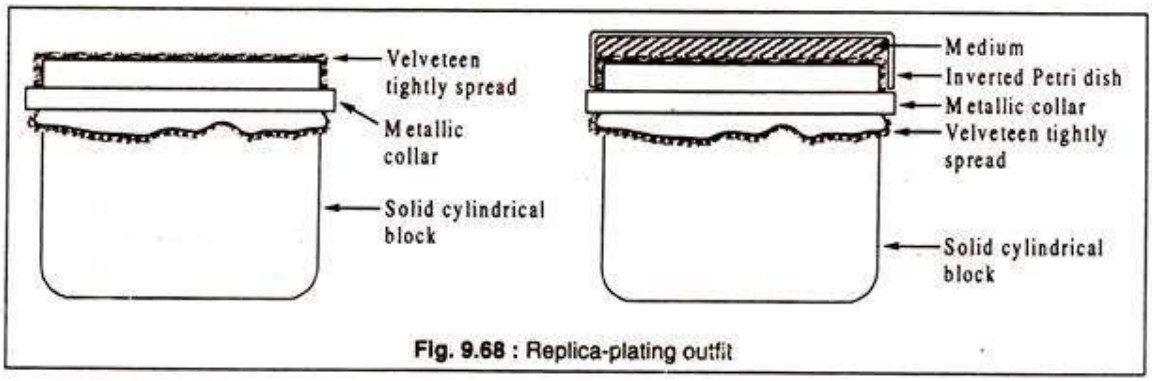


Fig. 9.68 : Replica-plating outfit

The master plate containing complete medium with 100-200 discrete colonies among which the majority colonies are wild type and few mutants is inverted over the velveteen covered cylindrical block and lightly pressed to transfer the impression of the colonies on to the bristles of velveteen.

After removing the master plate, a blank minimal agar plate is similarly placed on the velveteen surface to transfer the impression of the master plate to it. On incubation, colonies of only the wild-type grow on the minimal agar surface.When the two plates, i.e. the master plate and the minimal agar plate, are examined, it is observed that some colonies of the master plate are absent in the minimal agar plate indicating that they are probable mutants — because they grow in complete medium but fail to grow in the minimal agar.

These colonies are picked up from the master plate and purified by dilution plates Individual colonies are picked up from the dilution plates and tested for their growth requirement. Detection of the mutants by comparison of the master plate and the minimal agar plates can be facilitated by transfer of the colony images of the master plate on a photographic film. By correct positioning of the minimal agar plate against the negative and by illumination from bottom, it becomes easier to locate the mutant colonies which are absent in the plate.

A schematic representation of the master plate and a replica plate of minimal agar is shown in Fig. 9.69:

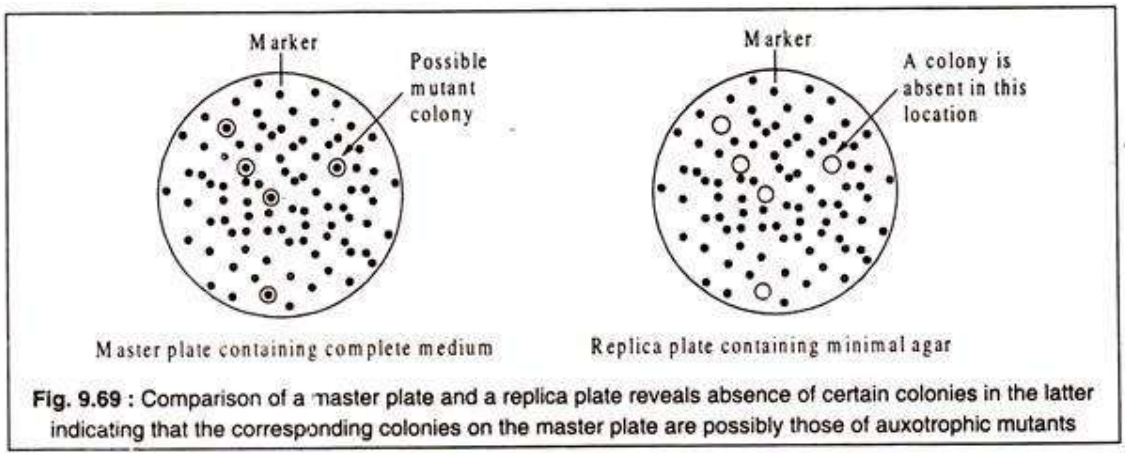


Fig. 9.69 : Comparison of a master plate and a replica plate reveals absence of certain colonies in the latter indicating that the corresponding colonies on the master plate are possibly those of auxotrophic mutants

Resistance Selection Method:

It is the other approach for isolation of mutants. Generally the wild type cells are not resistant either to antibiotics or bacteriophages. Therefore, it is possible to grow the bacterium in the presence of the agent (antibiotics or bacteriophage and look for survivors. This method is applied for isolation of mutants resistant to any chemical compounds that can be amended in agar, phage resistant mutants.

Substrate Utilization Method:

This method is employed in the selection of bacteria. Several bacteria utilize only a few primary carbon sources. The cultures are plated onto medium containing an alternate carbon sources. Any colony that grows on medium can use the substrate and are possibly mutants. These can be isolated.

Sugar utilization mutants are also isolated by means of colour indicator plates. A popular medium (EMB agar) is used for this purpose. The EMB agar contains two dyes eosin and methylene blue in the medium. Colour of these dyes is sensitive to pH. This medium also contains lactose sugar as carbon source and complete mixture of amino acids.

Therefore, both lactose wild type (Lac^+) and lactose mutant (Lac^-) cells can grow and form colonies on EMB agar plates. The Lac^+ cells catabolize lactose and secrete acids, therefore, local pH of the medium decreases. This results in staining of colony to dark purple.
On the other hand, Lac^- cells are unable to utilize lactose and use some of the amino acids as carbon source. After utilization of amino acid, possibly ammonia is produced that increases the local pH and decolorizes the dye resulting in white colony.

Carcinogenicity Test:

An understanding has developed to identify the environmental carcionogens that cause mutation and induce cancer in organisms. This method is based on detecting potential of carcinogens and testing for mutagenicity in bacteria.

Ames (1973) developed a method for deletion of mutagenicity of carcinogens which is commonly known as Ames test. It is widely used to detect the carcinogens. The Ames test is a mutational reversion assay in which several special strains of Salmonella typhimurium are employed. Each strain contains a different mutation in the operon of histidine biosynthesis.

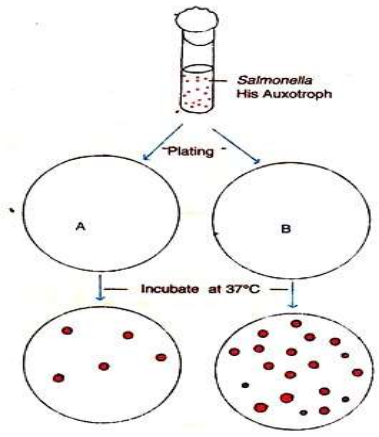


Fig. 9.16 : The Ames test for mutagenicity; A, complete medium containing a large amount of histidine; B, medium containing test mutagen and a small amount of histidine.

The Ames test follows the following steps

(i) Prepare the culture of Salmonella histidine auxotrophs (His^{-}).

(ii) Mix the bacterial cells and test substance (mutagen) in dilute molten top agar with a small amount of histidine in one set, and control with complete medium plus large amount of histidine.

(iii) Pour the molten mix on the top of minimal agar plates and incubate at 37°C for 2-3 days. Until histidine is depleted all the His^{-} cells will grow in the presence of test mutagens. When histidine is completely exhausted only the reverants (the mutants which have regained the original wild type characters) will grow on agar plate.The number of spontaneous reverants is low, whereas the number of reverants induced by the test mutagen is quite high. In order to estimate the relative mutagenicity of the mutagenic substance the visible colonies are counted and compared with control. The high number of colonies represents the greater mutagenicity.A mammalian liver extract is added to the above molten top agar before plating. The extract converts the carcinogens into electrophilic derivatives which will soon react with DNA molecule.
In natural way this process occurs in mammalian system when foreign substances are metabolized in the liver. Bacteria do not possess the metabolizing capacity as liver does; therefore, the liver extract is added to this test, just to promote the transformation.Therefore, several potential carcinogens that generally are not carcinogenic until modified in liver, for example aflatoxins viz., B_1, B_2, G_1, G_2, etc. The Ames test has now been used with thousands of substances and mixtures such as the industrial chemicals, food additives, pesticides, hair dyes and cosmetics.

Probable Questions:

1. Define mutation. Sate the major characteristics of mutation.
2. Classify mutations.
3. Describe four main classes of identifiable mutants with examples.
4. Define mutagens. State about different types of physical mutagens.
5. State about different types of chemical mutagens.
6. Describe CIB method for detection of mutation.
7. Describe Muller's method for detection of mutation.
8. Describe attached X method for detection of mutation.
9. Describe Curly Lobe-Plum Method for detection of mutation.
10. What is auxotrophic mutation?
11. Describe Ames Test for detection of carcinogens.
12. Describe replica plating technique for detection of mutant.

Suggested readings:

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7th Edition.
6. Gene cloning and DNA Analysis by T. A. Brown, Sixth Edition.
7. Genetics . Verma and Agarwal.

Unit-IV

DNA repair and retrieval; repair of spontaneous and induced mutations; mechanism of DNA repair; repair by direct reversion; excision repair; SOS response

Objective : In this unit you will know about DNA repair system. Repair of spontaneous and induced mutations, mechanism of DNA repair and repair by direct reversion.; You will also learn about excision repair and SOS response

Introduction:

DNA is a highly stable and versatile molecule. Though sometimes the damage is caused to it, it is able to maintain the integrity of information contained in it. The perpetuation of genetic material from generation to generation depends upon keeping the rates of mutation at low level. DNA has many elaborate mechanisms to repair any damage or distortion.

The most frequent sources of damage to DNA are the inaccuracy in DNA replication and chemical changes in DNA. Malfunction of the process of replication can lead to incorporation of wrong bases, which are mismatched with the complementary strand. The damage causing chemicals break the backbone of the strand and chemically alter the bases. Alkylation, oxidation and methylation cause damage to bases. X-rays and gamma radiations cause single or double stranded breaks in DNA.A change in the sequence of bases if replicated and passed on to the next generation becomes permanent and leads to mutation. At the same time mutations are also necessary which provide raw material for evolution. Without evolution, the new species, even human beings would not have arisen. Therefore a balance between mutation and repair is necessary.

Definition of DNA Repair:

One of the main objectives of biological system is to maintain base sequences of DNA from one generation to the other. Changes in DNA sequence arise during replication of DNA damage by chemical mutagens and radiation. During replication if incorrect nucleotides have been added, they are corrected through editing system by DNA Pol I and DNA Pol III.

The other systems also exist for correcting the errors missed by editing function. It is called mismatch repair system. Mismatch repair system edits the errors left by DNA Pol I and DNA III and removes the wrong nucleotides. Proof reading by Pol I and III.

DNA is always damaged and mutated by several chemicals and radiation. Only a few errors accumulate in DNA sequence. The stable errors cause mutation and the rest are eliminated. If errors in DNA sequence are corrected before cell division, no mutation occurs. However, there are some DNA damages which cannot be mutated because the damages are not replicated. Therefore, such damages cause cell death.

Types of Damage:

Damage to DNA includes any deviation from the usual double helix structure.

1. Simple Mutations:
Simplest mutations are switching of one base for another base. In transition one pyrimidine is substituted by another pyrimidine and purine with another purine. Trans-version involves substitution of a pyramidine by a purine and purine by a pyrimidine such as T by G or A and A by C or T. Other simple mutations are detection, insertion of a single nucleotide or a small number of nucleotides. Mutations which change a single nucleotide are called point mutations.

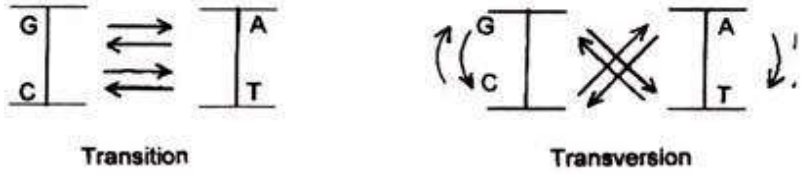


Fig. 5.1.

2. Deamination:
The common alteration of form or damage includes deamination of cytosine (C) to form uracil (u) which base pairs with adenine (A) in next replication instead of guanine (G) with which the original cytosine would have paired.As uracil is not present in DNA, adenine base pairs with thymine (T). Therefore C-G pair is replaced by T-A in next replication cycle. Similarly, hypoxanthine results from adenine deamination.

3. Missing Bases:
Cleavage of N-glycosidic bond between purine and sugar causes loss of purine base from DNA. This is called depurination. This apurinic site becomes non-coding lesion.

4. Chemical Modification of Bases:
Chemical modification of any of the four bases of DNA leads to modified bases. Methyl groups are added to various bases. Guanine forms 7- methylguanine, 3-methylguanine. Adenine forms 3-methyladenine. Cytosine forms 5- Methylcytosine.

Replacement of amino group by a keto group converts 5-methylcytosine to thymine.

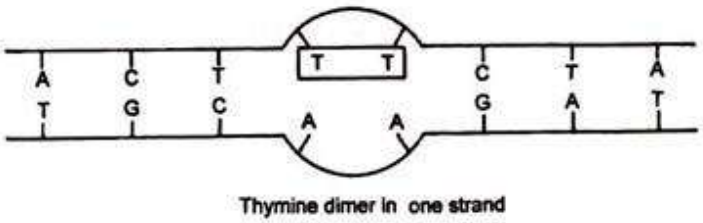


Fig. 5.2.

5. Formation of Pyrimidine Dimers (Thymine Dimers):
Formation of thymine dimers is very common in which a covalent bond (cyclobutyl ring) is formed between adjacent thymine bases. This leads to loss of base pairing with opposite stand. A bacteria may have thousands of dimers immediately after exposure to ultraviolet radiations.

6. Strand Breaks:
Sometimes phosphodiester bonds break in one strand of DNA helix. This is caused by various chemicals like peroxides, radiations and by enzymes like DNases. This leads to breaks in DNA backbone. Single strand breaks are more common than double strand breaks.

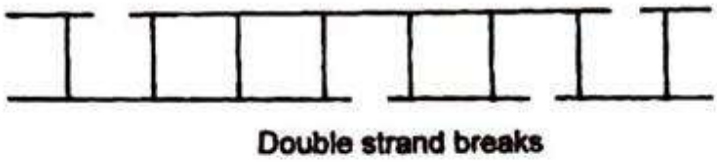


Fig. 5.3.

Sometimes X-rays, electronic beams and other radiations may cause phosphodiester bonds breaks in both strands which may not be directly opposite to each other. This leads to double strand breaks.

Some sites on DNA are more susceptible to damage. These are called hot-stops.

Repair Mechanisms:

A. Photo Reactivation:

We know that exposure of UV-irradiated bacteria immediately afterwards to visible light restores to a considerable degree the viability of the UV-inactivated bacteria. This phenomenon mown as photo reactivation, is based on enzymatic cleavage of the thymine dimers.The enzyme, photolyase, binds to the thymine dimer and catalyses photochemical cleavage of the cyclobutane ring of the dimer to make the thymine's free. The enzyme uses visible light for the reaction. Besides thymine-dimers, other pyrimidine-dimers—like cytosine-cytosine and cytosine-thymine dimer—are also attacked by the enzyme. The enzyme is devoid of any species-specificity. Photolyases have been detected in both prokaryotes and eukaryotes.

The action of photolyase on UV-irradiated DNA containing thymine dimers is schematically represented in Fig. 9.70:

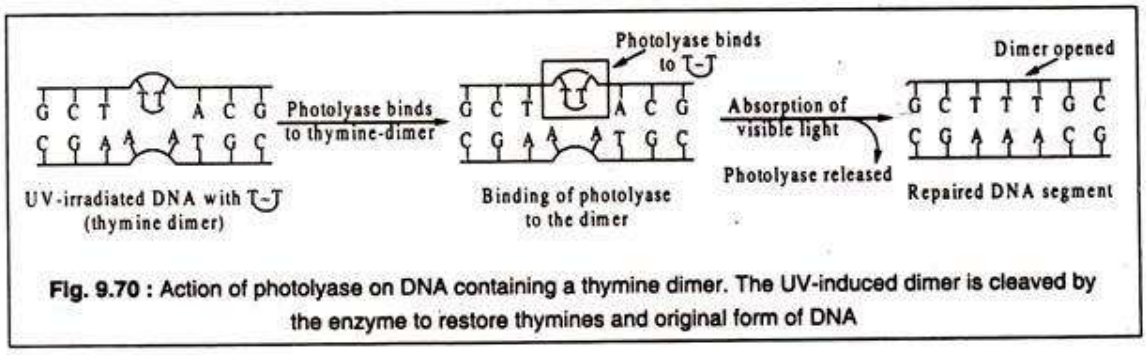


Fig. 9.70 : Action of photolyase on DNA containing a thymine dimer. The UV-induced dimer is cleaved by the enzyme to restore thymines and original form of DNA

It has been observed that UV-irradiated DNA containing 5-bromouracil — which is an analogue of thymine and is incorporated into replicating DNA replacing thymine — is resistant to photo reactivation. Such DNA binds the photolyase enzyme, but the enzyme neither dissociates from the dimer nor can liberate the free thymine molecules.

B. Excision Repair:

Apart from photo reactivation, there are also other mechanisms for repair of damaged DNA. One of these is excision repair which occurs in absence of light i.e. exposure to visible light is not required. It is also known as dark-repair.

The excision repair essentially consists of removal of a segment of DNA containing the damaged portion of one strand of DNA and new synthesis of the removed segment of the DNA strand using the undamaged strand as template. The first step, known as incision step, involves recognition of the damaged segment by an endonuclease.The enzyme cleaves the phosphodiester bond of the sugar- phosphate backbone at the 5′-end about eight nucleotides ahead of the damaged site producing a 3′-OH group free. The next step, known as excision step, involves a cut at a site 4 to 5 nucleotides downstream from the damaged site catalysed by a 5′-3′ exonuclease.

Thereby, a segment of DNA including the damaged site is removed. In the last step, known as the repair step, DNA polymerase I synthesizes a new stretch of DNA strand starting from the 3′-OH end using the intact complimentary strand as template. Finally, the newly synthesized strand is joined with the 5′-end by DNA ligase to complete the repair. The steps are diagrammatically shown in Fig. 9.71.Another variation of excision repair is catalysed by the enzyme glycosylase which cleaves the N-glycosidic bond between a thymine of the thymine dimer to the sugar-phosphate backbone of the DNA strand. At the next step, the phosphodiester bond is cleared by the endonuclease activity of the same enzyme which recognizes a blank deoxyribose without a base attached to it.

In the following step, DNA polymerase I initiates DNA synthesis at the free 3′-OH end displacing the thymine dimer along with a few more adjacent nucleotides as shown in Fig. 9.72. This type of excision repair occurs also in E. coli and several other bacteria like Micrococcus luteus.

Excision repair is observed when UV-treated bacteria are stored in dark for a few hours in a medium which does not support growth before returning them to the normal growth supporting medium (liquid holding recovery). In excision repair mechanism DNA polymerase I seems to play an important role.It has been shown that E. coli mutants which are deficient in this enzyme show extensive DNA damage following UV-irradiation, presumably because such mutants are unable to repair the damaged DNA by excision repair mechanism. It may be reminded that in normal DNA replication, polymerase III (pol III) catalyses DNA synthesis.

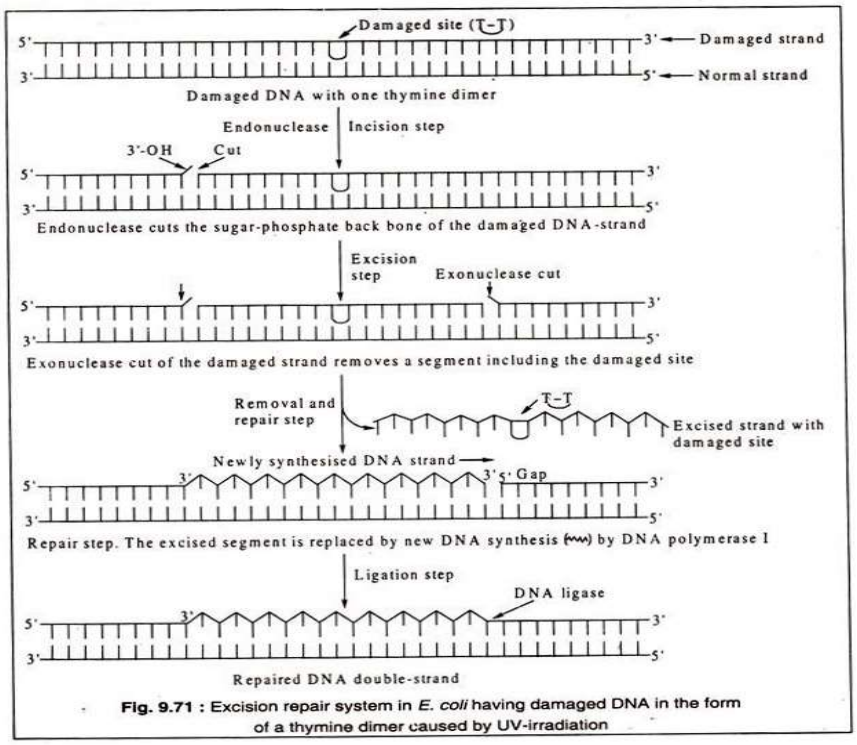


Fig. 9.71 : Excision repair system in *E. coli* having damaged DNA in the form of a thymine dimer caused by UV-irradiation

Excision repair are mainly two types:

(a) Base excision repair:

The lesions containing non-helix distortion (e.g. alkylating bases) are repaired by base excision repair. It involves at least six enzymes called DNA glycosylases.

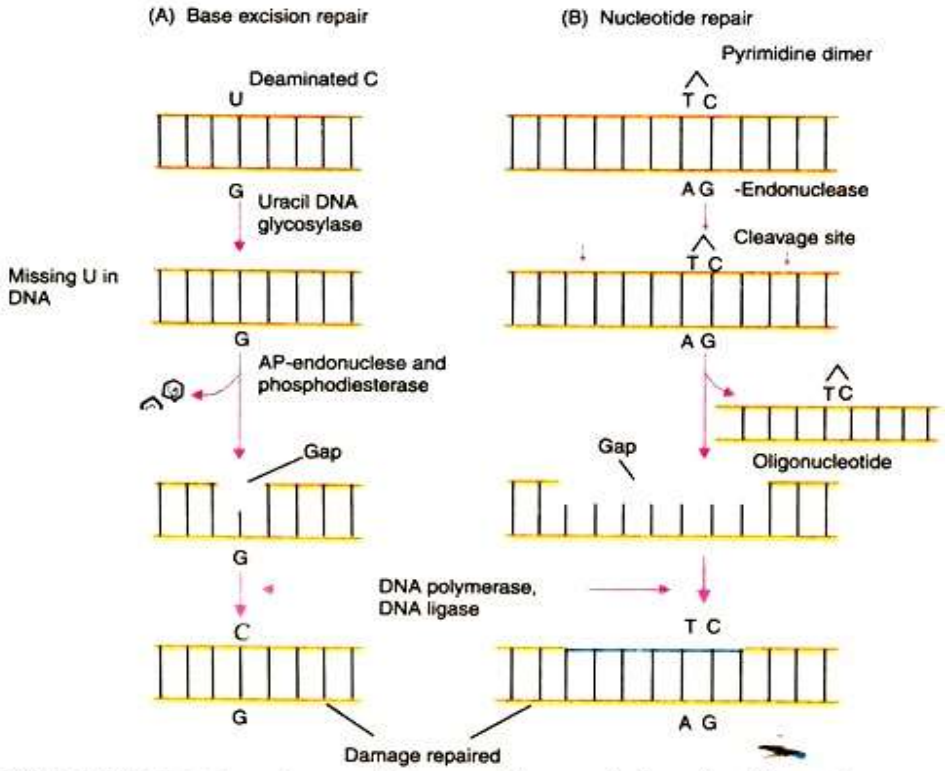


Fig. 9.18 : Excision repair pathways; A, base excision repair, B, nucleotide excision repair.

Each enzyme recognises at least bases and removes from DNA strand. The enzymes remove deaminated cytosine, deaminated adenine, alkylated or oxidised base. Base excision repair pathway starts with a DNA glycosylation. For example, the enzyme uracil DNA glycosylase removes the uracil that has wrongly joined with G which is really deaminated cytosine (Fig. 9.18A).

Then AP- endonuclease (apurinic or apyriminic site) and phosphodiesterase removes sugar-phosphate. AP-sites arise as a result of loss of a purine or a pyrimidine. A gap of single nucleotide develops on DNA which acts as template-primer for DNA polymerase to synthesise DNA and fill the gap by DNA ligase.

(b) Nucleotide excision repair:

Any type of damage having a large change in DNA helix causing helical changes in DNA structure is repaired by this pathway. Such damage may arise due to pyrimidine dimers (T-T, T-C and C-C) caused by sun light and covalently joins large hydrocarbon (e.g. the carcinogen benzopyrene).

In E. coli a repair endonuclease recognises the distortion produced by T-T dimer and makes two cuts in the sugar phosphate backbone on each side of the damage. The enzyme DNA helicases removes oligonucleotide from the double helix containing damage. DNA polymerase III and DNA ligase repair the gap produced in DNA helix (Fig. 9.18B).

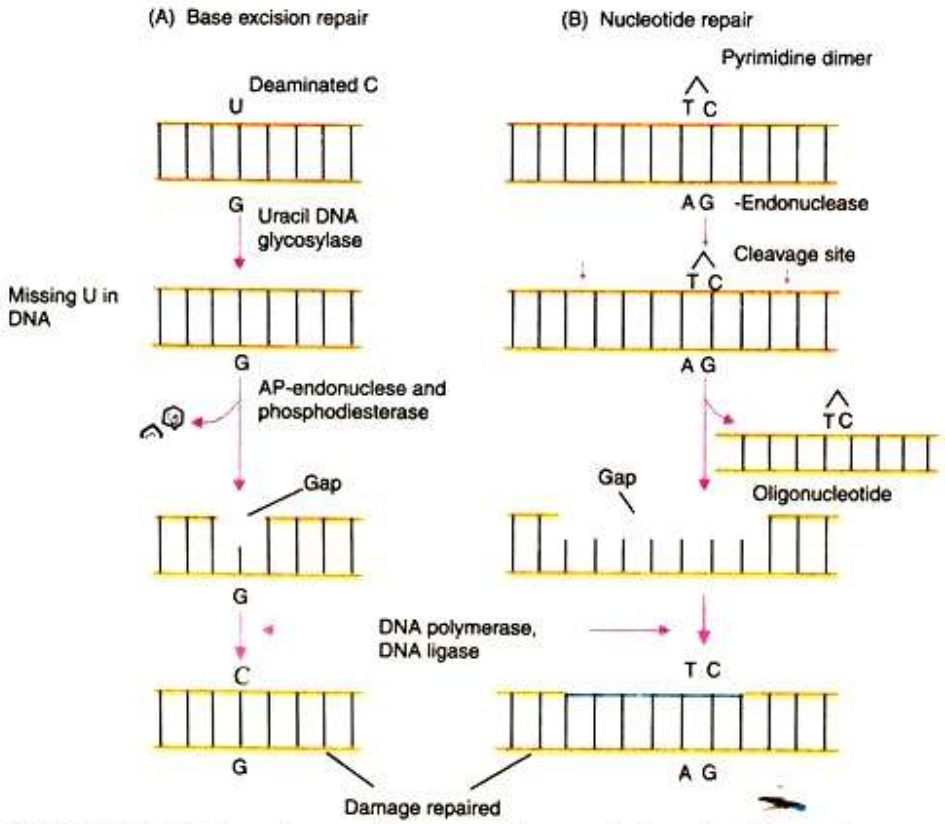


Fig. 9.18 : Excision repair pathways; A, base excision repair, B, nucleotide excision repair.

C. Re-Combinational Repair Mechanism:

This is another mode of repair of damaged DNA. It consists essentially of an exchange of a damaged segment of one DNA molecule by an undamaged segment of another one. As such exchange takes place only after replication of the damaged DNA has taken place it is also known as post- replication repair.

When a damaged DNA molecule — e.g. DNA containing thymine-dimers induced by UV-irradiation— begins replication with the help of DNA polymerase III, the enzyme stops synthesis as it reaches a dimer, because of the distortion caused by the dimer in the regular double helix.

As a result, the progress of the replication fork halts temporarily as it reaches a dimer. DNA synthesis is then reinitiated at a new site, few nucleotides past the thymine-dimer site. Thus, a gap is created opposite the dimer site and a few adjoining nucleotides. The newly synthesized daughter strand is produced with several gaps i.e. in short pieces, if several dimers occur in the same template strand (Fig. 9.73).

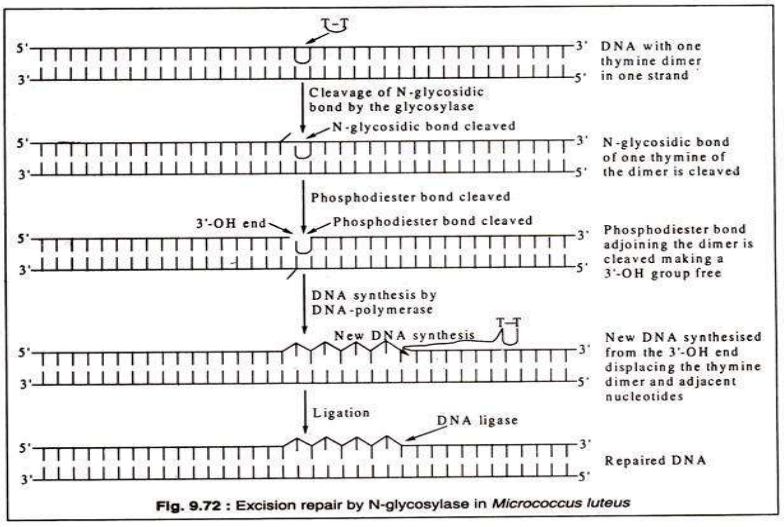


Fig. 9.72 : Excision repair by N-glycosylase in *Micrococcus luteus*

The gaps in the daughter strand are then filled up by exchanging undamaged homologous segments from a sister DNA double helix. The gaps produced in the donor strand are then filled up by new DNA synthesis with the help of DNA polymerase I and sealed by the DNA ligase as shown in Fig. 9.74. Thus, in re-combinational repair system, parts of DNA strand missing in one strand are retrieved from another normal DNA strand of a sister double helix.

The damaged DNA strand will continue to have the damaged site and will replicate to have gaps in the daughter strand which will be repaired by re-combinational repair mechanism. Ultimately, the damaged strand will be outnumbered by normal DNA and will be insignificant.In re-combinational repair mechanism, recA gene plays an important role. It has been observed that recA mutants are extremely sensitive to lethal effects of radiations and chemical mutagens. The recA gene is known to play a very important role in genetic recombination e.g. in conjugation where recA' recipient fails to show genetic recombination. The gene functions also in recombinational repair, where exchange of DNA strands is involved.

D. Repair of DNA by Homologous Recombination:

This type of repair is called for when both strands of DNA molecules are damaged at sites opposite each other. In such a case, the missing segments cannot be replaced from sister strands after replication by the usual recombinational repair mechanism. The lost portions have to be retrieved from another homologous DNA molecule.

In actively growing bacteria, each cell contains generally more than one copy of DNA. So, the lost portions of one damaged molecule can be repaired by crossing-over with a normal DNA molecule involving exchange of homologous segments. This type of DNA repair is known as homologous recombinational repair. Double-stranded breaks of DNA can be induced by exposure to X-rays. Rec A protein plays an important role also in this type of repair.

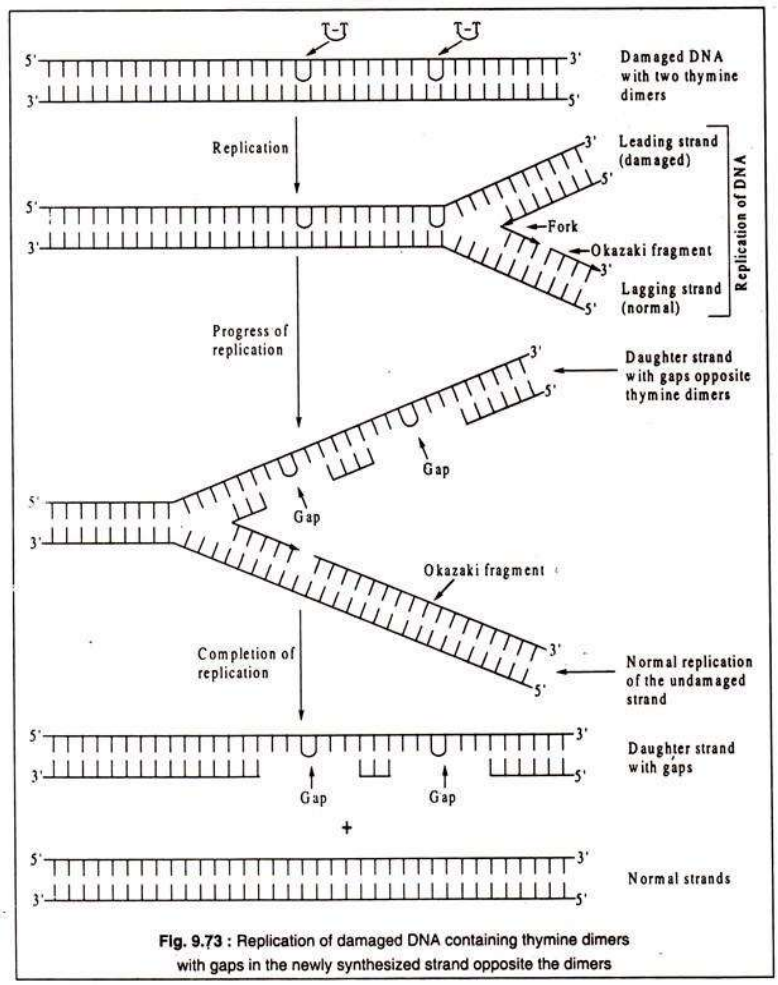


Fig. 9.73 : Replication of damaged DNA containing thymine dimers with gaps in the newly synthesized strand opposite the dimers

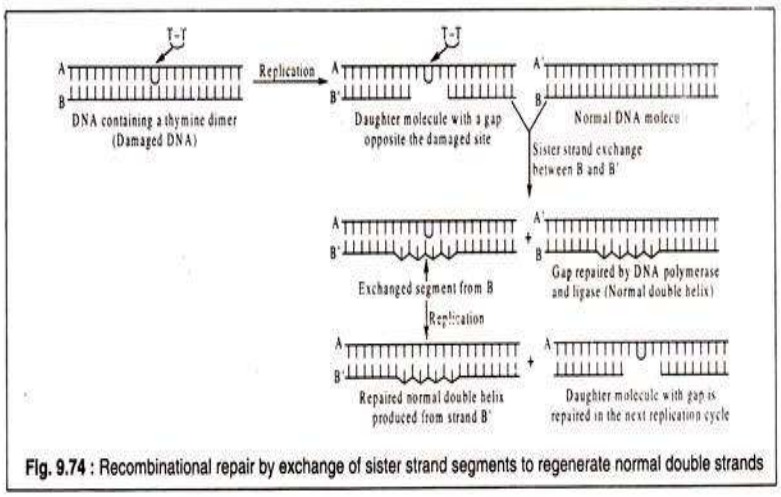


Fig. 9.74 : Recombinational repair by exchange of sister strand segments to regenerate normal double strands

The repair process begins with the production of single-stranded segments at the 3′-OH end of each strand through the action of a nuclease and binding of the Rec A protein to the single strands. The binding of Rec A protein initiates strand exchange between homologous segments of the normal DNA duplex and the

damaged one. The crossing-over results in the formation of two DNA molecules, each having an intact strand and a strand with gaps.

The gaps are then filled up by new DNA synthesis catalysed by DNA polymerase and sealing by DNA ligase. The intact strand is used as template. Thus, crossing-over which is normally a mechanism for creating genetic diversity by mixing up genes located on homologous chromosomes, can also function as a means for repairing damaged DNA. Rec A protein plays vital roles in both the processes (Fig. 9.75).

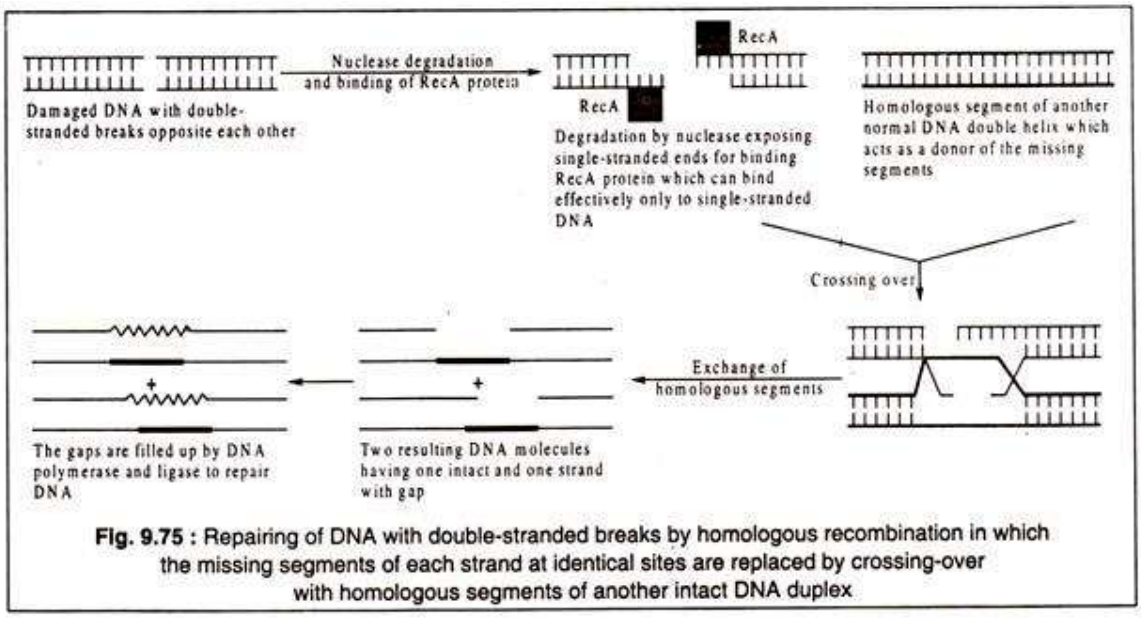


Fig. 9.75 : Repairing of DNA with double-stranded breaks by homologous recombination in which the missing segments of each strand at identical sites are replaced by crossing-over with homologous segments of another intact DNA duplex

E. SOS Repair of Damaged DNA:

The SOS repair mechanism functions in a more complicated way. Damages inflicted on DNA by mutagenic agents induce a complex series of changes which are collectively known as SOS response. The response leads to increased capacity to repair damaged DNA by excision repair and recombinational pathways.

The SOS response is set in action by the interaction of two proteins, — Rec A protein which is a product of the recA gene and Lex A protein, the product of lexA gene. The Rec A protein in addition to having a role in genetic recombination and recombinational repair also has a protease function. The Lex A protein acts as a repressor for a number of genes, known as SOS genes including the recA gene. Under normal conditions i.e. when the SOS response is not necessary, these genes remain repressed by the Lex A repressor.

The initial event in the SOS response is the activation of RecA protease activity induced by DNA damage. The activation of Rec A protease activity occurs within a few minutes of DNA damage. The protease activity catalyses cleavage of the Lex A repressor making it inactive. As a result, the SOS genes can now be expressed to produce the enzymes required for DNA repair

The events are shown in Fig. 9.76:

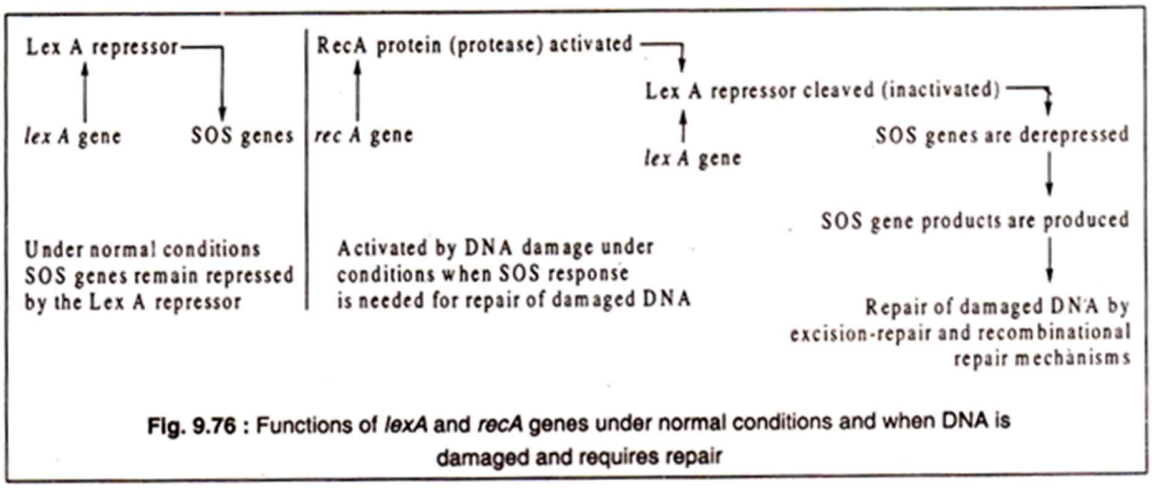


Fig. 9.76 : Functions of *lexA* and *recA* genes under normal conditions and when DNA is damaged and requires repair

The SOS response, as the name suggests, is an emergency measure to repair mutational damage. It makes it possible for the cell to survive under conditions which would have been otherwise lethal. However, the possibility of generating new mutations increases in the repair of DNA molecules. This is because the SOS repair system allows DNA synthesis bypassing the damaged site.

When the DNA polymerase III reaches a damaged site to which Rec A binds, the protein (Rec A) interacts with the epsilon subunit of the DNA polymerase molecule. This subunit is responsible for insertion of the correct base into the growing DNA strand. As a result, chain elongation continues bypassing the damaged site, but the chance of incorporation of a wrong base increases. SOS repair, therefore, enhances the chance of mutation due to mis-pairing of bases. This is known as error-prone bypass repair.

A more recent model based on SOS repair of UV-irradiated DNA in bacteriophages has been proposed. UV-irradiation is known to produce dimers of not only thymine, but also of thymine and cytosine and cytosine. During replication when T-C and C-C are reached, the SOS repair system is halted temporarily and cystosine is deaminated to uracil.
Uracil pairs with adenine, bringing about a transition mutation by changing C-G base pair to T-A. This is an error-free bypass repair thought it still causes a mutation. It is called error-free because the template DNA strand is faithfully copied in the newly synthesized strand. The change from C to U occurs in the template strand itself.

F. Methylation-directed very short patch repair:

Very short patch (VSP) repair is accomplished by involving methylation of bases especially cytosine and adenine. In E. coli methylation of adenine and in a sequence of -GATC- is done by the enzyme methylase (a product of dam gene) on both strands of DNA. After replication only A of -GATC- of one strand remains methylated, while the other remains un-methylated until methylase accomplishes methylation (Fig. 9.20 A-B).

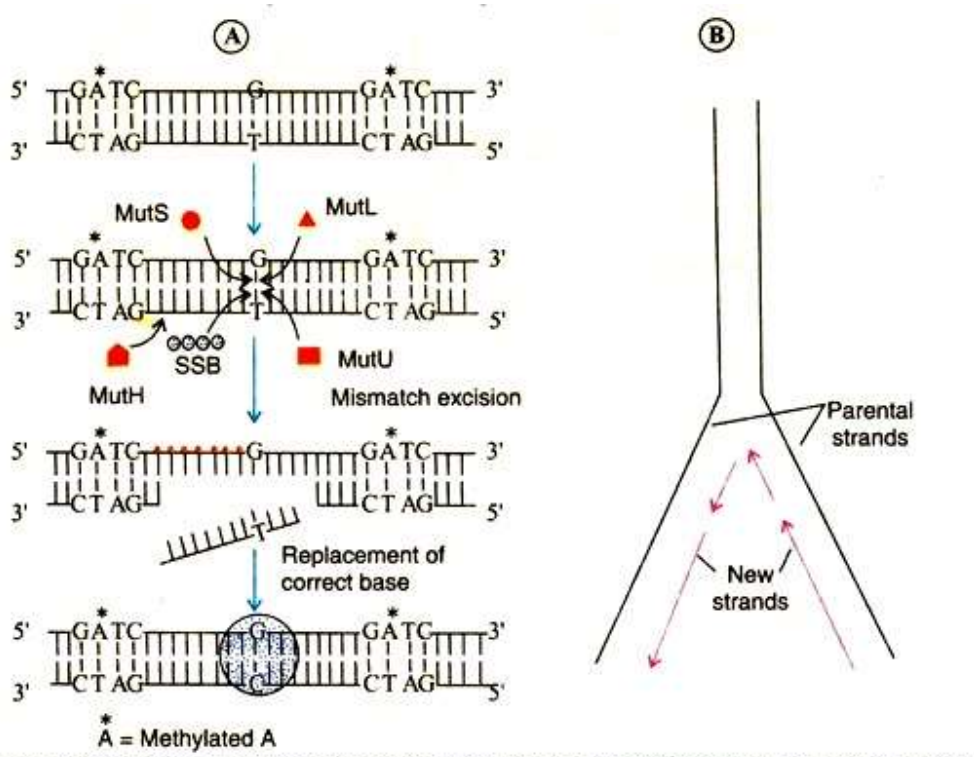


Fig. 9.20 : Mismatch repair. (A) excision of a newly synthesised strand and repair system; (B) arrows shows the region where methylation is not complete and dark region line shows the region where methylation is complete.

In E. coli repairing activity required four proteins viz., Mutl, MutS, MutU (UvrS) and MutH by the genes mutL, mutS, mutU, and mutH, respectively. The mut genes are the loci which increase the frequency of spontaneous mutation. The un-methylated -GATC allows the MutL to recognise the mismatch during transition period.

This helps MutS to bind to mismatch. MutU supports in unwinding the single strand and single strand DNA binding (SSB) proteins and maintain the structural topography of single strand. MutH cleaves the newly synthesised DNA strand and the protein MutU separates the mismatch strand (A).However, there is a gradient of methylation along the newly synthesised strand. Least methylation occurs at the replication fork. The parental strand is uniformly methylated.

The methylated bases direct the excision mechanisms to the newly synthesised strand containing the incorrect nucleotides (B). During this transition period, the repair system works and distinguishes the old and new strands and repairs only the new strands.

G. Mismatch Repair:

The rate of mutation varies usually in the range of 10^{-7} to 10^{-11} in bacteria. However, during normal DNA replication an error in inserting a correct base in the new strand occurs at a much higher rate, generally at a frequency the of 10^{-5}. This large difference indicates that bacteria possess an in-built mechanism to rectify most of errors during replication.
Most bacterial DNA polymerases, in addition to having the polymerase activity, have also an exonuclease activity which works in the opposite direction i.e. while polymerization proceeds in the 5′ —> 3′ direction, the exonuclease works in the 3′ —> 5′ direction. Whenever a wrong base is inserted into the polynucleotide

chain by mistake, the DNA polymerase stops and goes one step backward and the incorrect base is removed by the exonuclease activity. The polymerase then resumes its normal activity by inserting a correct base. This is known as the proof-reading function of the DNA polymerase. Mutants with an altered epsilon subunit of the DNA polymerase fails to perform the proof-reading function.

Although proof-reading by DNA-polymerase is an efficient way of removing many mismatched bases, a number of such errors may still persist after replication. Such mismatched base-pairs require removal. These are corrected by another repair mechanism, known as mismatch repair.In this repair mechanism, three gene-products (proteins) are involved — Mut S, Mut L and Mut H. The first step of this repair process consists of binding of the Mut S protein to the mismatched base- pair. The second step involves the recognition of a specific sequence of the template which is -GATC- m E. coli in which A (adenine) is methylated in N-6 position.

The proteins Mut L and Mut H which bind to the unmethylated -GATC- sequence of the new strand form a complex with Mut S which is bound to the mismatch pair. Thereby the mismatch pair is brought close to the -GATC- sequences. The Mut H protein then nicks the unmethylated DNA strand at the GATC site and the mismatch is removed by an exonuclease.The resulting gap is then filled by DNA polymerase III and DNA ligase (Fig. 9.77):

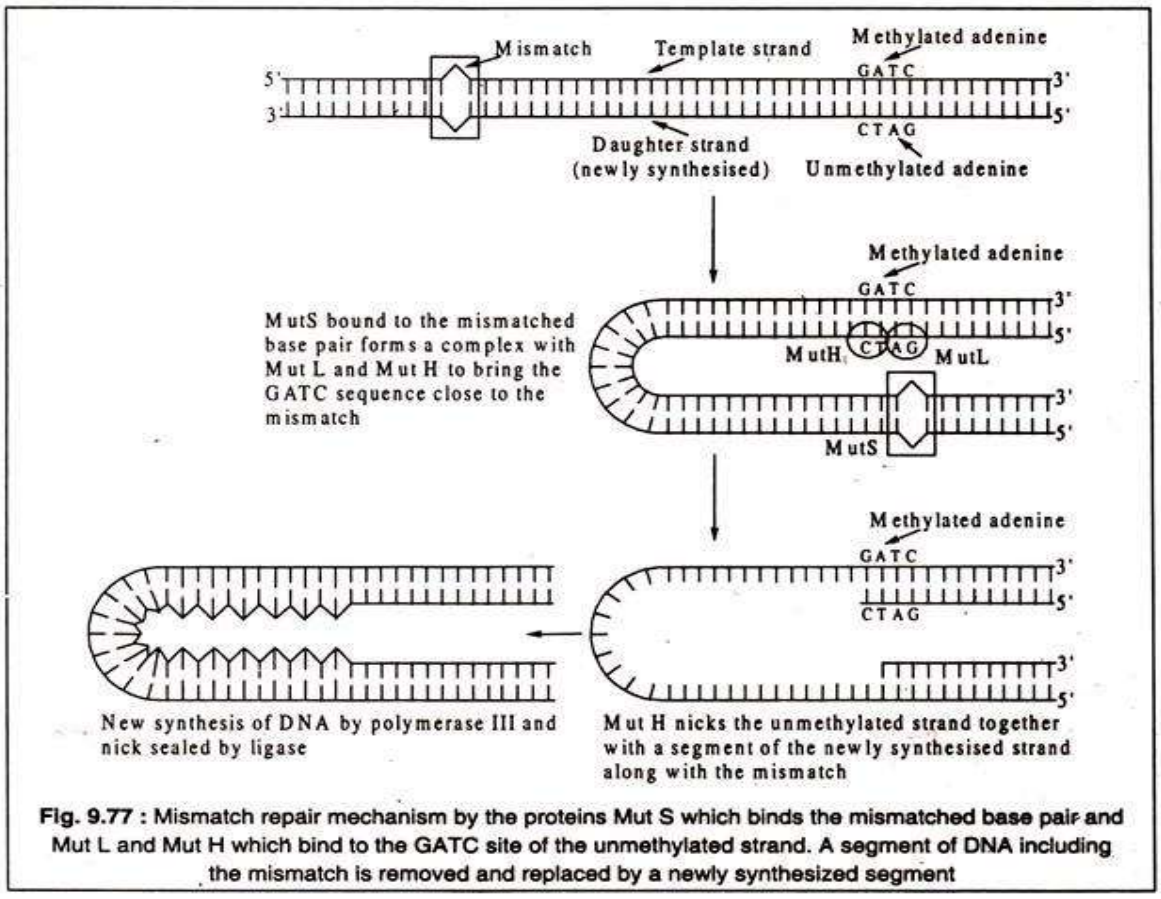


Fig. 9.77 : Mismatch repair mechanism by the proteins Mut S which binds the mismatched base pair and Mut L and Mut H which bind to the GATC site of the unmethylated strand. A segment of DNA including the mismatch is removed and replaced by a newly synthesized segment

Probable Questions:

1. State different types of DNA damage.
2. Describe photoactivation system of DNA repair with suitable diagram.
3. Describe mismatch system of DNA repair with suitable diagram.
4. Describe base excision repair system of DNA repair with suitable diagram.
5. Describe SOS repair system of DNA repair with suitable diagram.
6. What s the difference between base excision repair and nucleotide excision repair system?
7. Describe Re-Combinational Repair Mechanism of DNA with suitable diagram.
8. . Describe homologous recombination DNA repair system with suitable diagram.
9. Describe methylation-directed very short patch repair of DNA with suitable diagram.
10. What are the importance of DNA repair ?

Suggested readings:

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7th Edition.
6. Gene cloning and DNA Analysis by T. A. Brown, Sixth Edition.
7. Genetics . Verma and Agarwal.

Unit-V

Human genetics: karyotype and sex chromosomes; sex mosaics; sex chromosome anomalies; sex influenced and sex limited genes

Objective: In this unit you will learn about different aspects of human chromosome including sex chromosome. You will also learn about Sex chromosomal anomalies , sex influenced and sex limited genes in this unit.

Introduction to Chromosomes:

A chromosome can be considered a stainable threadlike nuclear component having special organisation, individuality and function. Their presence was first demonstrated in the eukaryotic cell by E. Strasburger in 1875 and these were first termed as chromosomes by W. Waldeyer in 1888.

This term is actually taken from Greek chromasoma which means "coloured bodies" (chroma = colour; soma = body) due to their marked affinity for basic dyes as a consequence of which they axe stained. This property is known as chromaticity.Staining the cell with certain types of stain (e.g., Aceto-orcein, Acetocamine, Feulgen's stain) shows that chromosomes are not visible in the interphase nucleus or metabolically active nucleus due to their high water content, but can be easily seen during cell division characteristics whether mitosis or meiosis.

During cell division, the chromosome undergoes dehydration, spinalisation and condensation. So they become progressively thicker and smaller and, accordingly, the satiability of chromosome also increases. Hence the chromosome becomes readily observable under microscope. Staining of chromosomes is generally carried out to make them visible under light microscope.Chromosomes are capable of duplication and maintaining their morphologic and physiologic properties through successive cell divisions. It has also been demonstrated that the chromosome contains DNA, which in turn, carries the genes and thus plays a major role in heredity.

When reproduction of organism takes place, they are passed on to the next generation through the gametes. Besides, they play an important role in variation, mutation and evolution, and in their control of morpho-genesis, multiplication and equilibrium of vital processes.The term chromosome is mainly used to describe the chromosome of eukaryotic cell. The naked DNA of prokaryotes and DNA or RNA of viruses are sometimes broadly called prokaryotic chromosome and viral chromosome, respectively, due to their similarity in fundamental properties with eukaryotic chromosomes.But the morphology and the organisation of eukaryotic chromosome is much more complex. The morphology of chromosomes in all eukaryotes is essentially similar—except some variations in number and size.

Most of the chromosomes in an eukaryotic cell are called autosomes which control all somatic characteristic of an organism [These are symbolized by 'A']. But, in addition, there are some other chromosomes which control some specialised characteristics of an organism and are called allosomes.Sex chromosome (X and Y) for determination of sex, B-chromosomes, L-chromosomes, M-chromosomes, S-chromosomes and E-chromosomes are examples of allosomes. Autosomes are universally present in all eukaryotic-organisms, but allosomes may or may not be present in all organisms.

Chromosome Number:

The number of chromosomes varies from species to species but it remains constant for a particular species. The number of chromosomes serves as an aid in the determination of phylogenetic status, such as taxonomic position of plant and animal species.In higher organisms, each somatic cell contains one set of chromosomes inherited from the maternal (female) parent or organ and a comparable set of chromosomes (homologous chromosomes or homologues) from the paternal (male) parent or organ.

The number of chromosomes in this dual set is called the diploid (2n) number. The suffix 'ploid' refers to chromosome "sets". Homologous chromosomes are two copies of a chromosome (one comes from the female and the other from the male parent or organ)—which are ordinarily identical in size and shape, gene content and gene order.Sex cells or gametes—which contain half the number of chromosome set found in somatic cell—are referred to as haploid cells (n). A genome is a set of chromosomes corresponding to the haploid set of a species. The number of chromosomes in each somatic cell is the same for all members of a given species.

Chromosome number varies widely and may be very low or high in both plant and animals. In animal kingdom *Ascaris megalocephala* var univalens shows a single pair of chromosomes in the cells of the germ line.But, since in the diploid soma the two chromosomes split into numerous small chromosomes, the single haploid chromosome has to be considered an aggregate chromosome or compound chromosome. It, for reasons unknown, maintains its unity under the conditions imposed by the cells of the germ line.

Again, the next lowest diploid chromosome number (2n) recorded so far in animals is four in *Mesotoma*(flat worm) and *Ophryotrochapuerilis* (Polychaeta). The highest diploid chromosome number (2n) in animals is 254 in *Eupagurusschotensis* (a hermit crab). Belar (1926) has, in fact, recorded that Aulacantha, a radiolarian has as a diploid number (2n) approximately 1,600 chromosomes.The somatic chromosome number generally remains constant among individuals of the same species. But in many species, somatic cells of the same individual may exhibit different (2n, 4n, 8n etc.) chromosome numbers.

In such species, cellular differentiation is often accompanied in some cells with a phenomenon of endomitosis. Endomitosis means the duplication of chromosomes without division of the nucleus, resulting in increased chromosome number within a cell. Chromosome strands separate but the cell does not divide.Endomitosis leads to the production of endopolyploid cells having 2n, 4n, 8n etc. chromosomes. In natural polyploid individuals, it becomes necessary to find out the ancestral chromosome number which is represented by x and is called as the basic number.For example in common wheat Triticum aestivum 2n = 42; n = 21 and x = 7 showing that common wheat is a hexaploid (2n = 6x). The whole collection of chromosomes in the nucleus of an organism is referred to as chromosome complement.

Chromosome Size:

The size of chromosome of a cell shows a remarkable variation depending upon the stage of cell division. Chromosomes are longest and thinnest during interphase. But on the onset of prophase there is a progressive decrease in size associated with an increase in thickness.Chromosome are smallest during anaphase. But the measurement of chromosome size are practically taken during mitotic metaphase when they are very thick, quite short and well-spread.

The size of mitotic metaphase chromosome of various plants and animals varies from 0.5μ to 32μ in length and 0.2 μ to 3.0 μ in diameter. The giant chromosome found in the cells of salivary gland of Diptera are permanently in pre-metaphase stage and are easily visible in the interphase nucleus. These chromosomes are 300 μ in length and 10 μ in diameter.The size of each chromosome is 32 μ long. Again, chromosome size of Trillium is hundred times bigger than its closely related genus Medeola, size differences may be seen in the

different species of a genus. For instance, the chromosomes of Allium Porum are half the size of the chromosome of Allium sativum.

The size of chromosome may vary in the different tissues within a single organism. For example, in plant Medeola, the root tip chromosomes are 50% bigger than the shoot tip chromosomes.Among animals, grasshopper, crickets, mantids, newts and salamanders have big chromosomes. In animals, size variation of chromosome has also been reported in different varieties within a species.

For example, the chromosome size of *chironomous thumiithumii* (fly) differs from its closely related varieties. During embryogenesis of certain marine insects the size of chromosomes of the early blastula are smaller than those of the later stage of development.The size, shape and number of the metaphase chromosomes constitute the karyotype which is distinctive for each species. When all chromosomes of a species are more or less equal in size, the karyotype is called symmetrical karyotype.

Asymmetrical karyotype refers to the chromosome of different size. In most organisms, all cells have the same karyotype. However, species that appear quite similar can have very different karyotypes—indicating that similar genetic potential can be organised on chromosomes in very different ways.

Variation in the size of the chromosome can be induced by some factors:
i. When the cell divides at low temperature, the size of chromosomes become short and more compact.

ii. When the pre-treatment of cells is done with colchicine, the chromosomes become slightly shorter in size.

iii. Repeated and rapid cell divisions tend to result in smaller chromosomes.

Morphology of Chromosome:

It has been observed that the morphology of chromosome changes with the stage of cell division. During the prophase of meiosis, homologous chromosomes pair with each other at zygotene, the cell then enters the stage of pachytene where chromosomes become shortened and coiled.

Pachytene stages are very useful for the study of chromosome morphology because they are longer than the chromosomes in mitotic metaphase, so that the structural details of chromosomes can be easily resolved.But the meiotic cell division as well as the pachytene stages are not readily available at any time for experimental purpose. On the other hand, mitotic metaphase is easily available by arresting the divisional cycle with some chemical agents. Further, mitotic metaphase is also suitable as convenient stage for studies on chromosome morphology and some of the features are more clear during mitotic metaphase.At metaphase, each chromosome is made of two symmetrical structures, the chromatids. They are also called sister chromatids. Each chromatid contains a single DNA molecule. The chromatids are held together closely by the

centromereFig. 13.1) and become separated at the start of anaphase when the sister chromatids move to

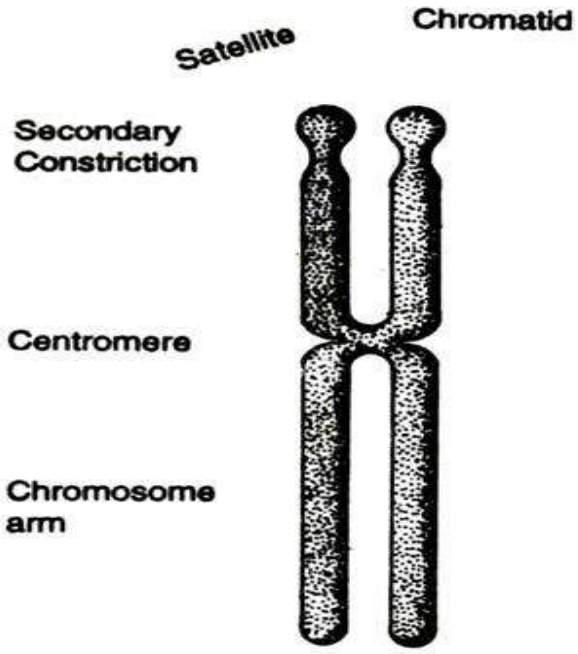


Fig. 13.1: Structure of a typical chromosome.

opposite poles.

Therefore, until two sister chromatids share the common centromere, they are called chromatids. But as soon as they are separated at anaphase and possess their own individual centromere, they are called chromosome instead of chromatid. Hence, from anaphase to next G_1 phase, chromosomes have only one chromatid while from S phase to metaphase chromosomes have two (Fig. 13.2).

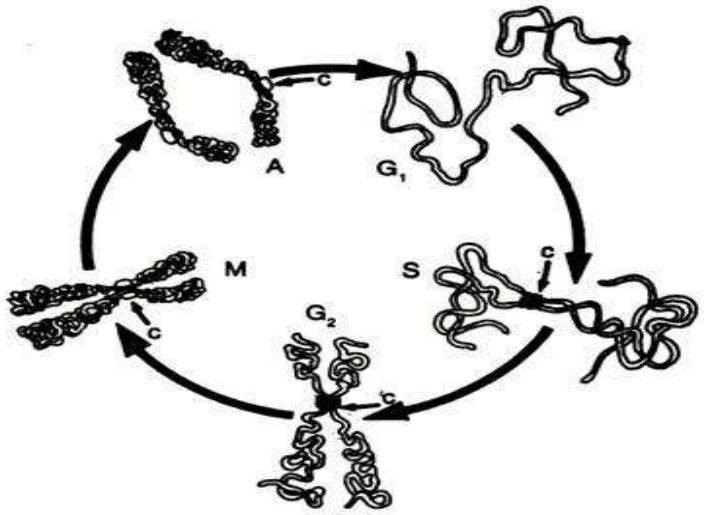


Fig. 13.2: The condensation and decondensation cycle of chromosomes.

The DNA present in each chromosome (made of a single chromatid) replicates during S phase to produce an identical copy of itself so that during G_2 prophase and metaphase each chromosome is composed of two chromatids.During prophase, and sometimes during interphase, the chromosomal material becomes visible as very thin threads which are called chromonemata and which represent chromatids in early stages of condensation. 'Chromatid' and 'chromonemata', therefore, are two names of the same structure. It is now accepted universally that chromatid is the structural and fundamental unit of chromosomes.
The region where two sister chromatids are held together is called the centromere. This region generally appears as a constricted or narrowed zone in the centromere, hence it is also known as primary constriction. Sometimes centromere appears as gap during metaphase because it does not take up any stain.Centromere has a clear zone in which the fibrils remained uncoiled or less coiled than those in the rest chromosomes. The reduced similarity of centromere" is understandable as the chromosome region in centrosome is less coiled or uncoiled and is composed of heterochromatin.At or near the centromere of each chromosome, the centromere is associated with a specialised structure called kinetochore. The structure of kinetochore is complex and is seen during late prophase. In ultra-thin sections of chromosomes, the kinetochore is seen as a stack of three-layered proteinaceous disc like plates (Fig. 13.3).

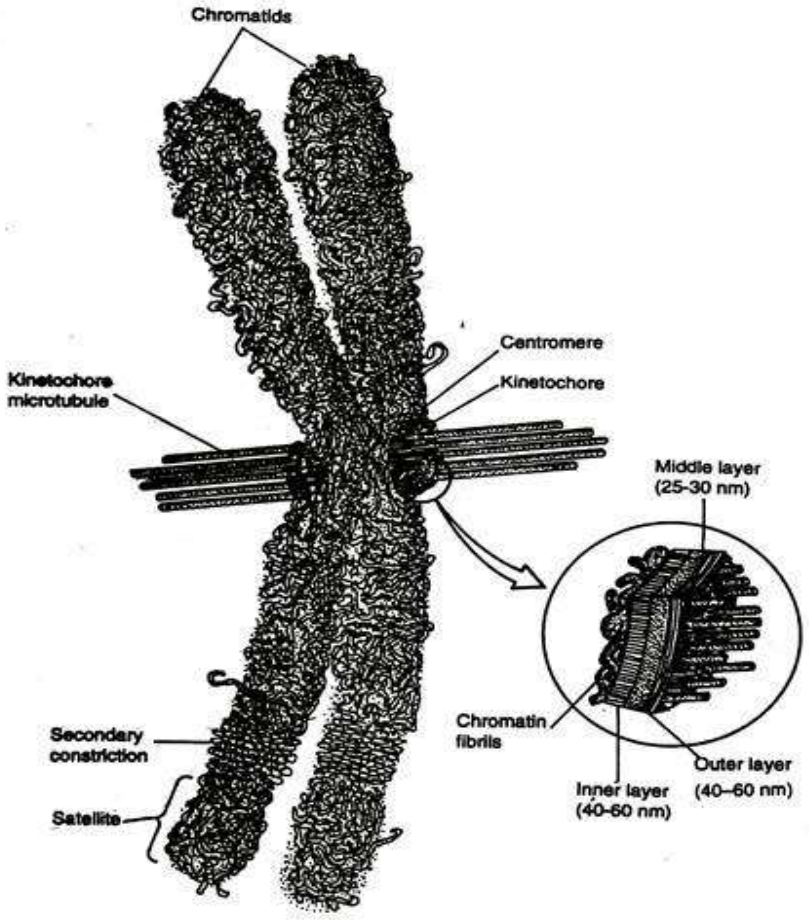


Fig. 13.3: Schematic diagram of metaphase chromosome showing the kinetochore.

The innermost disk (40-60 nm) probably consists of chromatin that is condensed differentially than the surrounding heterochromatic chromatin. The outer disk is a fibrous structure where kinetochore microtubules are attached by their ends.A 25 to 30 nm layer—the middle layer separates the inner and outer disk. A series of fine filaments which bridge the middle layer may help to hold the two disks together. The kinetochore microtubules—that extend toward the spindle pole of the cell play an active role in movement of chromosomes toward the poles during anaphase.The location of centromere and, hence, that of the kinetochore is directly controlled by a unique segment of chromosomal DNA termed centromeric DNA. Much of the information about the structure of auto mere has been obtained from genetic studies of the simple centromere in yeast.

Yeast centromere is very small and binds, a single microtubule. Sequence analysis of cloned centromeric DNAs (CEN DNAs) from yeast chromosomes shows that they are generally organised into three regions—centromeric DNA elements (CDEs) I, II and III (Fig. 13.4).Out of the three regions CDF II and CDF III appear to mediate interactions with microtubules through centromeric binding factors (CBF) 2 and 3, respectively, and through the kinesin-related protein Kar^{3}p. These proteins are an important link between kinetochore microtubules and the centromere CDE I is conserved in sequence but is not required.

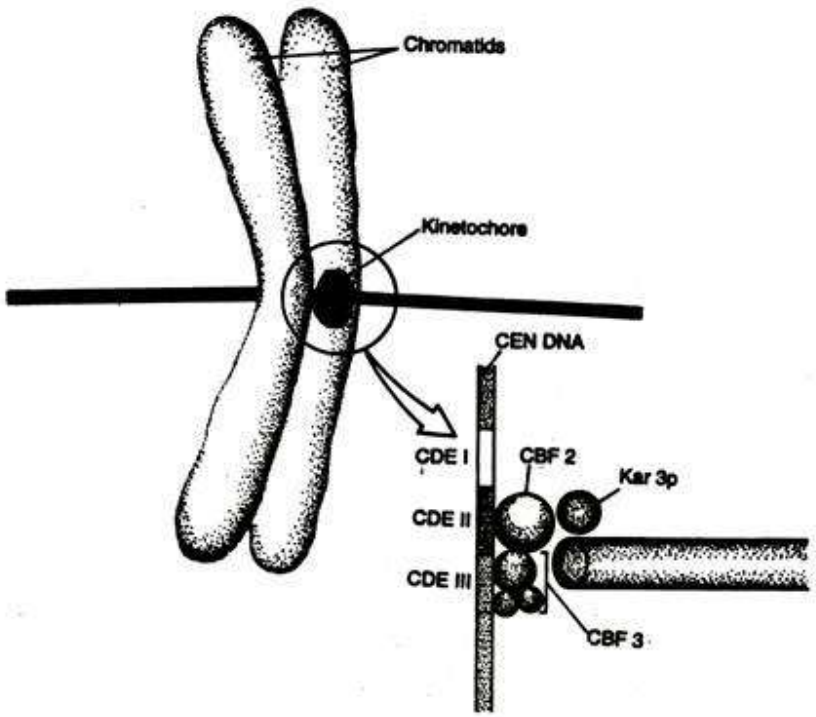


Fig. 13.4: The structure of a yeast centromere and kinetochore.

Each chromosome in a genome can be distinguished on the basis of the position of centromere which divides the chromosome into two arms of varying length. The portion of the chromosomes on either side of the centromere is called the arm of chromosomes—which may be equal or unequal.In case of unequal chromosome arms, one arm of a chromosome is longer than the other, hence they are termed long arm or q arm, and short arm or p arm, respectively.

Depending on the position of the centromere, chromosomes may be divided into four categories:metacentric,sub-metacentric,acrocentric andtelocentric chromosome (Fig. 13.5).

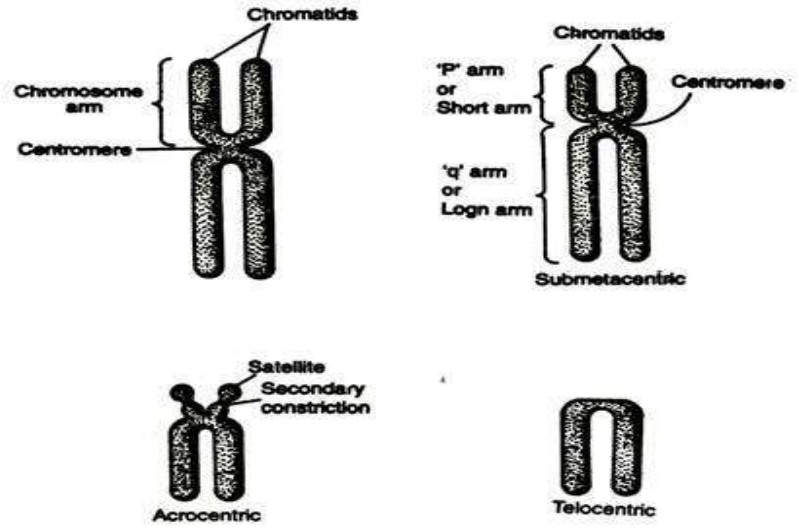


Fig. 13.5: Chromosomes are classified according to the position of the centromere.

In a metacentric chromosome it occurs at the centre, i.e., the centromere is median so that the two arms of such chromosomes are equal. The metacentric chromosomes look 'V'-shaped during anaphasic movement. In Trillium and Tradescantia, all the chromosomes are metacentric.The sub-metacentric chromosomes look 'L'-shaped in anaphase and the centromere is located on one side of the centred point, i.e., the centromere is sub-median so that it divides the centromere into two unequal arms.In acrocentric or sub-telocentric or sub-terminad centromere, the centromere is situated almost near one end of the chromosome, i.e., centromere is sub-terminal in position and it gives two arms—one exceptionally short and the other long. Acrocentric chromosomes look 'J'-shaped in anaphase. Chromosomes of locust and some grasshoppers are acrocentric.

In some chromosomes, however, centromeres appear to be located at one end of the chromosome, i.e., in the position normally occupied by one of the two telomeres. In this type, one arm is more or less equal to the length of the chromosome, and, other arm beyond the centromere is represented simply by dot.Acrocentric chromosome may appear 'rod- shaped' or T-shaped in anaphase. Telocentric chromosomes are of rare occurrence. Telocentric chromosome exist normally in certain species of holomastigote protozoa.

Telocentric chromosomes arising through a transverse fracturing of the centromere are believed to be unstable due to the centromere's irregular manner of division. This type of division is also known as misdivision of centromere—a process which leads to the formation of iso-chromosome (those in which the two arms are of equal length and are genetically homologous with each other). Misdivision of centromeres has been observed in Pea, Datura, Wheat and Fritillaria. Telocentric chromosomes have been experimentally produced in wheat, maize etc. Usually, each chromosome has only one centromere but, in some species, each chromosome has more than one centromere. Again, in some cases the centromere is absent.

Hence, depending on the number of centromeres, chromosomes are classified as given:

(a) Acentric:

The chromosome is without any centromere. Acentric chromosome is very rare occurrence. It may arise due to unequal breaking of chromosome arm into two so that only one part has the centromere while, in the other part, centromere is absent.The part of chromosome having no centromere is called acentric fragment. Due to

lack of centromere, acentric chromosomes are not able to attach with spindle fibres and they do not take part in cell division. Ultimately, the cell eliminates the acentric fragments.

(b) Mono-Centric:

Mono-centric chromosomes have only one centromere. It is a very common occurrence in most of the species.

(c) Dicentric:

A chromosome has two centromeres. Dicentric chromosome may be produced as a result of translocation, paracentric inversion etc. If the two centromeres tend to move to opposite poles during anaphase, the chromosome breaks. Rarely a new centromere may appear on the chromosome resulting in an abnormality. Such a centromere is called a neo-centromere. Dicentric chromosome is reported in the cells of wheat.

(d) Polycentric:

In addition to the shapely localised type of centromere described above, there exists a type of non-localised centromere where each chromosome has more than two centromeres. Such chromosomes are called polycentric.

Polycentric chromosomes are found in plant *Luzulapurpurea* (Fam Juncaceae), in the generative tissue of Ascaris *megalocephalla univalens*, and in Thyanta. In both the above cases, the centromeric property is confined to one or more definite locus of the chromosome so that such centromere is Called localised centromere.However, in many insects—e.g., most homopteran and hemipteran insects—the centromere activity is non-localised and spread over the entire chromosome length. In such cases, the centromere is called a diffused centromere.

Polycentric chromosome often break into a number of smaller fragments. Each small segment functions independently. For instance, in case of Ascaris megalocephala univalens, the zygotic cell contains only two chromosomes.But during embryonic development these chromosomes break in the somatic cells so that the cell may have up to 42 chromosomes. However, the cell that will give rise to the generative cell contains only two chromosomes.Non-staining gaps are seen in certain chromosomes in addition to the primary constriction regions. These regions are called secondary constrictions (Sc). Generally, secondary constrictions are located in the short arm of chromosomes near end but in many chromosomes they are located in the long arm and sometimes they may be present on both arms.

Secondary constrictions are constant in their position and extent. These constrictions are useful in identifying particular chromosomes in a set.The number of Sc in a genome varies from species to species. In some species, a somatic cell contains at least a pair of chromosomes with Sc while other chromosomes within the same cell are without Sc. In some other species, the number of chromosomes with Sc may be four (e.g., Viciahajastana), six, eight, ten (e.g., human somatic cell).

Secondary constrictions are distinguished from primary constriction:

i. Sc is without kinetochore.

ii. Sc is not able to attach with spindle fibre during anaphasic movement.

iii. Sc shows the absence of marked angular deviation of the chromosomal segments during anaphase.

Certain Sc contains the genes coding for 18S and 28S ribosomal RNA and that induce the formation of nucleoli. Since they are usually sites for the organisation of the nucleolus they are also called nucleolus organising regions or NOR.

The region of the chromosome separated from the rest of the chromosomes by NOR or Sc is a rounded body called satellite or trabant. If a fine thread is seen between satellite and the rest of chromosome, it is the satellite stalk and the chromosome is a SAT-chromosome.

The satellite and the constriction are constant in shape and size for each particular chromosome. In man, the nucleolar organizers are located in the secondary constrictions of chromosomes 13,14,15, 21 and 22—all of which are acrocentrics and have satellites.

The eukaryotic chromosome terminates at either end in a structure called the telomere. Telomeres have special properties when chromosomes are broken, the free ends without telomeres become “sticky” and tend to fuse with other broken chromosomes. However, the intact ends of unbroken or normal chromosomes are stable and show no tendency to fuse with other chromosomes or other ends.

The telomere differs in structure and composition from the rest of the chromosome. Telomere structure has been studied in a number of organisms. All telomeres so far studied have multiple (30-70) repeats of short species-specific sequences such as TTAGGG in humans, in Tetrahymena thermophila, TTTAGGG in plant Arabidopsis thaliana etc.Although these sequences are. somewhat variable in different species the basic repeat unit in all species has the pattern 5’Ti$_{1_4}$ A$_{0-1}$G$_{1_8}$3’Telomeres have either two strands of DNA covalently linked with a sub-teiminal nick or have a single- stranded 3′ end, i.e., the G-rich strand extends by 12-16 nucleotides beyond the C-rich strand. The protruding single-stranded DNA portion is also known as DNA primer.Telomere sequences are added by a special enzyme called a telomere terminal transferase or telomerases. Telomerase is a ribonucleoprotein. It contains a short RNA component, 159 bases long in Tetrahymena, 192 bases long in Euplotes. This RNA provides the template for synthesizing the G-rich repeating sequence to which it is complementary.

Centromeric Index

The position of centromere can be determined from microscopic studies by centromeric index. Centromeric index is the percentage of a quotient of the length of short arm of chromosome divided by the length of the chromosome. It is calculated from the following formula:

$$\text{Centromeric index or F\%} = \frac{\text{length of the short arm}}{\text{length of the chromosome}} \times 100.$$

The nature of primary constriction corresponding to the value of centromeric index is given in Table 13.1:

Table 13.1: Centromeric Index (F%)

Centromeric Index (F%)	Nature of constriction	Centromeric Index (F%)	Nature of constriction
50.00	median	18.74-12.51	nearly sub-terminal
49.99-37.51	nearly median	12.50	sub-terminal
37.50-25.01	nearly sub-median	12.49-6.25	nearly sub-terminal
25.00	sub-median	6.24-1.00	nearly terminal
24.99-18.75	nearly sub-median	1.00	terminal

Arm Ratio

Arm ratio is another useful numerical calculation for determining the position of centromere. It is the ratio of length of the long arm to the short arm of a chromosome. The position of centromere corresponding to the arm ratio is given in Table 13.2.

Table 13.2: Arm ratio value and centromere position

Arm ratio	Centromere Position
1.0	Median *sensu stricto*
1.7	Median
3.0	Submedian
7.0	Subterminal
0.0	Terminal

Three nucleotides (say … A AC …) of RNA template within the enzyme telomerase pairs with 3′ terminal three nucleotides (… TTG) of DNA primer, as shown in Fig. 13.6. RNA template directs and adds nucleotides to the 3′ end of DNA primer.It adds G and T bases one at a time to the primer as directed by the template and polymerisation continues to end of template region. The enzyme moves to new 3′ end of template before another round of addition takes place. The cycle starts again when one repeating unit has been added.

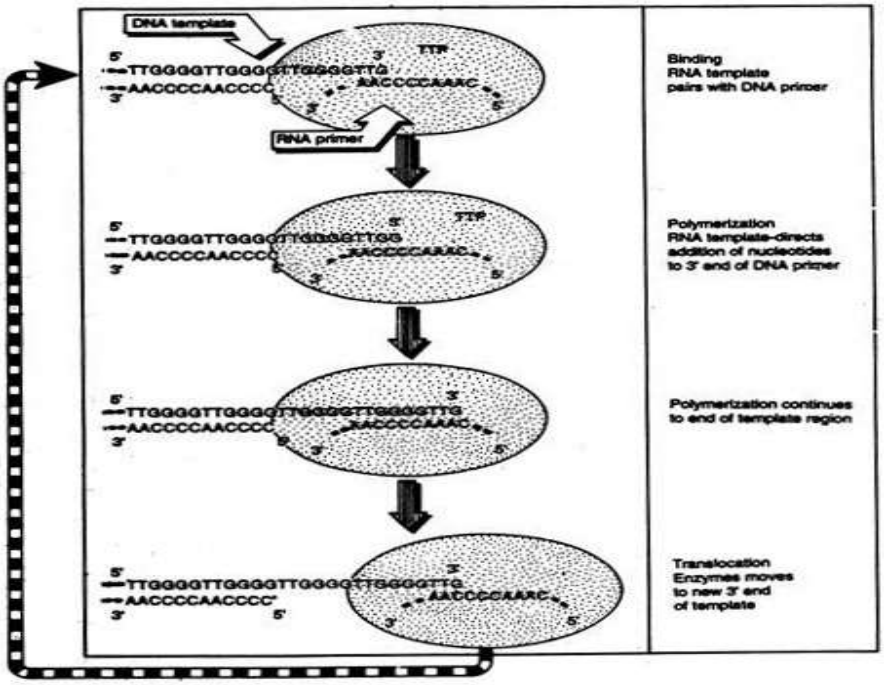


Fig. 13.6: Model for telomerase action in Tetrahymena.

The telomerase is a specialised example of a reverse transcriptase, an enzyme that synthesizes a DNA sequence using an RNA template. The added G-rich sequence can fold back on itself to form a novel hairpin or four stranded DNA loop.This involves G : G base pairing in which one or two of the G bases has the syn configuration (Fig. 13.7). Although such secondary structures may stabilise the ends of the chromosomes, they may also interfere with telomerase action.

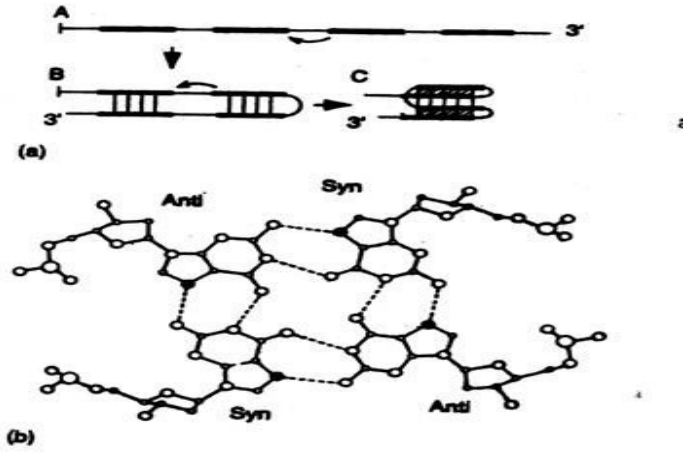


Fig. 13.7: (a) A model of how the G-rich strand could fold upon itself to form two- or four- stranded structure. (b) Shows a G tetrad involving in the Syn and anti configuration.

The unique structure of telomere of eukaryotic chromosomes performs three important functions:

i. It prevents exonucleases from degrading the ends of the linear DNA molecules.

ii. It facilitates replication of the ends of the DNA molecules without loss of termini.

The unusual behaviour of telomeric fractions are:

i. It consists of a basic repeat unit having the general pattern 5′ T_{1_4} A_{0_1} G_{1_8} 3′.
ii. In some species the telomeres terminate with a single-stranded G-rich region of the DNA strand with the 3′ end (a so-called 3′ overhang).

iii. The enzyme telomerase conditions the RNA component which provides the template for synthesizing the G-rich strand. This enzyme acts as a specialised Reverse transcriptase.

iv. The terminal DNA is folded and forms either a duplex hairpin by G-G pairing or a G quartet when one G is contributed by each of the 4 repeating units.

v. DNA polymerases are not able to replicate the terminal segment.

In the meiotic prophase and early mitotic prophase, the chromatin material is seen to have dense thickened areas at regular intervals giving the appearance of a string of beads. These bead-like areas are known as chromo- meres.

The distribution of chromomeres in a chromosome is highly characteristic and constant. Homologous chromosomes show an identical pattern. Chromomeres are specially obvious in polytene chromosomes where they become aligned laterally constituting the chromosome bands.Chromomeres of a single chromosome show a considerable variation in size. Once it was believed that genes were located within chromomeres and one chromosome may possibly represent a single gene.

But this idea has been a controversial one and available cytological evidence does not support this view. The chromomere can best be considered as a unit of functional coordination. Chromomere represents simply the tightly coiled or folded regions of DNA than in the neighbouring regions of chromosome called inter-chromomeric region.So they are visible in the phase of cell division. At metaphase, the chromosome is tightly coiled and chromomeres are no longer visible.

Chromosomes in Nucleoprotein:

(a) Chromatin:

Eukaryotic chromosomes in metaphase are generally known as chromosomes but in interphase the term chromatin is more generally used to describe the nucleoprotein fibres in the cell nucleus.

On the basis of stain-ability with basic dyes during various stages of cell cycle, chromatin is sub-divided into two main classes:(i) Euchromatin and(ii) Heterochromatin.

Interphase chromosome cannot be distinguished individually. The interphase nucleus is seen to contain scattered chromosome material and certain highly condensed bodies or chromo- centre. The euchromatin region takes light stain in the interphase nucleus.It takes comparatively deep stain during cell division. On

the other hand, heterochromatin takes deep stain during interphase and prophase while during metaphase it takes light stain (Fig. 13.8).

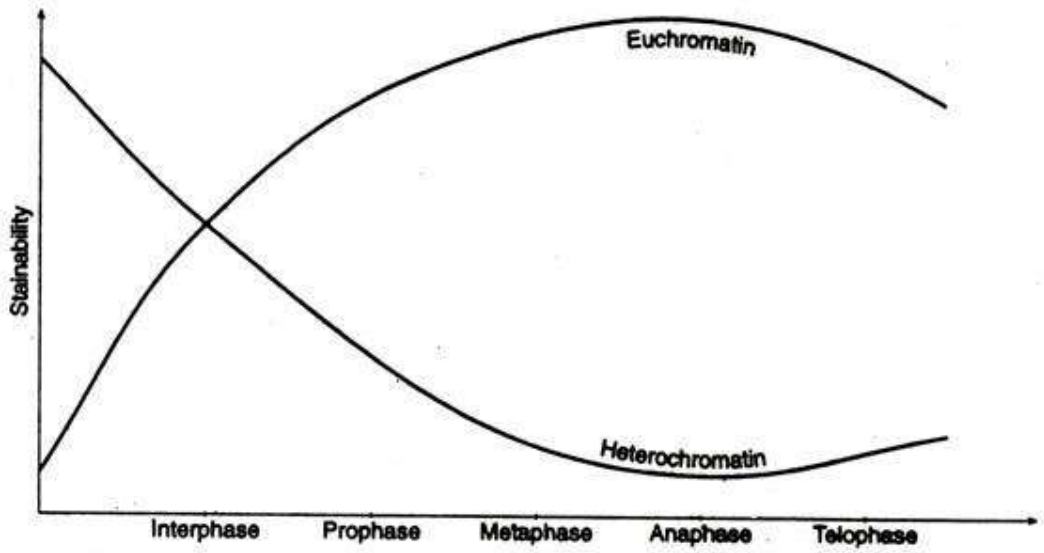


Fig. 13.8: Relationship between euchromatin and heterochromatin.

The changes of stain-ability can be correlated with the changes of condensation and de-condensation property of the chromosome during various stages of cell division. It is obvious that condensed state takes deep stain while extended form takes light stain.

The distribution of heterochromatin of chromosomes has been studied extensively. Heterochromatin has been found to be located at some specific regions such as centromere, chromomere, nucleolar organising region, satellite etc. (Fig. 13.9). But besides these regions there are some other regions where heterochromatin may be present.

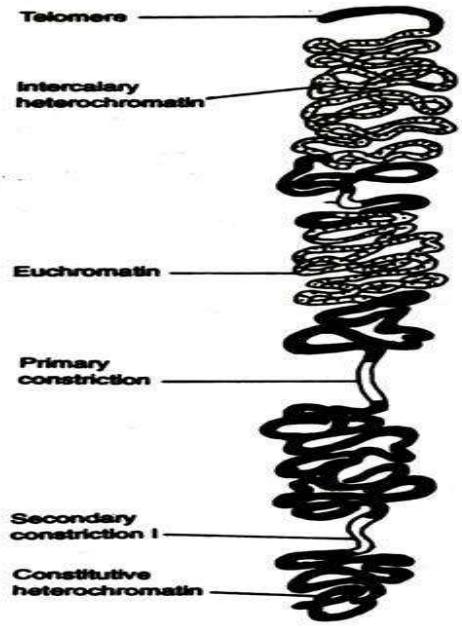


Fig. 13.9: Showing distribution of heterochromatin.

Heterochromatin may be classified into:(i) Constitutive, and(ii) Facultative.

Constitutive heterochromatin remains permanently in the heterochromatic state, i.e., it does not turn back to the euchromatic state. It tends to have a constant position on homologous chromosomes, e.g., centromere.In contrast, facultative heterochromatin is essentially euchromatin that has converted into heterochromatin (heterochromatinisation) whenever it needs and, again, it may be changed into euchromatin state (euchromatinisation).

Facultative heterochromatin is seen to develop during the development of an organism. Heterochromatinisation may involve a segment of a chromosome, a whole chromosomes (for example one X chromosome of human females) or one whole haploid set of chromosome (for example, full male set of chromosomes of mealy bug).

Within a chromosome, constitutive heterochromatin has been found to be located at certain regions:(i) Centromere,(ii) Telomere,(iii) Nucleolus organising regions and (iv) Intercalary segment of chromosome.

Entire chromosome may show heterochromatic behaviour, e.g., sex chromosomes in some plants, supernumerary chromosomes etc..

Heterochromatin shows different properties. All types of heterochromatin do not exhibit similar properties. Again, all such properties are not associated with a particular heterochromatin. Earlier workers considered that all heterochromatins were largely genetically inactive since addition or loss of heterochromatin did not have an appreciable phenotypic effect.It has been also suggested that highly condensed heterochromatin is generally not available for transcription and this condition leads to genetic inactivity. But these concepts are not of universal application. Heterochromatin may not be completely devoid of genes or genetic activity.

Muller demonstrated the presence of the “bobbed eye” gene on the Y-chromosome of Drosophila which was heterochromatic. Gates suggested the location of the gene for hairy earlobes in man on the presumed inert Y-

chromosome.In tomato, a gene has been localised on heterochromatic segment. Again, the proportion of genes on heterochromatic and euchromatic regions in salivary gland chromosome was found to be same on a length to length basis.

When a heterochromatin segment is trans-located next to euchromatin segment, the activity of euchromatic genes is heterochromatinised. However, similar properties are seen when euchromatic part is shifted next to heterochromatin. This is called position effect.Temporary genetic inactivity has been observed in certain chromosome segment, which are facultatively heterochromatic. This property is very useful for explaining the process of differentiation. For example, in certain coccids (insects) a given chromosome becomes heterochromatic during embryonic development and, at the later stage, it turns back to the euchromatic state.

Heterochromatic regions are not able to synthesise RNA in vitro. It indicates that chromatin fibres in heterochromatic segments are more tightly packed than euchromatic regions.Experiment with the rate of incorporation of tritiated thymidine into different cells at different stages of development reveals that heterochromatic regions are seen to replicate their DNA in synthetic phase later and early than the euchromatic region. This phenomenon can be correlated with transition from euchromatin to heterochromatin.

In certain cases, heterochromatic region contains a high content of repetitive DNA. This explains the low phenotypic effect following the loss or gain of segment. Heterochromatic regions are easily broken by ionising agents and radiomimetic chemicals. Allocycly or heteropycnosis is another property of heterochromatin. Certain segments of the chromosome are more condensed than the rest of the chromosomes during various stages of the cell cycle. Such differences in thickening has been called heteropycnosis. It is exhibited principally by the heterochromatin on the two sides of the centromere. This property is not universal because many forms do not show complete allocycly.

Karyotype:

Karyotype represents the chromosome constitution of a cell or an individual. It deals with the length of chromosome, the position of centromere, presence of secondary constriction, and the size of satellite of the somatic chromosome complement. Generally, karyotype is prepared from well-scattered chromosomes of mitotic metaphase plate.

The information regarding chromosome constitutions are obtained from hand drawing of the microscopic view of chromosomes with the help of camera Lucida or drawing prism. Photomicrographs of metaphase plate are also used for the preparation of karyotype.

Karyotypes are presented by arranging the chromosomes in a descending order of length in a straight line. The longest chromosome is always placed on the right side and the smallest one of the right. All chromosomes in a karyotype do not bear the centromere at the same position.

So, in a karyotype, chromosomes are grouped on the basis of the position of centromere and, in each group, descending order of length of chromosome is also maintained. Each chromosome of a karyotype is marked by a serial number from the extreme left to extreme right.

Broadly, karyotypes of different organisms may be classified into two categories:i. Symmetrical Karyotype and ii. Asymmetrical Karyotype.

A symmetrical karyotype has all metacentric chromosome of the same length.

In case of asymmetrical karyotype (Fig. 13.10) variation of length of chromosome complement is found and the position of centromere may or may not be identical.

In certain case, karyotype is asymmetrical but the length of chromosome is sharply two types—some chromosomes are very long and some are very short. This type of asymmetrical karyotype is known as bimodal karyotype.

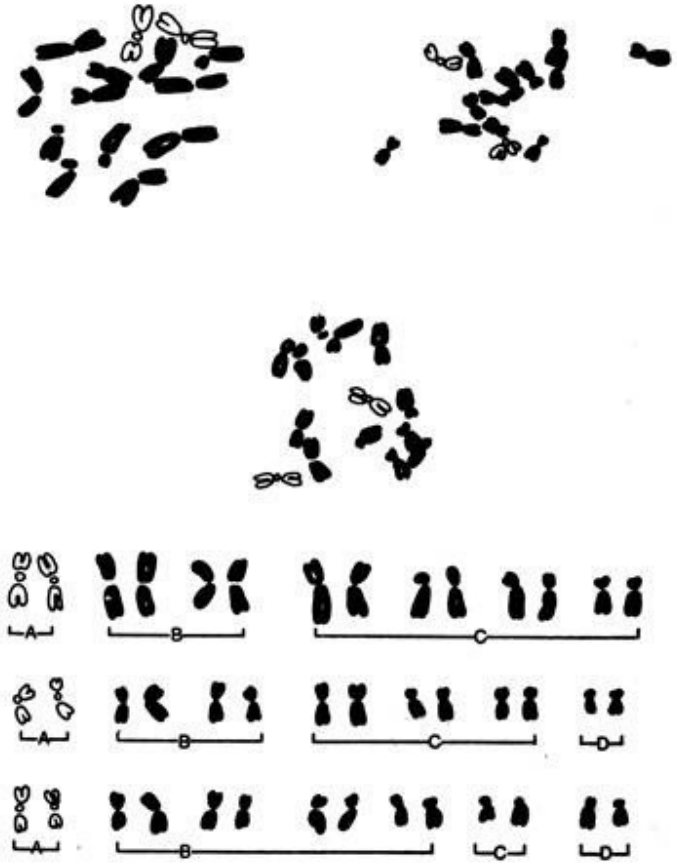


Fig. 13.10: Camera lucida drawing of chromosomes at metaphase and their Karyotype (asymmetric).

It is believed that symmetrical karyotype is the primitive form from which more advanced asymmetrical karyotype has been evolved. The karyotype of a species may be represented on graph or plain paper by bar diagram showing all morphological features of the chromosome. Such diagram is known as Idiotype or Idiogram (Fig. 13.11). Idiogram is prepared from haploid chromosome complement of an organism. An idiogram gives the identical information like that of karyotype.

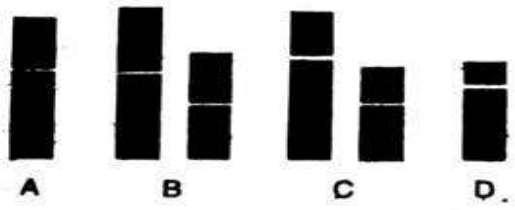


Fig. 13.11: Showing the idiogram of haploid set of chromosomes represented by bar.

Chromosomes Containing Single DNA Molecule:

All DNA viruses and bacterial cells contains a single chromosomal DNA molecules. The general belief is that all eukaryotic chromosomes also contain a single long linear DNA molecule. Extraction of longest DNA experimentally from lower and higher eukaryotes leads to hypothesis that each chromosome contains a single DNA molecule.

For example, physical analysis of the largest DNA molecules extracted from several genetically different species and stains of Drosophila exhibits that they are from 6×10^7 to 1×10^8 base pair long.
These sizes match the DNA content of single stained metaphase chromosomes of Drosophila melanogaster, as measured by the amount of DNA specific stain absorbed. Hence, each chromosome possibly contains a single linear DNA.

The correspondence between the number of chromosomes and the number of DNA molecule per cell has been demonstrated in yeast cells. The length of yeast chromosomal DNA ranges from about 1.5×10^5 or 10^6 base pairs.The DNA of yeast chromosome can be separated and individually identified by pulse-field gel electrophoresis. The result of this technique shows that the number of separated DNA molecules equals to the number of chromosome in yeast.
The strongest evidence in the support of unineme (having a single DNA double helix per chromatid) concept is provided by studies on lamp brush chromosome. The loops of a lamp brush chromosome represent single chromatid in a fully extended state.In electron micrographs of RNAase and protease treated lamp brush chromosomes, the loops have a diameter of 20A which is the diameter of a DNA double helix. Overwhelming evidence form a variety of studies supports the theory that each chromatid contains a single giant DNA molecule.

Types of Chromosomes:

In a vast majority of plants and animals, besides autosome, the cells of individual may contain one or more than one chromosome(s). These chromosomes are collectively referred to as allosomes. They differ from autosomes by their specialised functional role.Autosomes are present in all cells of all organism but the existence of allosomes is not always universal. Allosomes are of different types. All types are not necessarily present in one organism.

(a) Sex Chromosomes:

Chromosomes that are connected with the determination of sex, are called sex chromosomes. There are two types of sex chromosomes; X and Y. X chromosome is found in both males and females although one sex has only one while the other sex have two X-chromosomes.

Y-chromosome occurs only in one of the two sexes of a species, e.g., male fruit fly, human, male mice, some male plants and female birds, reptiles. Y-chromosome contains mostly heterochromatin and only few genes are located in it. On the other hand, X-chromosome is made of euchromatin and many genes are located on it.

(b) B Chromosomes or Super Numerary Chromosome or Accessory Chromosome:

In some organisms, chromosomes in addition to normal autosomes are present as an extra chromosomes that are not genetically necessary for the individual and not homologous to any of the normal chromosomes. These chromosomes differ from the normal ones in their variable number, smaller size and greater degree of heterochromatinisation. They are found more commonly in plant than in animals (Locustamigratoria, Camunla pellucida, Helix pomatia, Myrmeleo tettix maculatus).

B-chromosome do not usually have any effect on the phenotype and, hence, they are not genetically desirable. In some plant their presence results some deleterious effect, i.e., loss of vigour. Though they are found to be deleterious, yet the occurrence of such chromosomes through generations indicates that they must have some positive adoptive role as well.

B-chromosomes do not usually pair with normal chromosome during meiosis though they may pair with each other without the formation of chiasmata when present in even number.B-chromosomes may be eliminated from certain tissues or organs during embryogenesis.

The origin of B-chromosome is rather obscure. They may have been originated from the ordinary chromosome. It has been suggested that the centric heterochromatin part of an autosome is gradually converted into B-chromosome by the elimination of euchromatin part (Fig. 13.12).

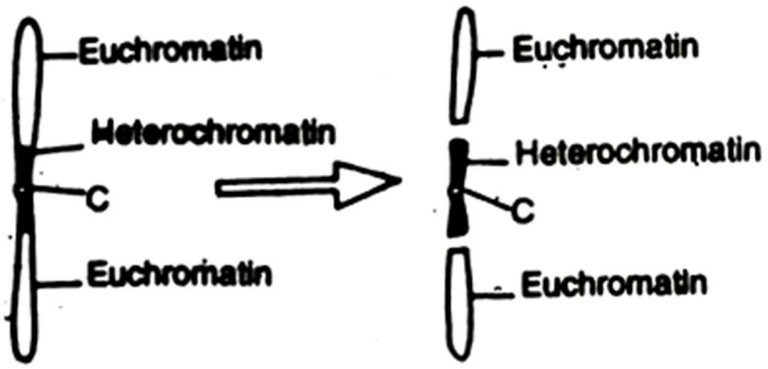


Fig. 13.12: Derivation of B-chromosome (C = centromere).

(c) Micro-Chromosome:

These chromosomes are also known as minute or m- chromosomes. They are so-called because of their extremely small, dot like size (about 0.5 μm). Micro-chromosomes are known both in plant (in many species of bryophyte) and animals [in insects of coreidae (Heteroptera), birds etc.].

They have been found mainly during meiosis and occasionally during mitosis. Micro-chromosomes are seen along with large chromosomes or bivalents.

They contain DNA and undergo pairing into bivalents which are sometimes arranged in a rectangle like a quadripartite group. In a peat moss sphagnum there are 19 large bivalents and two m-chromosomes consisting of univalents and four m-chromosomes arranged in quadripartite fashion (Fig. 13.13).

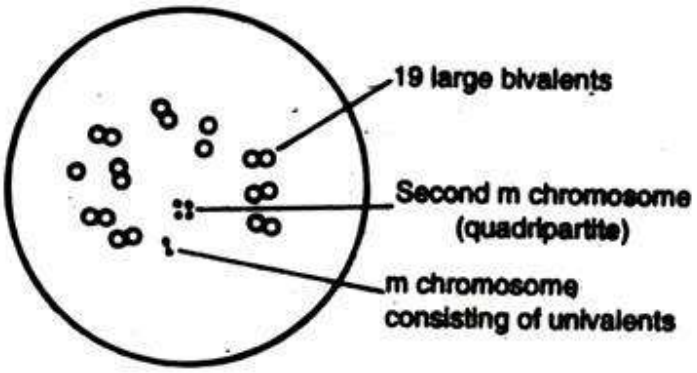


Fig. 13.13: Diagram of microchromosome (m chromosome).

In certain exceptions sat chromosomes are relatively rare and the property of organising nucleoli has been ascribed to specific micro-chromosomes as in bird.

(d) Mega-Chromosomes:

Mega-chromosomes are so called because they are non polytenic and many times longer than the length of normal chromosomes. They are not found in all cells and occur only in a small population of somatic cells. Generally, there is only one mega-chromosome per cell. Sometimes more than one mega-chromosomes have been reported.

Mega-chromosomes may be mono-centric, dicentric or acentric. They are found in the successive generations but they are not transmitted through the gametes. Hence mega-chromosomes are inheritable but the cells are able to produce them. Mega-chromosomes have been reported in a few species of Nicotiana hybrids.

(e) Limited Chromosomes:

Limited chromosomes are large in size and limited in distribution, i.e., they are found only in the germ cells. Limited chromosomes are also known as L-chromosome. They are found in insects of the family Sciaridae (Diptera).

During the embryonic developmental stage particularly the fifth and sixth cleavages limited chromosomes are eliminated from the somatic tissue but are retained in the germ line cells.

In somatic cells of both male and female L-chromosomes are absent. Because L-chromosomes are present in all individuals of species in which they are found they are considered to be B-chromosomes.

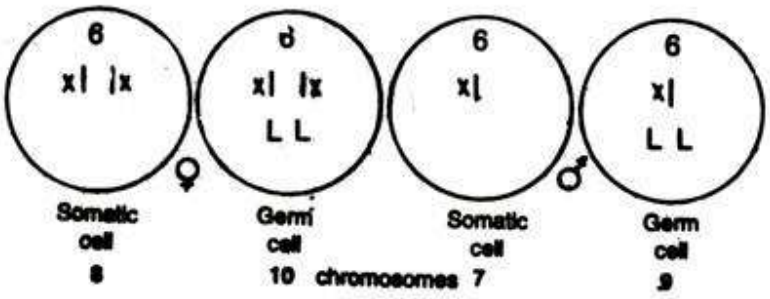


Fig. 13.14: Diagram of limited chromosome (L chromosome).

Fig. 13.14 shows the schematic representation of L-chromosome in the Sciaridae where the germ line cells of both male and female contain six autosome, sex chromosomes (two X-chromosomes in female and one X-chromosomes in male) and two L-chromosomes.

(f) Somatic Chromosome and Eliminated Chromosome:

Somatic chromosome or S-chromosome and eliminated chromosome or E-chromosome are so called because some chromosomes are retained in both somatic and germ line cells but other chromosomes are eliminated only in somatic cell during early cleavage stages of the embryo.

S and E chromosome have been found in gall insects (fam. Cecidomyiidae) and the insects belonging to the family chironomidiae (Fig. 13.15). In case of Maistor a gall insect both male and females have 48 chromosome in their germ cell and there is no loss of chromosomes. But in somatic cell, 36 chromosomes are lost in female and 42 chromosomes from male.Hence out of 48 chromosomes, 12 chromosome are present in somatic cell female and 6 chromosomes in male. Chromosomes which axe retained in both germ line cells as well somatic cells are referred to as S-chromosome. Those which are lost or eliminated from the somatic cells but Eire retained in germ cell are known as eliminated chromosome or E-chromosome.

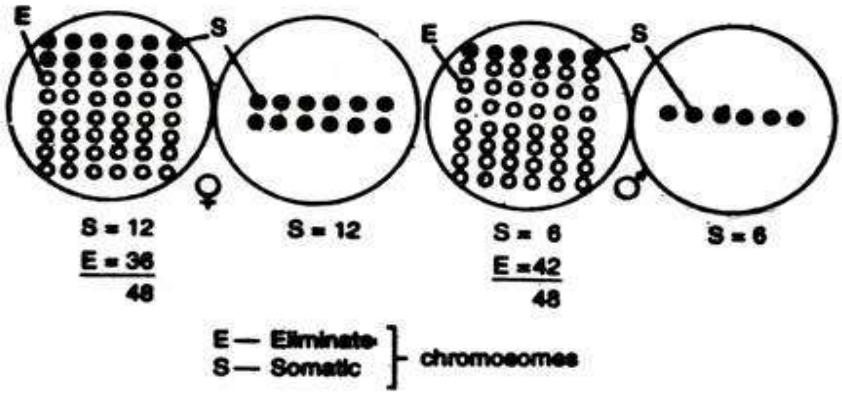


Fig. 13.15: Diagram of S and E chromosome.

(g) Special Type of Chromosomes/Giant Chromosomes:

In certain eukaryotic organisms there are special tissues where the chromosomes are of special structures not found in other cells of the same organism. These chromosomes attain their largest size in the nuclei of their respective cell. Hence these are also called giant chromosomes.

The giant chromosomes are found in the suspensors of the embryo of certain plant, cells of salivary glands of Drosophila and Chironomous, in the cells of Malpighian tubules, epithelium lining of gut of Drosophila, in the cells of fat bodies of larval stage of certain Diptera, oocyte nuclei of certain vertebrate, and invertebrate.

Special types of chromosome have been classified into two categories:
(A) Polytene chromosome and(B) Lampbrush chromosome.

A. Polytene Chromosome:

Polytene chromosomes are those giant chromosomes in which DNA is replicated in such a way that the daughter chromatids do not separate. In more details, polytene is achieved by replication of the DNA many times without nuclear division (endomitosis) and the resulting daughter chromatids do not separate and remain aligned side by side to form a giant multi-stranded chromosome.

Polytene chromosome first provided the evidence that eukaryotic gene is regulated at the level of RNA synthesis. These chromosomes are the valuable material for the study of gene regulation because their gene transcription can be seen directly in the microscope.Polytene chromosome differs from polyploidy, in which there is also excess DNA per nucleus, but in which the new chromosomes are separated from each other.

(a) Polytene Chromosomes in Animal Cells:

Polytene chromosome in animal was first observed by E. G. Balbiani in 1881 in the salivary glands of chironomous (a dipteran fly) larva. That is why these chromosomes are also known salivary gland chromosome.

These chromosomes are easy to see in light microscope as large coiled bodies about 150- 200 times as large as gonad cell chromosome. Due to their enormous large size compared to that of normal chromosome they are also called giant chromosomes.In course of investigations of polytene chromosome in other animal cells it is observed that such chromosomes also exist frequently in the other tissues such as the living cells of the gut, Malpighian tubules muscles, fat cells in some other dipteran like flies, mosquitoes and midges.

The most prominent ones are located in the salivary gland larva of Drosophila melanogaster (fruit fly). These are easily and readily available for cytogenetical study. Hence the salivary gland chromosomes become an ideal material for the purpose of practical as well as research work.In Drosophila melanogaster these chromosomes—observed in the salivary glands to late larval (3rd instar) stages—are over 100 times the length of the somatic metaphase chromosomes which measure about 7.5 μ. According to Bridges (1938) the salivary gland chromosome of Drosophila measure up to 1180 μ or even up to 2,000 μ.

Those of a related genus *Rhyncosciara*are even larger and may reach even greater dimensions as a result of pathological disturbances.The salivary gland chromosomes show somatic pairing at interphase because of their multi-stranded giant nature. Hence the number of these chromosomes in the salivary gland cells always appear to be half of the normal somatic cells (2n = 8).

Another characteristic feature of the polytene chromosome is that along the length of chromosome there is a series of dark bands alternating with other clear zones called inter-bands (Fig. 13.23).

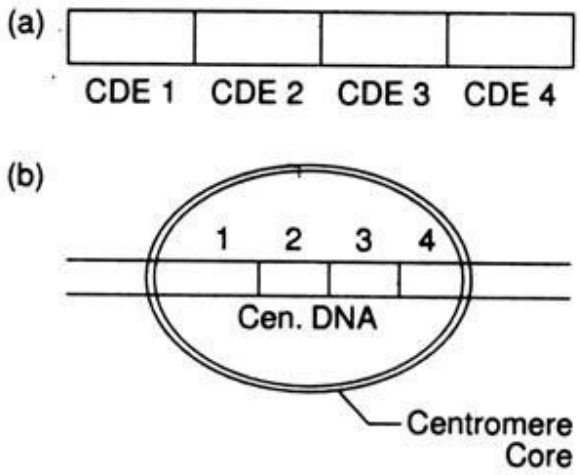


Fig. 13.23: (a) Diagram of Centromeric DNA in yeast; (b) Position of the Centromeric core particle.

The dark bands are heterochromatic in nature and stain intensely and are Feulgen positive. Furthermore they absorb ultraviolet light at 600A^0. There are about 5,000 bands in the Drosophila genome. About 85% of polytene chromosome is in bands and 15% is an inter-bands.Burke Judd et al have reported about 1,000 bands only on the X-chromosome. The bands of polytene chromosome is thought to represent a looped domain (loops of chromatin that extend at an angle from the main chromosome axis) that is highly folded as shown schematically in Fig. 13.24.

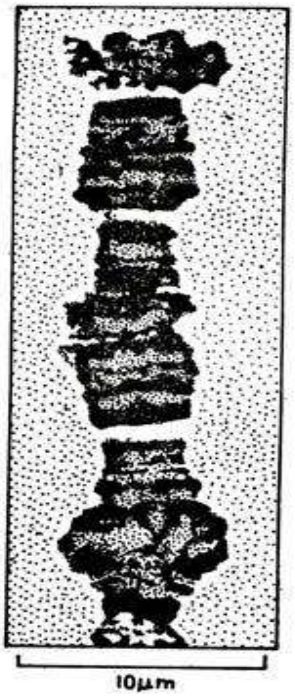


Fig. 13.24: Light micrograph of a portion of a polytene chromosome from Drosophila salivary glands showing the distinct patterns recognizable in different chromosome bands.

Depending on their size, individual bands are estimated to contain 3,000 to 300,000 nucleotide pairs per chromatin strand. Since the bands can be recognised by their different thickness and spacing's, each one has been given a number to generate a polytene chromosome "map".In polytene cell the chromosomes appear as five long strands and one short strand, attached to a central amorphous mass known as chromo Centre to which the single large nucleolus is attached (Fig. 13.25). The pericentromeric heterochromatin of all the Drosophila chromosomes coalesces in a chromo Centre.Of the six strands the short one represents chromosome 4 and the larger one represents the X-chromosome while the remaining four are the left and right arms of V shaped chromosome 2 and chromosome 3.

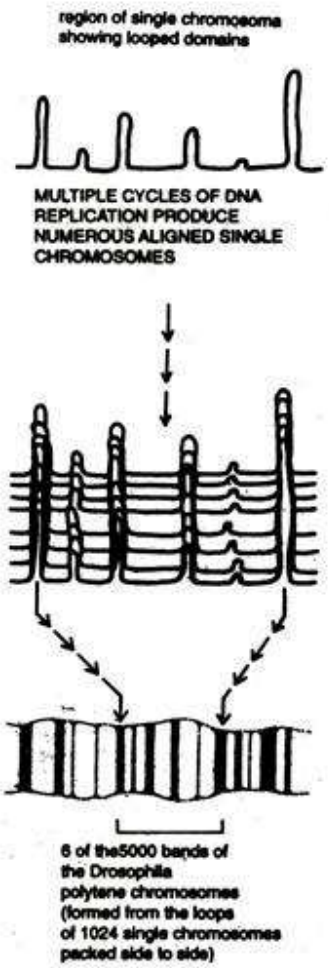


Fig. 13.25: Diagram showing how bands of polytene chromosome are thought to be generated by the side-to-side packing of homologous looped domains.

The 4th chromosome, being quite small, is almost completely inserted in chromo Centre and appears as a dot. In female flies, a pair of the X-chromosome appear as a single structure due to somatic pairing. But in male flies 'X' chromosome is single. The Y chromosome is fused within the centromere. Hence Y chromosome is not seen as a separate strand.The DNA in each of the four Drosophila chromosomes has been replicated through 10 cycles without separation of the daughter chromosomes so that $2^{10} = 10^{24}$ identical strands of chromatin are lined up side-by-side (Fig. 13.26). Other Dipteran species have more DNA molecules per polytene chromosomes, for example, *Chironomus* has 16,000.

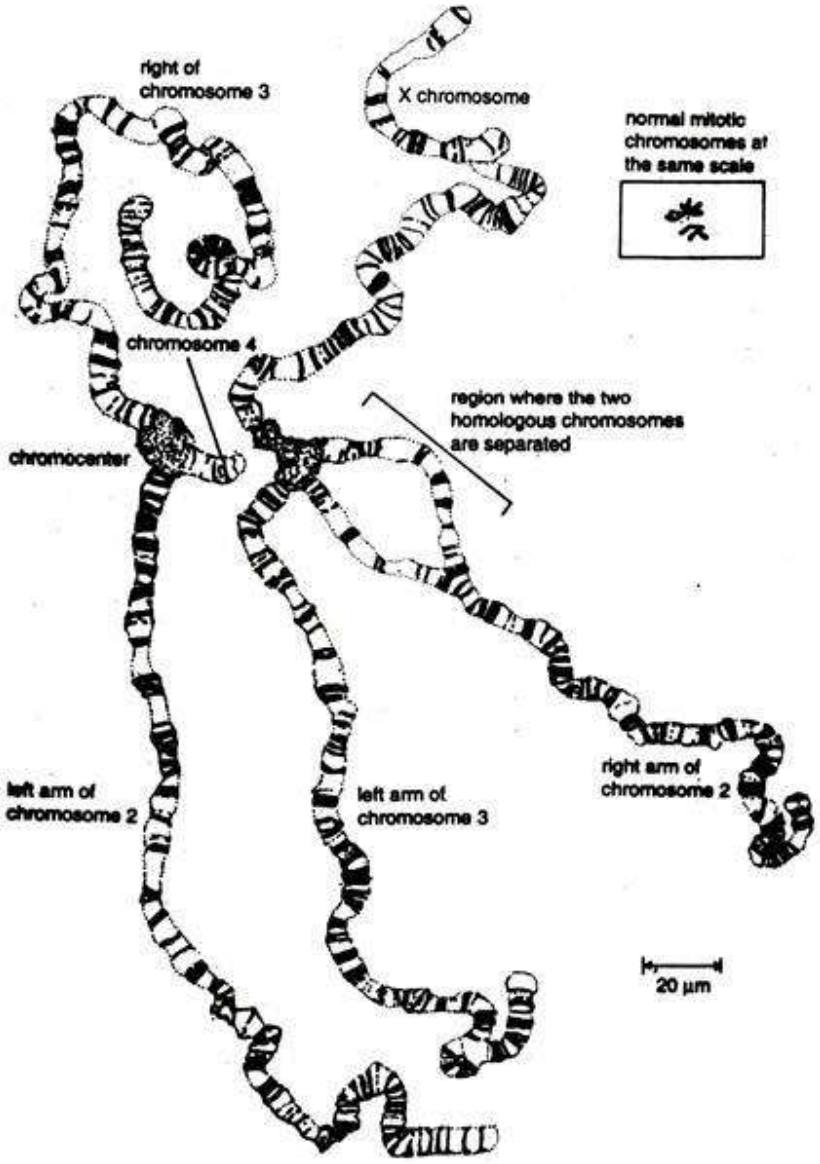


Fig. 13.26: A detailed sketch of the entire set of polytene chromosome of Drosophila.

One of the most important characteristics of polytene chromosome is that it is possible to see in them the genetic activity of particular chromosomal site at local enlargements. This is known as puffs or chromosomal puffs or Balbiani rings which are associated with differential gene activation.

A puff is considered a band in which the DNA unfolds into open loops as a consequence of intense gene transcription, i.e., RNA synthesis. Puffing is a cyclic and reversible phenomenon at definite time and in different tissues of larvae.Puffs may appear, grow and disappear. Puff formation can be identified by labelling the cell briefly with the radioactive RNA precursor 3H uridine and locating the growing RNA transcripts by autoradiography.One of the main factors controlling the activity of genes in polytene chromosome is the insects' steroid hormone ecdysone. During larval development the level of ecdysone goes

up and down—that induces the transcription of various gene coding for proteins that the larvae requires for each moult and for pupation. As the larvae progresses from one developmental stage to another new puff forms and old puff disappears as transcription units are activated and deactivated and different mRNA and proteins are made.

Electron microscopy of thin sections of a puff (Fig. 13.27) shows that the DNA is less condensed. This observations suggest that a looped domain can de-condense as a unit during transcription. It is also observed that puffing can be induced by heat shock when Drosophila larva, normally grown at 25°C, are transferred to a temperature at 37°C, a series of specific gene is activated while most other genes are deactivated.

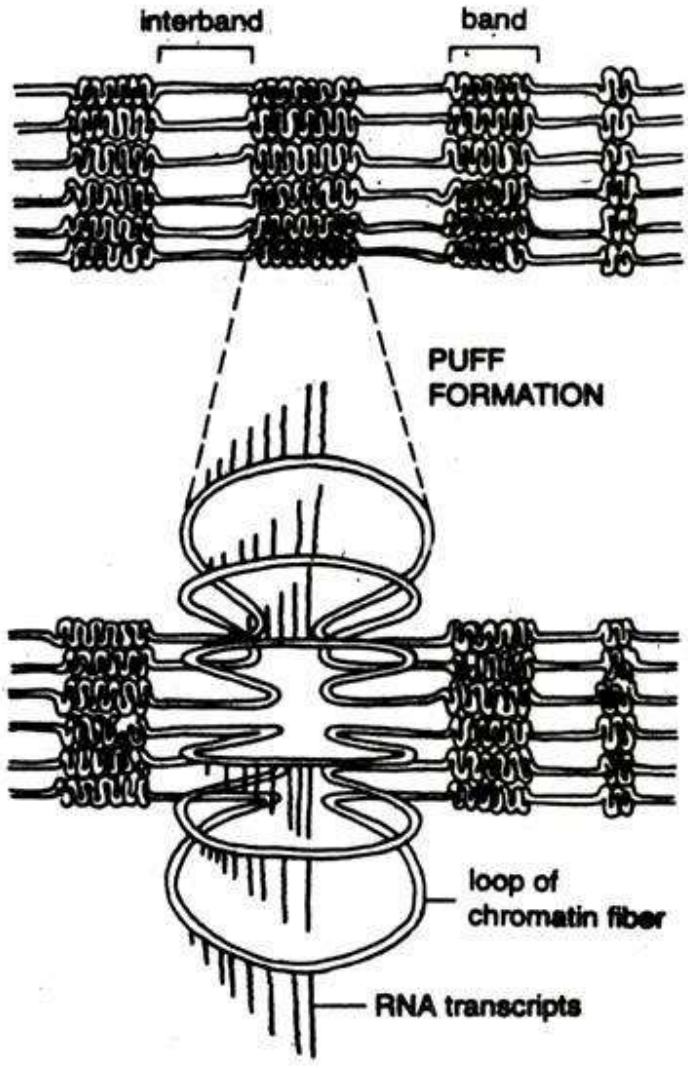


Fig. 13.27: Schematic diagram showing the process of puff formation in polytene chromosome.

B. Lampbrush Chromosomes in Animal Cell:

Lampbrush chromosome is another type of large diplotene chromosomes present in oocyte nuclei particularly conspicuous in urodele amphibians. These chromosomes have also been reported in the diplotene of oocytes of some fishes, sharks, molluscs, reptiles, birds and spermatocyte nuclei of Drosophila. Typical lampbrush chromosomes are made of a well- defined chromosomal axis, chromomeres and numerous thin lateral loop extensions. The organisation of the lampbrush chromosome is shown schematically in Fig. 13.29.

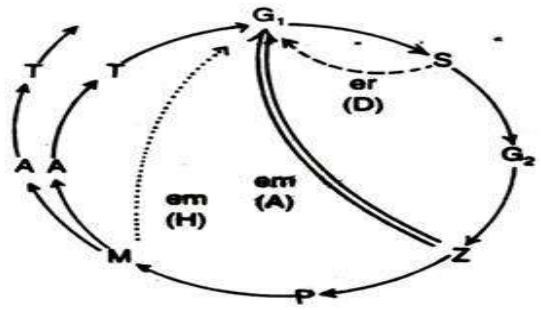


Fig. 13.29: Diagram of the mitotic cell cycle and its alteration (short-cut) in the course of endoreduplication (er) and endomitosis (em).

Lampbrush chromosomes were first observed by Fleming in 1882 and were described in detail by Ruckert in 1982. The name 'lamp- brush' was given because it is similar in appearance to the brushes used to clean the chimneys of oil lamp.

Lampbrush chromosomes have many fine lateral loops, giving them the characteristic 'hairy' appearance. Lampbrush chromosomes are found in meiotic prophase, they are present in the form of bivalents in which the homologous chromosomes axe held together by chiasmata. Each bivalent has four chromatids, two in each homologue.The axis of each homologue consists of a row of granules or chromomeres from each of which one to nine lateral loops may arise. The loops are always symmetrical, each chromosome having two of them—one for each chromatid. Lampbrush chromosome are up to 300µm long. There are about 10,000 per chromosome set. The size of loops varies from an average 9.5 µ to 200µ. An average sized loop can be estimated to contain roughly, 100,000 nucleotide pair of DNA.About 5 to 10% of the DNA is present in the lateral loop. The loops may vary in size, thickness and other morphological characteristics. Each loop has an axis formed by a single DNA molecule that is unfolded from the chromosome as a result of intense RNA synthesis.

Models of Chromosome Structure:

The chromosome of eukaryotic organism is basically made of two major components such as protein and nucleic acid like DNA. So chromosome is a nucleoprotein complex. But how the DNA protein complex builds up the chromosome structure is not clearly understood.So initially it was under speculation and several models have been proposed time to time to explain the association of proteins with DNA. After then various studies and experiments have been done on chromosome structure to understand its biological architecture and again a new lots of model have been proposed. But all models are not universally accepted.

The various chromosome models may be grouped under three heads:

a. Multi-stranded model;b. Single-stranded modelc. Nucleosome-Solenoid model.

(a) Multi-Stranded Model:

In the multiple strand model, the chromosome is supposed to be made of several nucleoprotein strands. Cytologists on the basis of their observation have proposed a number of multi-stranded models. According to some, each chromosome consists of two chromatids which is divisible into two half chromatids.

Each half chromatid is again composed of two quarter chromatids. Each quarter chromatid is composed of four chromatin fibres.Again each chromatin fibre is made of two strands. Each strand consists of a single DNA molecule plus the associated histone and non-histone proteins. DNA and proteins are held together by divalent cations like Ca^{++} and Mg^{++}.

Thus one chromatid is made of 4x2x2x2 = 32 DNA molecule. Hence a chromosome with two chromatids is composed of 32 x 2 = 64 DNA molecules. According to this model the chromosome consists of 64 double helices of DNA arranged in a parallel manner and twisted together like the strands of rope (Fig. 13.16).

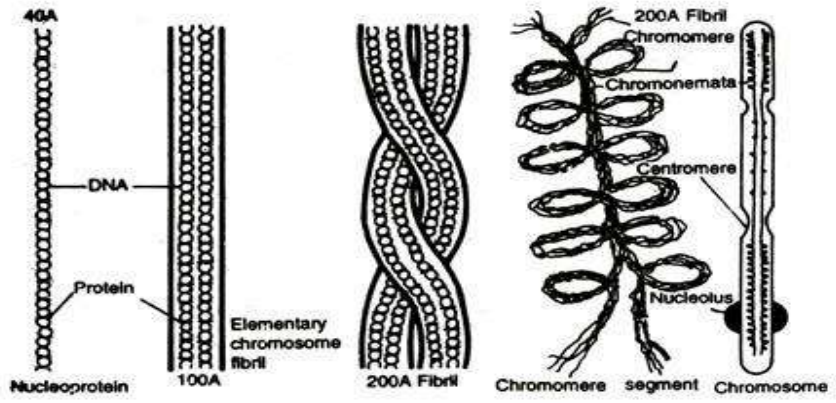


Fig. 13.16: Diagram of multistranded model.

According to Ris histone is associated with DNA in some regular but unspecific fashion to form a DNA-histone or nucleoprotein fibril. Two nucleoprotein fibrils make up the elementary chromosome fibril. Two elementary chromosome fibrils wind spirally with each other to form a fibril.

Two fibrils constitute the chromonemata which forms several loops called chromomere. Each chromatid is made of four chromonemata. Hence each chromatid apparently has 16 elementary fibrils. Most of the evidence now indicate that chromosomes are not multi-stranded except giant polytene chromosome.

(b) Single-Stranded Model:

According to this model, chromosomes are single-stranded. Taylor (1957) proposed a single-stranded model according to which the chromosome is made of a long protein back bone from which DNA coils branch-off like the legs of a centipede.

Hence this model is known as Taylor's centipede model (Fig. 13.17). The protein backbone is composed of two parallel layers of proteins and these layers can be pulled apart during replication.

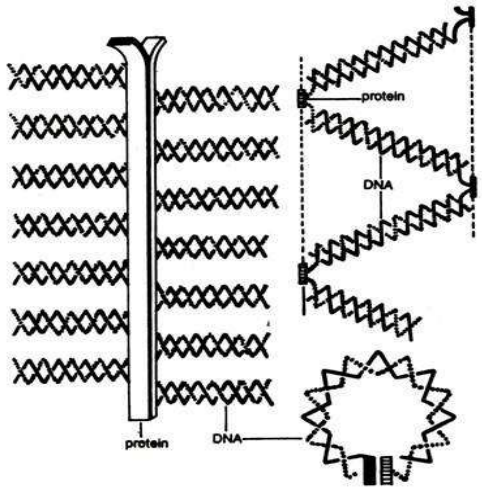


Fig. 13.17: Diagram of Taylor's centripede model.

It is thought that each layer has one strand of DNA helix on separation. On such a separated chromatid, a new chromatid could then be formed. The greatest demerit of this model is that it ignores the fact that genes are arranged in a linear fashion along the entire length of the chromosome. It is also inconsistent with genetic recombination data.

A second model was proposed jointly by Taylor and Freese. According to this model there are two protein spines instead of one. The DNA chains stretch between them like a Zigzag stair. In effect the DNA molecules are kept in position by the protein linkers (Fig. 13.14).If the linkers become closely put together they would form the axis of chromosome and the DNA would be in the form of lateral loops. The only merit of this model that it can satisfy the concept that the genes are arranged in linear fashion.Ris (1967) postulated a modified single- stranded model. According to this model DNA double helix binds with histone protein to form nucleoprotein fibrils. Folding of this fibrils takes place because of Ca^{++} bridge to form basic fibrils. The basic fibrils undergoes still further folding to form the chromosome (Fig. 13.18).

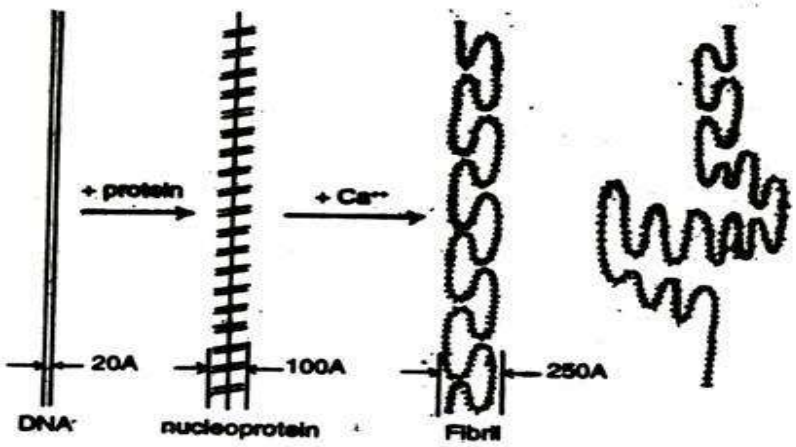


Fig. 13.18: Diagram of R is' modified model.

Du Praw, on the basis of his studies on human leucocytes under electron microscope, proposed a 'Folded-Fibre Model'—to describe the structure of chromosome. According to this model chromosomes are made of chromatin fibres. Each chromatin fibre contains only one DNA double helix which is spirally coiled and coated with histone and non-histone proteins.

The fibre then becomes folded back longitudinally and transversely and thus intertwined and forms the body of a chromatid. Two sister chromatids remain held at the centromere. The folded fibre model is applies to both the interphase and the metaphase chromosomes.During interphase folding is less and it is more at metaphase. This model is widely accepted and has been proved by various cytochemical, auto-radiographic and electron microscopic observation.

(c) The Nucleosome Model:

The nucleosome model is proposed by Roger Kornberg (1974). This model clearly explains the relationship of DNA and protein (Histone) as present within the chromosome. According to this model histones form core particle and DNA molecules coils around them so that the nucleoprotein fibre has beads on a string appearance.

(i) Histones:

There are five major types of histone molecules in the eukaryotic chromosome. These have been classified as histones H_1,H_2A,H_2B,H_3 and H_4. The histones Eire the basic proteins of low Mr (mobility rate on electrophoresis) and account for just about the same mass as the DNA.
Histones are readily isolated by salt or acid extraction of chromatin. Each histone molecule consists of a hydrophobic core region with one or two basic arms.

Histone Hi is a very lysine rich protein of about 215 amino acids. Histones H_2A and H_2B are highly conserved and are known as the slightly lysine-rich histones. The most conserved of all are the arginine rich histones H_3 and H_4.A special type of histone known as histone H_5 is found in the nucleated erythrocyte of fish, amphibians and birds. It bears many similarities to histone Hi and is thought to maintain the highly repressed state of the chromatin in these non-dividing cell. In non-dividing cells of mammals histones $H_1°$ and H_{1e} are present whereas histones H_{1a} and H_1b are present in large amounts only in dividing cells.Histones may be methylated, phosphorylated, acetylated or ADP-ribosylated and some of these modifications of histone may take place by altering the charge on the molecule which may affect the interactions of histones with each other or with DNA.
For example there are six subtypes of histone H_1 (H_{1a} – e and $H_1°$) giving rise to 14 different phosphorylated forms. Acetylation of histone H_4 in particular causes unfolding of the nucleosome core his tones and is associated with transcriptionally active segment of chromatin.
About 20% of H_2a histone is covalently linked with ubiquitin, a 76 residue polypeptide and forms a branched- chain protein known as UH2A which possibly control the gene expression. In sperm cell histones are replaced by other small basic proteins known as protamine's.

(ii) Non-histone proteins:

Besides histones, some non-histone proteins are present in chromatin in an amount approximately equal to the histone. About 100 types of different non-histone proteins have been isolated from the chromatin. Some of these are the enzymes involved in replication and transcription or to form part of the nuclear envelope.

Other non-histone proteins can be classified into two categories like low mobility group (LMG) and high mobility group (HMG) of protein on electrophoresis.Non-histone proteins are also basic protein like histone

and they are present in multiple copies in the chromatin, i.e., they play a structural role. These proteins are not tightly associated with chromatin. The N-terminal and C-terminal part of non-histone proteins are separated by a short region which is rich in serine, glycine and proline. The most characterised are HMG_1, HMG_2, HMG_{14} and HMG_{17}.

(d) Experimental Evidence in Favour of Nucleosome Structure:

Several experimental studies have been made to prove the existence of nucleosome in chromatin structure.

The studies are:

(i) X-ray Diffraction Pattern Studies:

X-ray diffraction pattern of chromatin indicated the presence of a structure repeating every 10 nm.

(ii) Electron Microscopic Studies:

i. Electron microscopy of ruptured nuclei showed the presence of a series of spherical particles connected by a fine filament—the so called beads on a string. The beads have a diameter of 7-10 nm but the length of the filaments is variable.

Much work on the structure of sets nucleosome has been carried out with the virus SV40 (Simian Virus 40). DNA of Simian Virus 40 is a circular double- stranded molecule. When added to normal culture of cells, the DNA of SV40 may become integrated into the genome of the host. Normally viral DNA is devoid of nucleosomes. But under integrated condition viral DNA may form nucleosomal organisation.

Electron micrographs of SV40 infected cells also indicated the presence of nucleosome on viral DNA. The nucleosomal form of viral chromosome is known as minichromosome. Normally, the length of naked SV_{40} DNA is 1590 nm and that of the minichromosome is about 250 nm, it is clear that there has been six to seven fold packing of the DNA into the mini-chromosome.

(iii) Digestion of Chromatin with Micrococcal Nuclease:

It is already stated that the nucleosomes grossly appear as beads on string. It is obvious that beads are connected by non-beaded string or linker DNA which holds the nucleosomes. When a small fragments of DNA containing 4-5 nucleosomes are treated with micro-coccal nuclease, it gradually digests the linker DNA but nucleosome remains partially resistant to nuclease action.

An analysis of the size of the DNA showed that the spacing between successive nucleosomes was about 200 bp. On further digestion the size of the mono-nucleosome with about 200 bp DNA is reduced first to 166 bp and finally to 146 bp and $H_1$is lost.

(iv) Crosslinking Studies:

Cross linking studies using di-methyl-suberimi-date have shown that in chromatin an octamer of histone composed of two molecules of H_2A, H_2B, H_3 and H_4 are present. Further studies have shown that one octamer is present per 200 bp DNA.

(v) Chromatin Digestion with Nuclease:

DNAse I (Nuclease) treatment makes nicks all along the length of DNA in chromatin. The nicks occur at ten base intervals. It means that the DNA is wrapped around a core of histones at a regular interval. Further studies involving the analysis of stoichemistry and X-ray crystallography have shown that one octamer is present per 200 bp DNA, i.e., per nucleosome. Each nucleosome is shallow, v-shaped structure around which a 146 bp core of DNA is wrapped making about one and three quarter turn.

(e) Nucleosome Structure:

All eukaryotic chromatin consists of nucleosomes. When interphase nuclei are ruptured by dipping them in a solution of low ionic strength, the chromatin fibres spill out of lysed nuclei. When isolated chromatin fibre is examined by electron microscope, it is seen that the chromatin fibre consists of a series of compactly organised ellipsoidal bead like particles.

The particles are joined by thin threads, a duplex of DNA. Actually a continuous duplex thread of DNA runs through the series of particles. The diameter of each particle is 110 A and the height is 60A. The beads or chromatin sub-unit is called nucleosome or Nu body.Individual nucleosome (Fig. 13.19) consists of a 146 nucleotide pair length of core DNA. Core DNA wraps round core histone by one and three quarter turns (1 3/4).

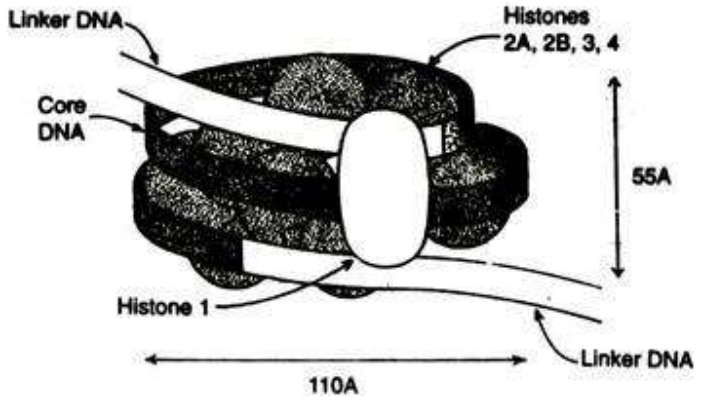


Fig. 13.19: Schematic diagram of a region of chromatin containing a nucleosome.

Each core histone is composed of octamer (Fig. 13.20) containing two copies of the four histones H_2A, H_2B, H_3 and H_4. Histone H_1 is present at one copy per nucleosome sealing the DNA entry/exit points to form a chromatosome of 166 bp and the remaining DNA forms the linker joining nucleosomes together to form oligonucleosomes. The length of the linker varies from species to species and even within tissue. Linkers as short as 8 nucleotide pairs and as long as 114 nucleotide pairs have been reported.

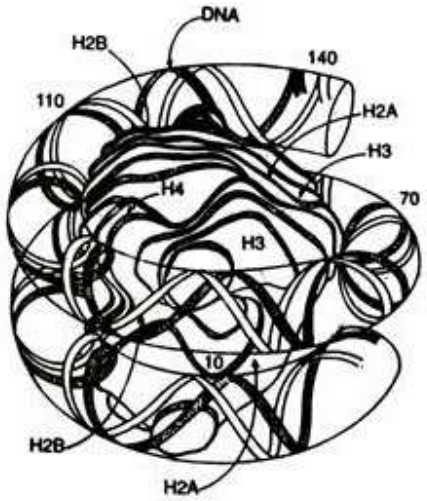


Fig. 13.20: Model of a nucleosome core showing DNA wound in a left-handed super-helix around the histone octamer.

A chromatin fibre is, therefore, made of a linear array of repeated nucleosome units plus a linker between every two nucleosomes (Fig. 13.21). Such a structural organisation constitutes a Poly-nucleosome. Under biological conditions, the nucleosome appear to be stable in position and to have little tendency to move along a length of DNA.The nucleosome play a significant role in gene expression. Gene expression is related to the transcription which involves the unwinding of DNA and may require the fibre to unfold in restricted regions of chromatin that constitute a particular gene.

The linker DNA has no problem to unwind but the unwinding may be prevented where nucleosomes are present. It, therefore, seems inevitable that transcription of active gene must involve a structural change to unwind DNA.

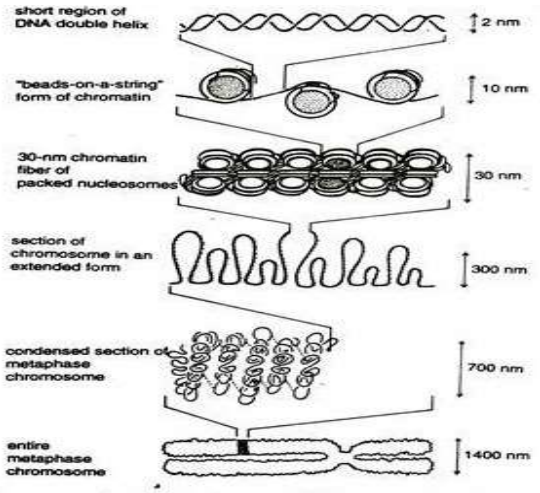


Fig. 13.21: Schematic representation showing the steps of DNA to metaphase chromosome organisation according to nucleosome model.

Again during transcription, the enzyme, RNA polymerase is essential to move along the length of template. On the basis of observation it is clear that an important structural change occurs when a gene is intensely transcribed.In case of the rRNA genes the nucleosomes are entirely displaced. Hence it seems that RNA polymerases displaces the nucleosome at the point of transcription but that the histone octamer immediately recaptures its position unless another RNA polymerase is present to prevent it from doing so.During

replication the DNA is free of nucleosomes. Once DNA has been replicated, nucleosomes are quickly generated on both the duplicates.

The diameter of a double helix of DNA is 2 nm whereas the diameter of metaphase chromatid is much thicker. Hence it is obvious that DNA undergoes a higher order of supercoiling (Fig. 13.22). The diameter of a nucleosome is about 10 nm. Therefore, in the first state of condensation, the nucleosomes are packed into a spiral or solenoid arrangement with six nucleosome per turn.

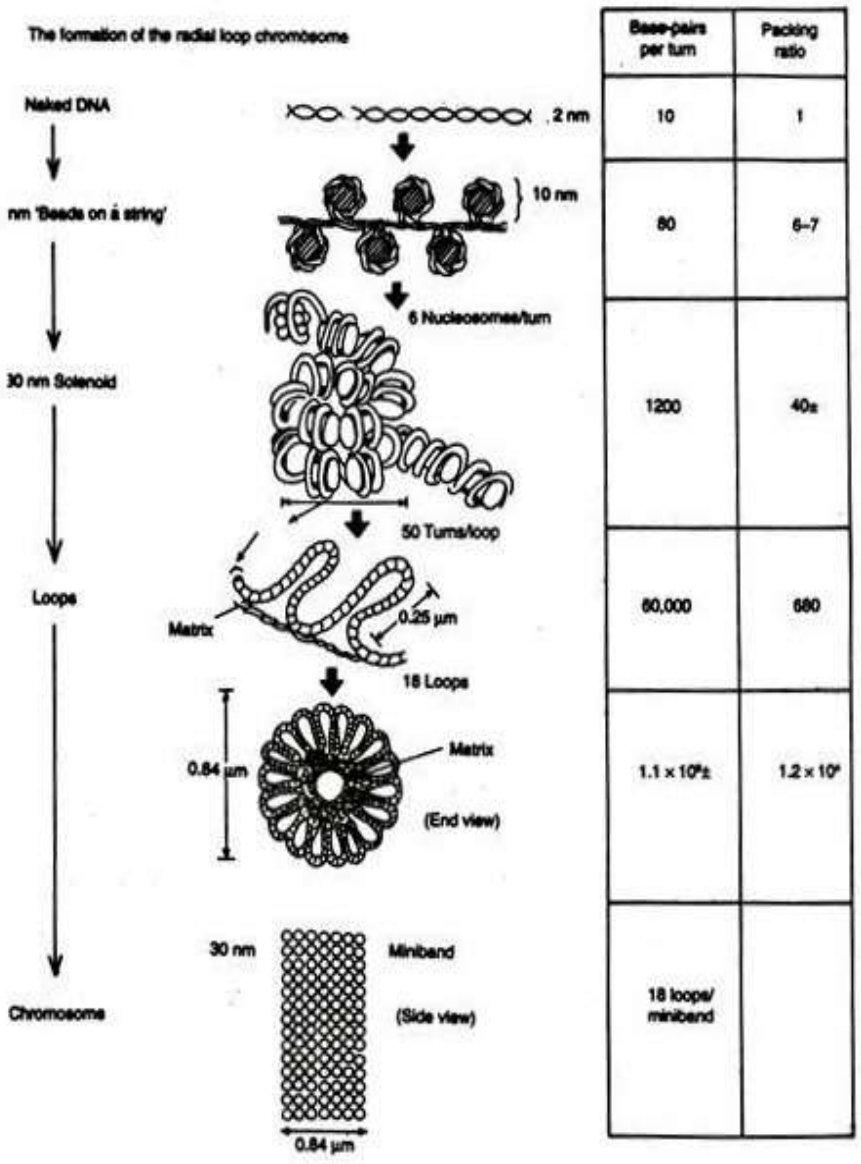


Fig. 13.22: A schematic diagram showing the higher order organisation of a chromatid. (Courtesy of RLP Adams, J. T. Knowler and D. P. Leader)

The pitch of the solenoid is 11 nm and the faces of the nucleosome are approximately parallel to the solenoid axis. The fifth histone H_1, i.e., is bound to the DNA on the inside of the solenoid. The solenoid structure then forms a number of loops around a central core or scaffold or a matrix which is made of an ill-defined fibrous protein network.The scaffold proteins also include two abundant proteins of Mr (Mobility rate) 1,70,000 and 1,35,000. The larger is DNA topoisomerase II and the smaller binds MARs (Matrix attachment regions) in co-operative fashion.
Both initiation and continued replication of DNA occur in association with matrix proteins and topoisomerase II binding sites Eire found on matrix associated DNA. The binding sites for topoisomerase II are called Scaffold Associated Regions (SARs).

Chemical Structure of Chromosomes:

Chemical analysis of eukaryotic chromosomes has shown that they are composed of deoxyribonucleic acid (DNA), ribonucleic acid (RNA) histone and non-histone proteins and certain metallic ions like Ca^{++}, Mg^{++}, etc. Primarily, chromosome contains about 90% DNA—basic protein forming a nucleoprotein complex and 10%.
RNA non-histone protein, although it is variable according to the metabolic state of nucleus. Nucleoprotein complex constitutes the backbone of chromosome while RNA non-histone protein complex is sometimes regarded as residual chromosome. The ratio of DNA— basic, protein in chromatin—is nearly 1 : 1 and remains constant over a wide range of plants and animals.

The histones axe joined with phosphate of DNA as salt linkage. The protamine's are bound to the DNA by ionic bonds. Besides this Mg^{++},Ca^{++} ions are supposed to maintain the chemical architecture of chromosome intact.

Biological Importance of Chromosome:

The chromosomes are considered the very important biological organisation because of the following reasons:

i. The genetic material DNA is localised in the chromosome and its contents are relatively constant from one generation to the next.

ii. The chromosomes retain their structure, individuality and continuity throughout the life-cycle of organism.

iii. The chromosomes maintain and replicate the genetic information contained in their DNA molecule and this information is transcribed at the right times in proper sequence into the specific types of RNA molecules which directs the synthesis of different types of proteins to form a body- form like the parents.

iv. The chromosomes form the only link between two generations and plays a significant role in the development of an organism from the zygote.

Sex Chromosomes:

Generally a genetic mechanism regulates the determination of sex in various organisms. There may be a single gene or gene complex that governs sex determination, e.g., in papaya, Asparagus and several fishes. One of the two sexes is homogametic in that it produces a single type of gametes, while the other sex is

heterogametic and produces two types of gametes, while the other sex is heterogametic and produces two types of gametes. Thus the progeny in any generation consist of both the sexes in equal ratio.

In maize, male and female flowers (unisexual flowers) are produced on the same plant (monoecious condition). A simple system involving two pairs of genes (Ba ba and Ts ts) converts this monoecious plant into a dioecious one, i.e., the male and female flowers are produced on different plants.The dominant gene Ba produces normal female flowers in the cob, but its recessive allele ba in homozygous state interferes with the cob development and produces rudimentary female flowers. Thus baba plants are functionally male.

The dominant allele Ts of the other gene produces normal male flowers in the tassel, but its recessive allele ts (tassel seed) in homozygous condition causes seed setting in the tassel making the plant functionally female.When both the dominant genes Ba and Ts are present, the plant is monoecious. The double recessive plant (babatsts) is functionally female. Similarly, the plant of genotype baba Ts/Ts develops into male, while Ba Batsts plants develop into females. The plants of baba Ts ts genotype are heterogametic male. On crossing a babatsts (female) and baba Ts ts (male) plants, male and female plants are obtained in 1: 1 ratio in the progeny. Thus maize plant can be made dioecious by two genes ba and ts.

In many cases, there operates a chromosomal mechanism of sex determination. There are specific chromosomes which carry the genes responsible for sex determination. Such chromosomes are called allosomes or sex chromosomes, while the remaining chromosomes are called autosomes (symbolized by "A"). One of the sexes is homogametic while the other is heterogametic.The sex chromosome of the homogametic sex is designated as the X-chromosome. One sex chromosome of the heterogametic sex is the same as the X-chromosome, while the other (if present) is different from the X; it is designated as Y-chromosome.Several different chromosomal mechanisms of sex determination exist in the nature (Table 7.1). In certain plant species, such as, Humulus lupulus and *Rumexacetosa*, compound sex chromosomes are known to occur.

TABLE 7.1. Chromosomal mechanism of sex determination in different organisms

Mechanisms	Homoga-matic sex	Gametes of homogametic sex	Hetero-gametic sex	Gametes of heteroga-metic sex	Example
Simple Sex Chromosomes					
XX–XO	Female (XX)	X	Male (OX)	X, O	Animals : *Protenor*, spiders, grasshopper, Orthoptera Plants : *Vallisneria spiralis*, *Dioscorea sinuata*
XX-XY	Female (XX)	X	Male (XY)	X, Y	Animals : Human, Diptera (*Drosophila*, house fly etc.), Hemiptera, Coleoptera, Some fishes and some amphibia Plants : *Melandrium (Silene)*, *Rumex*, *Humulus*, *Salix*, *Cannabis*, *Bryonia*
OX – XX (ZO – ZZ)	Male (XX)	X	Female (XO)	X, O	Animals : *Fumea* (a moth)
XY – XX (ZW – ZZ)	Male (XX)	X	Female (XY)	X, Y	Animals : Birds, reptiles, silk-worm. Plants : *Frageria* elatior
Compound sex Chromosomes					
$XX - XY_1Y_2$	Female (XX)	X	Male (XY_1Y_2)	X, Y_1Y_2	Plants : *Rumex acetosa*, *Humulus japonicus*
$X_1X_1X_2X_2$ $X_1X_2Y_1Y_2$	Female ($X_1X_1X_2X_2$)	X_1X_2	Male ($X_1X_2Y_1Y_2$)	X_1X_2, Y_1Y_2	Plants : *Humulus lupulus* var. *cordiofolius*.

The X chromosome also carries genes that have no role in sex determination; they are called sex-linked genes. In some organisms, e.g., human, the Y- chromosome is smaller, while in others, such as, *Drosophila, Melandrium*, it is larger than the X-chromosome.Further, the Y chromosome is often more heterochromatic than the X. The Y chromosome carries genes for maleness in several organisms, e.g., human, *Melandrium* and *Cocciniaindica*. Genes located on the Y chromosome are inherited from father to son; such genes are called holandric genes.

In the system where female is heterogametic (XY) and male is homogametic (XX), the Y chromosome is inherited from mother to daughter, and the genes located on this chromosome are called hologynic genes. A few genes have been located on the human Y chromosome, e.g. histocompatibility gene (H-Y) and the testis determining factor (TDF).

Origin of sex chromosomes:

The accepted hypothesis of XY and ZW sex chromosome evolution is that they evolved at the same time, in two different branches. However, there is some evidence to suggest that there could have been transitions between ZW and XY, such as in *Xiphophorusmaculatus*, which have both ZW and XY systems in the same population, despite the fact that ZW and XY have different gene locations. A recent theoretical model raises the possibility of both transitions between the XY/XX and ZZ/ZW system and environmental sex determination. The platypus' genes also back up the possible evolutionary link between XY and ZW, because they have the DMRT1 gene possessed by birds on their X chromosomes. Regardless, XY and ZW follow a similar route. All sex chromosomes started out as an original autosome of an original amniote that relied upon temperature to determine the sex of offspring. After the mammals separated, the branch further

split into Lepidosauria and Archosauromorpha. These two groups both evolved the ZW system separately, as evidenced by the existence of different sex chromosomal locations. In mammals, one of the autosome pair, now Y, mutated its SOX3 gene into the SRY gene, causing that chromosome to designate sex. After this mutation, the SRY-containing chromosome inverted and was no longer completely homologous with its partner. The regions of the X and Y chromosomes that are still homologous to one another are known as the pseudoautosomal region. Once it inverted, the Y chromosome became unable to remedy deleterious mutations, and thus degenerated. There is some concern that the Y chromosome will shrink further and stop functioning in ten million years: but the Y chromosome has been strictly conserved after its initial rapid geneloss.

There are some species, such as the medaka fish, that evolved sex chromosomes separately; their Y chromosome never inverted and can still swap genes with the X. These species are still in an early phase of evolution with regard to their sex chromosomes. Because the Y does not have male-specific genes and can interact with the X, XY and YY females can be formed as well as XX males.

Meiotic behaviour:

A very small homologous segment is present at one end of the X and Y chromosomes in which pairing occurs; as a result, the X and Y chromosomes form a bivalent during meiosis and move to opposite poles during Al.

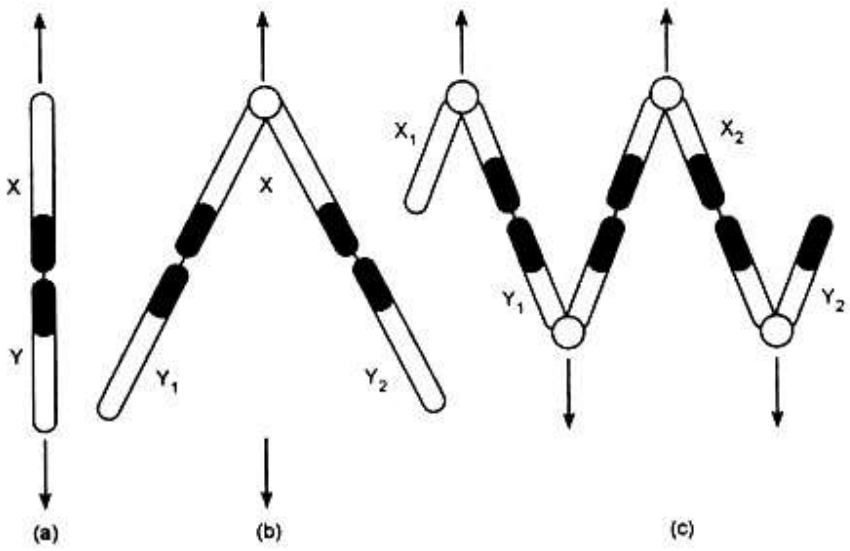


Fig. 7.6. Diagram showing orientation of the X and Y chromosomes at MI. **(a)** X and Y chromosomes oriented towards opposite poles. **(b)** The single X is oriented towards one pole, while the two Y chromosomes Y_1 and Y_2 are oriented towards the other pole. **(c)** X_1 and X_2 are oriented towards one pole, while Y_1 and Y_2 are oriented towards the other pole. The black regions denote the pairing regions in the X and Y chromosomes.

In case of compound sex chromosomes, say, $XY_1 Y_2$ system, both the arms of the X chromosome possess pairing segments. The Y_1 and Y_2 chromosomes pair in such a way that the X chromosome moves to one pole, while both the Y chromosomes move to the opposite pole (Fig. 7.6). In the X, Y,X_2Y_2 system, both the X chromosomes move to one pole, while the Y chromosomes move to the opposite pole.

In the XX-XO mechanism, the XO individuals have a single X chromosome. Their X chromosome orients itself at the metaphase plate and moves to one pole at Al, leaving the other pole devoid of this chromosome.

At All, the X chromosome divides and its chromatids move to the opposite poles. Thus only 50% of the gametes possess an X chromosome, while the remaining 50% of the gametes have no X chromosome.

Genie Balance Theory:

This theory was given by Bridges in 1921 based on his study of the progeny of crosses between triploid females and diploid males of D. melanogaster. According to this theory, the X chromosome of Drosophila carries the genes for femaleness, while the autosomes carry the genes for maleness. A balance between the number of X chromosomes and the number of sets of the autosomes determines the sex.If the ratio "X/autosomal set (A)" is equal to 1.0, the fly develops into a normal female, while if the ratio is 0.5, it develops into a normal male. The ratios falling between 1.0 and 0.5 lead to development of intersexes (Table 7.2).

Later, it was shown that the male determining factors are carried on the 2nd and 3rd chromosomes of Drosophila. The Y chromosome has no role in determination of sex but it is essential for male fertility; as a result, XO flies are phenotypically males but they are sterile.

TABLE 7.2. Chromosome constitutions and their sex expressions in *Drosophila melanogaster* and *Silene* (*Melandrium album*)

DROSOPHILA			SILENE (MELANDRIUM)		
Chromosome constitution	**X/A* ratio**	**Sex****	**Chromosome constitution**	**X/Y ratio**	**Sex****
2A+ XXX	1.5	super ♀	2A + XX	0.0 ♀	
2A + XX	1.0	normal ♀	2A + XYY	0.5 ♂	
2A + XXX	1.0	normal ♀	2A + XY	1.0 ♂	
3A + XXX	1.0	normal ♀	3A + XY	1.0 ♂	
4A + XXXX	1.0	normal ♀	4A + XY	1.0 ♂	
3A + XX	0.67	intersex	4A + XXYY	1.0 ♂	
3A + XXY	0.67	intersex	4A + XXXYY	1.5 ♂	
4A + XXX	0.75	intersex	2A + XXY	2.0 ♂	(occasional ☿ blossom)
2A + X	0.5	sterile ♂	3A + XXY	2.0 ♂	(occasional ☿ blossom)
2A + XY	0.5	normal ♂	4A + XXY	2.0 ♂	(occasional ☿ blossom)
2A + XYY	0.5	normal ♂	4A + XXXXYY	2.0 ♂	(occasional ☿ blossom)
4A + XX	0.5	sterile ♂	3A + XXXY	3.0 ♂	(occasional ☿ blossom)
3A + XY	0.33	super ♂	4A + XXXY	3.0 ♂	(occasional ☿ blossom)
			A4 + XXXXY	4.0 ♂	(occasional ♂ blossom)
			4A + XXXX	0.0 ♀	

*A = set of autosomes ; **♂ = male; ♀= female; ☿ = hermaphrodite (bisexual)

X-Y balance:

In several organisms male determining genes are present on the Y chromosome while the female determining genes are located on the X chromosome; sex is determined by the balance between the X and Y chromosomes.In human, Y chromosome is strongly male determining (Table 7.3). In the absence of Y chromosome, the phenotype of the individual is female. Thus a female phenotype develops in individuals of XO, XX, XXX, XXXX, XXXXX constitution. However, in presence of a single Y chromosome, they all (XY, XXY, XXXY, XXXXY, XXXXXY) develop into males.

TABLE 7.3. Sex determination in human

Chromosome complement	Sex phenotype
2A + X	female, sterile (Turner's syndrome)
2A + XX	female, normal
2A + XXX	female, fertile (metafemale)
2A + XXXX	female, fertile ? (metafemale)
3A + XXX	female, (triploid)
2A + XY	male, normal
2A + XYY	male, fertile
2A + XXY	male, sterile (Klinefelter's syndrome)
2A + XXXY	male, sterile (Klinefelter's syndrome)
2A + XXYY	male, sterile
3A + XXY	male, (triploid)

Y chromosome:

The Y chromosome is the sex chromosome confined to the heterogametic sex in the XX-XY system of sex determination (in contrast, X chromosome is found in both the sexes although the heterogametic sex has only one copy).

The size and function of the Y chromosome vary in different organisms. In Drosophila, the Y chromosome is slightly larger than the X chromosome; it is heterochromatic and does not carry the genes for maleness.However, it is necessary for the fertility of the males since XO male Drosophila are sterile. In contrast, in the case of humans and several plants like Melandrium, the Y chromosome possesses male determining genes. In human, the Y chromosome is very small, but it is strongly male determining so that individuals with XXXXY and mosaics with XXXY/XXXXY/XXXXXY constitution develop the male phenotype.

Genes for maleness are located on the short arm of human Y so that XY individuals with deleted short arm of the Y chromosomes develop the female phenotype. The segments bearing male determining genes in the short arm of Y may be trans-located to the X chromosome; in this case the XX individuals develop the male phenotype.In Silene (Melandrium), Y chromosome is much larger than the X chromosome. It possesses the following four distinct regions (Fig. 7.7).

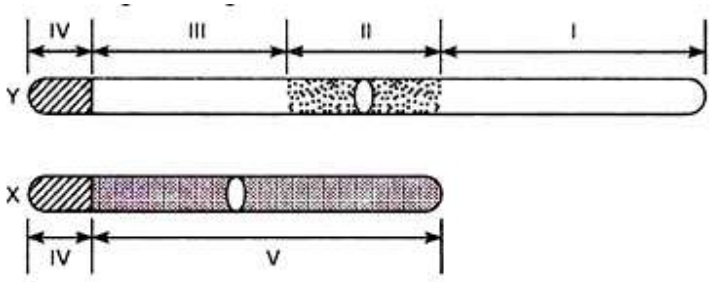


Fig. 7.7. Diagram of Y and X chromosomes of *Silene* (*Melandrium*) showing regions having different activities. Region I : female suppressor region (when absent, leads to bisexual development). Region II : essential male promoting region (when absent, leads absent, leads to female development). Region III : essential male fertility region (when absent, anthers abort). Region IV : Pairing region in both, X and Y. Region V (on X chromosome) : differential portion of the X (genes for femaleness). Segment lengths in this diagram are arbitrary).

(I) Female suppressor region:
It is located at the end of the Y chromosome and carries the genes for suppression of the female reproductive organs. In the absence of this region, hermaphrodite (bisexual) flowers are produced on the XY plant.

(II) Male promoting region:
This region lies next to the first (female suppressor) region and carries genes for initiation of anther development. Absence of this region causes the production of female flowers on the XY plant.

(III) Male fertility region:
It lies next to the second (male promoting) region and carries the genes for male fertility. When this region is absent, anthers abort and the plant is male sterile.

(IV) Pairing region:
It is located at one end of the Y chromosome and is homologous to a region in the X chromosome. During meiosis, the X and Y chromosomes pair in this region which ensures their proper separation during AI. The X chromosome of Silene possesses a differential region (V) which carries genes for the development of female reproductive organs (Fig. 7.7).

Dosage compensation:

In the sex determining system where XX individual is female and XY individual is male, females contain two X chromosomes, whereas males contain only one X chromosome. Apart from the genes governing the sex, other genes are also present in the X chromosome; they are called sex-linked genes.

These genes are in homozygous or heterozygous condition in the females (XX), but they are in hemizygous condition in the males (XO, XY) since they do not have corresponding alleles in the Y chromosome. But males and females are morphologically and physiologically similar in expression of these genes.The mechanism by which the effects of sex- linked genes in males (XO, XY) are equalized to their effects in females (XX) is known as dosage compensation.

In Drosophila, this mechanism operates by enhancing the activity of the X-linked genes in males, while the activity of these genes in the two X chromosomes of females is restrained. The mechanism of dosage compensation in man and other mammals differ from that in Drosophila.One of the two X chromosomes of the females is inactivated through heterochromatinization so that only one X chromosome remains active. Thus there is a balance of X-linked gene activity in the females and males.

Single active X-hypothesis or Lyon hypothesis of dosage compensation: The dosage compensation in human and other mammals is regulated by the inactivation of one X chromosome in the females. This is known as single active X hypothesis or Lyon hypothesis proposed by Lyon in 1961 and elaborated subsequently.The main genetic evidence for this hypothesis comes from the mosaic phenotype of female mice heterozygous for sex-linked recessive genes that affect coat colour.

According to this hypothesis:

(i) One of the two X chromosomes in the cells of normal female mammals is genetically inactive.

(ii) Inactivation occurs early in embryonic development.

(iii) The inactive X chromosome may be maternal paternal one in the different cells of the same animal.

(iv) The decision as to which X chromosome becomes inactive is taken at random. Once an X chromosome is inactivated in a cell, the same X chromosome will always be inactivated in all its progeny cells.

(v) The inactivation occurs due to heterochromatinization. The hetero-chromatinized X chromosome forms the sex chromatin observed during interphase, and is late replicating.

There occurs a preferential heterochromatinization of abnormal X chromosome (Table 7.4). If one X is normal and the other is an iso-X-chromosome, the iso-chromosome is always heterochromatic. In man, inactivation of sex-linked genes has been demonstrated at cellular level, for example, glucose-6-phosphate-dehydrogenase (G6PD), Hunter-hurler syndrome, Juvenile hyperuricaemia.

TABLE 7.4. Chromosome constitution, sex chromatin and sex phenotype in human

Chromosome constitution**	Maximum number of sex chromatin bodies	Maximum number of late replicating X chromosomes*	Sex phenotype
45, X	0	0	♀ Turner's syndrome/ovarian dysgenesis
46, XX	1	1	♀ Normal
46, XXP–	1	1	♀ Turner's syndrome/ovarian dysgenesis
46, XXq–	1	1	♀ Turner's syndrome/ovarian dysgenesis
46, XXpi	1	1	♀ Turner's syndrome/ovarian dysgenesis
46, XXr	1	1	♀ Turner's syndrome/ovarian dysgenesis
47, XXX	2	2	♀ Normal, mentally deficient
48, XXXX	3	3	♀ Mental deficiency
49, XXXXX	4	4	♀ Mental deficiency
46, XY	0	0	♂ Normal
47, XYY	0	0	♂ Fertile, tall, aggressive
47, XXY	1	0	♂ Klinefelter's syndrome
48, XXXY	2	2	♂ Klinefelter's syndrome
48, XXYY	1	1	♂ Klinefelter's syndrome, tall, aggressive
49, XXXXY	3	3	♂ Infertile, somatic anomalies
46, XY#	0	0	♂ testicular feminization or intersex
46, XY##	1	1	♂ Klinefelter's syndrome or intersex (sex reversed females)

* Abnormal X chromosome is invariably the late replicating one.

** Xp– = deletion in the short arm of X ; Xq– = delection in the long arm of X ; Xpi = iso-chromosome for short arm of X; Xr = ring X chromosome.

Sex reversed genetic males produced due to some genes acting on the target organs making them insensitive to testosterone. It causes the development of female phenotype.

The XX males may be either (i) potential mixoploids (46, XX/47, XXY) from which the 47, XXY line was lost after sex determination, or (ii) XXY syndrome from which Y chromosome was lost, or (iii) male determining factors of Y were translocated to one X or to some autosome, or (iv) sex reversed females caused by some autosomal sex reversal gene.

♀= female; ♂ = male.

Sex chromatin (Barr body) Drum sticks:

Sex chromatin is the hetero-chromatinized X-chromosome observed as a condensed body in interphase nuclei of mammalian females. It was discovered by Barr and Bartram in 1949 in the neurons of cat and was called Barr body after M.L. Barr.

Generally the sex chromatin is observed as a planoconvex body lying adjacent to the inner surface of the nuclear membrane. A detailed study shows that it has V or U-shaped structure and its apex points towards the centre of the nucleus.The size of sex chromatin ranges from 0.7 x 1.0 μm to 1.0 x 1.4 μm with an average of 0.8 x 1.1 μm in the different tissues and species. Sex chromatin is not visible in all the interphase nuclei of females; the frequency of cells showing sex chromatin varies in the different tissues of the same species.The frequency of **"sex chromatin positive"** nuclei is 85% in the nervous tissues, 96% in the whole mounts of amnion epithelium and from 20-25% to 60-70% in oral smears. It has been found that the number of haploid autosome complements influences the number of late replicating X chromosomes.

The relationship between numbers of sex chromatin, autosomal set and the X chromosome has been expressed by the following formula:

$$B = X - (p/2) \quad (7.1)$$

where, B = number of sex chromatin bodies

X = number of X chromosomes

P = number of autosomal sets

The above relationship holds for all even degrees of ploidy, viz., 2n, 4n, 6n, 8n, etc. Thus in human, a tetraploid cell (2n = Ax = 92, XXXX) has two sex chromatin bodies. But a tetraploid cell with XXYY constitution (92, XXYY) will not show any sex chromatin. In triploids (69, XXX), some cells have one and others have two sex chromatin bodies (average 1.5 per cell).

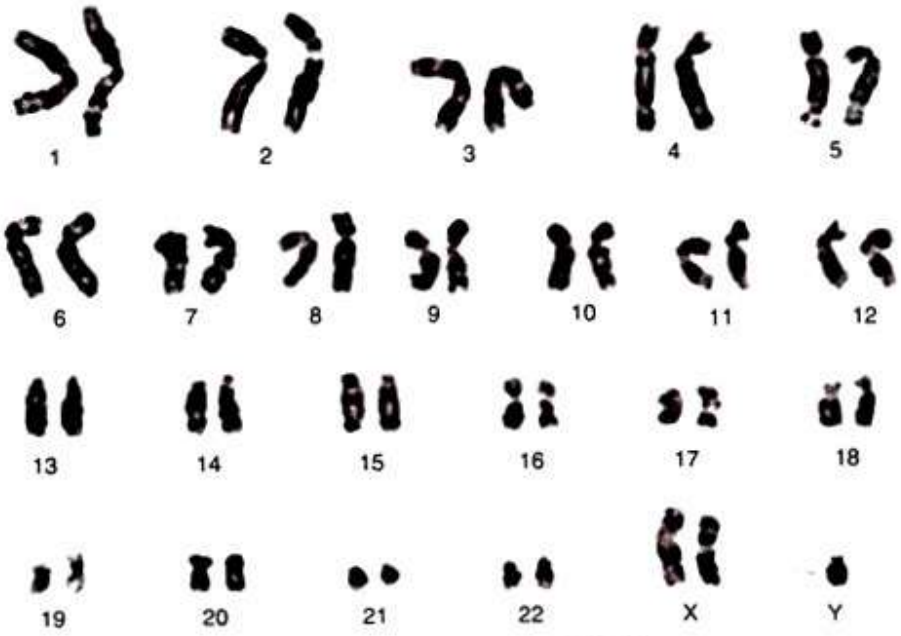
The abnormal males, such as, XXY (Klinefelter's syndrome) also show sex chromatin in their cells (Table 7.4). Abnormal X chromosome is, as a rule, always hetero-chromatinized. The Y chromosome has no role in sex chromatin formation; XYY males do not show any sex chromatin. Thus sex chromatin can be used as a direct clinical test to determine the number of X chromosomes in an individual.Davidson and Smith in 1954 found some bodies similar to sex chromatin in the circulating polymorphonuclear neutrophil leucocytes of human blood. This body is like a drum stick and is attached to one lobe of the polymorph nucleus.However, the frequency of drum sticks is very low, viz., 1 in 40 leucocytes in normal females and < 1 in 500 leucocytes in normal males. The drum sticks probably represent the hetero-chromatinized X chromosome.

Sex Chromosome Anomalies:

i. Klinefelter's Syndrome:

In 1942 Klinefelter described a condition in phenotypic males which turned out to be due to an extra X chromosome (47, XXY). The affected individuals appear normal in childhood, the abnormalities becoming visible only in adult males. The syndrome is characterised by absence of spermatogenesis, gynaecomastia, and excessive secretion of gonadotropins in the urine.

Since buccal smears of Klinefelter's males show Barr bodies, they are referred to as chromatin-positive males. Most of the patients are mentally retarded and develop a variety of psychiatric problems. Although many have the karyotype 47, XXY, some may have 48, XXXY, 49, XXXXY, or 48, XXYY, or they may be cytogenetic mosaics.



Klinefelter syndrome—47,XXY

ii. Turner's Syndrome:

This is shown by females characterised by a short stature, gonadal dysgenesis, sexual infantilism, webbed neck, prominent ears, cubitus valgus (increased carrying angle of the arms) dystrophy of the nails and hypoplastic nipples.

Their sex chromosome constitution is XO and they have only 45 chromosomes. They are chromatin-negative females as they do not show Barr bodies. They are frequently mosaics with more than one cell line such as XO/XXX, XO/XX/XXX, and others. The incidence is one in about 5,000 births.

iii. The XYY Male:

In 1965 Jacobs et al found that many of the men kept in institutions for the retarded due to aggressive and antisocial behaviour have 47 chromosomes with XYY sex chromosomes. They are usually tall but not always mentally retarded, frequently show hypogonadism and are sterile.Males inherit an extra Y chromosome--their genotype is XYY. As adults, these "super-males" are usually tall (above 6 feet) and generally appear and act normal. However, they produce high levels of testosterone. During adolescence, they often are slender, have severe facial acne, and are poorly coordinated. They are usually fertile and lead ordinary lives as adults. Many, if not most, are unaware that they have a chromosomal abnormality. The frequency of XYY syndrome is not certain due to statistical differences between different studies. It may be as common as 1 in 900 male births to as rare as 1 in 1500 or even 1 in 2,000. XYY syndrome is also referred to as Jacobssyndrome. arly studies of XYY syndrome done in European prisons initially led to the erroneous conclusion that these men were genetically predisposed to antisocial, aggressive behaviour, below

average intelligence, and homosexuality. Contributing to the early view that XYY syndrome men have serious personality disorders was the case of Richard Speck. In 1966, he coldly murdered 8 nurses in a Chicago dormitory. At his trial, his lawyer claimed that he was innocent due to uncontrollable urges caused by his XYY genotype. This novel appeal was akin to claiming insanity or severely diminished mental competence. The jury was not convinced and found him guilty of murder. He was sentenced to life in prison where he eventually died. In fact, Richard Speck did not have an XYY genotype. However, some researchers suggest that the high testosterone levels of XYY men can make them somewhat more prone to violence and that this may cause higher rates of wifebeating. The presence of two Y chromosomes can be recognized as two brightly fluorescent bodies by proper staining. The discovery of this syndrome received publicity because of the possible association of a chromosome anomaly with human behaviour.

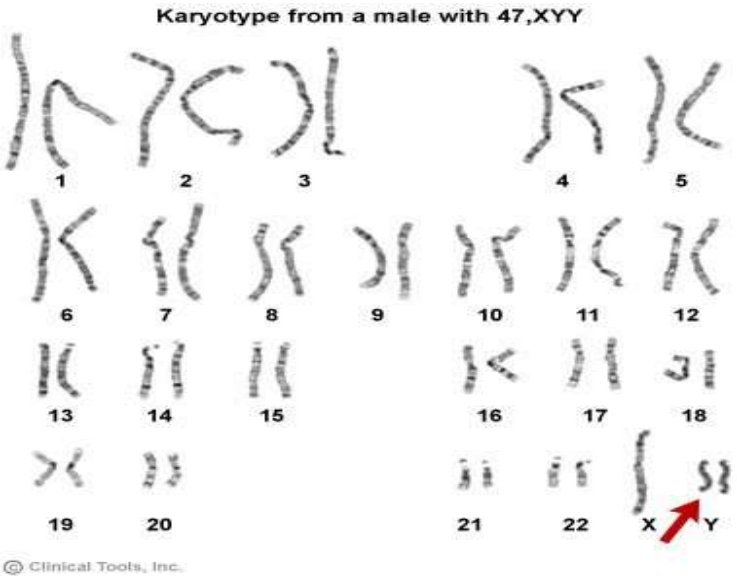


Fig : 47,XYY karyotype showing extra Y Chromosome at right side

iv. The Triple-X Syndrome:

These are individuals with 3X chromosomes designated super-females. They are mentally retarded, sexually normal and fertile. Although most triple-X females have 47, XXX karyotype, some may have 48, XXXX, 49, XXXX, and still others may be mosaics. They show 2, 3 or 4 Barr bodies in their buccal smears (always one Barr body less than the total number of X's).

It occurs in women who inherit three X chromosomes--their genotype is XXX or more rarely XXXX or XXXXX. As adults, these "super-females" or "metafemales", as they are sometimes known, generally are an inch or so taller than average with unusually long legs and slender torsosbut otherwise appear normal. They usually have normal development of sexual characteristics and are fertile but tend to have some ovary abnormalities that can lead to premature ovarian failure. They may have slight learning difficulties, especially in speech and language skills, and are usually in the low range of normal intelligence (especially the XXXX and XXXXX individuals). They frequently are very tall in childhood and tend to be emotionally

immature for their size. This sometimes results in teachers and other adults labeling them as troublemakers because they expect more maturity from bigger girls. However, they are usually as emotionally mature as other girls of their age. None of these traits prevent them from being sociallyacceptedasordinaryadultwomen.Individualswhoaregeneticmosaics(XX/XXX)have less noticeable symptoms. Triple-X syndrome is less rare than Turner syndrome, but little is known about it. The frequency is approximately 1 in 1,000 female infants and it occurs more commonly when the mother isolder.

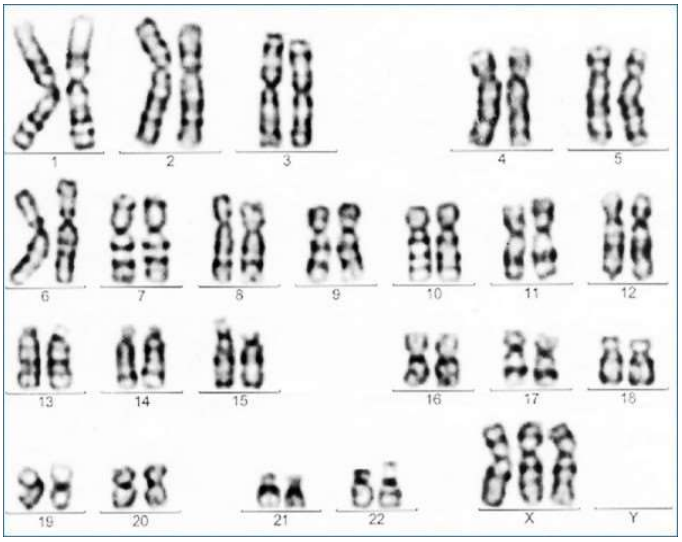


Fig : Karyotype of Triple XXX Syndrome

v. Intersex:

Individuals with both male and female gonadal tissues are called hermaphrodites (from Greek Hermaphrodites, the son of Hermes and Aphrodite). Their karyotype analysis shows that they are mosaics having both X and Y chromosomes in their cell lines. Their buccal smears may or may not show a Barr body. Their external genitalia are often ambiguous, and they are almost always sterile.

The condition of pseudo-hermaphroditism is also included among intersexes. Such individuals are cytogenetically normal with 46, XY (male pseudo-hermaphrodites) or 46, XX (female pseudo-hermaphrodites) chromosomes and normal buccal smears for one sex only. But phenotypically they show both male and female characters.There are two classes. Male pseudo-hermaphrodites that have testes and either ambiguous or female-like external genitalia. The female pseudo-hermaphrodites have ovaries and either ambiguous or male like external genitalia. The pseudo-hermaphrodites have some defect in the biosynthesis of testosterone in the testes or in the adrenal glands or in both.

Sex Mosaics:

In genetics, a mosaic, or mosaicism, involves the presence of two or more populations of cells with different genotypes in one individual who has developed from a single fertilized egg. Mosaicism has been reported to be present in as high as 70% of cleavage stage embryos and 90% of blastocyst-stage embryos derived from in vitro fertilization.

Genetic mosaicism can result from many different mechanisms including chromosome non- disjunction, anaphase lag and endoreplication. Anaphase lagging is the most common way by which mosaicism arises

in the preimplantation embryo. Mosaicism can also result from a mutation in one cell during development in which the mutation is passed on to only its daughter cells. Therefore, the mutation is only going to be present in a fraction of the adult cells.

Genetic mosaics may often be confused with chimerism, in which two or more genotypes arise in one individual similarly to mosaicism. However, the two genotypes arise from the fusion of more than one fertilized zygote in the early stages of embryonic development, rather than from a mutation or chromosome loss.Most people have 46 chromosomes in each of their cells, and two of those 46 chromosomes are sex chromosomes. Most girls and women have two X sex chromosomes (so we say their chromosomal component is "46,XX"). Most boys and men have an X sex chromosome and a Y sex chromosome ("46,XY").

But some people have "mosaic" chromosomes, meaning that not all their cells have the same component of chromosomes. This is called "mosaicism" because it is sort of like the body is made up of a varied set of colored tiles, rather than a single-colored set of tiles. When a person
has more than one component of sex chromosomes, the person is said to have "sex chromosome mosaicism."So, some people have 46,XX in some cells with 46,XY in other cells. Some may have 46,XY in some cells and 47,XXY in other cells. Some may have 45,X in some cells and 46,XX in others. Many other variations are possible.

In some insects sometimes one part of the body is male, the other part female producing sex mosaics known as gynandromorphs or gynanders. These have been studied most thoroughlyin Drosophila which has no sex hormones so that tissues develop autonomously. The sexual phenotype is determined by the number of X chromosomes against sets of autosomes. If during mitosis in early embryogenesis there is nondisjunction between the two X chromosomes, some cells will have XX, others XO constitution.The descendants of XX containing cells will result in the development of female tissues while descendants of XO cells will produce male tissues. The resulting individual will be a spectacular mosaic. Gynandromorphs are usually bilateral with one side of the body male, the other side female.

There are irregular gynandromorphs also in which the proportions of male to female tissues are variable depending upon the time and stage of embryo development when XX nondisjunction took place. Sex mosaics are sometimes mistaken for intersexes. In a gynandromorph the boundary between male and female regions is always sharp and distinct, whereas in intersexes, all parts of the body may have a mixture of male and female characteristics showing a condition intermediate between maleness and femaleness. Sex mosaicism occurs in humans also. Chromosome preparations from peripheral blood may show XO/XX or XO/XXY cells frequently observed in Turner's and Klinefelter'ssyndromes.

Sex Limited Genes:

Sex limited genes are those which produce characteristics that are expressed in only one of the sexes. They are often confused with sex linked genes, but are entirely different in their mode of inheritance. Sex limited genes may be located in any of the chromosomes, while the sex linked genes are located only in the X or Z chromosome. Sex limited genes are responsible for secondary sexual characteristics as well as primary characters.

The beard in males is a good example in man. Both the males and the female carry all the genes necessary to produce a beard, but only man shows this trait. However, in rare cases, hormone imbalance in a woman results in a bearded lady. Similarly, breast development is normally limited to women, but hormone imbalance may cause breast development in men.In vertebrates, the sex limited characteristics depend upon

the presence or absence of one of the sex hormones. For instance, the genes for masculine voice and masculine musculature depend on the presence of male hormones.

A castrated male will have female voice even though no female hormones are present. The genes for feminine voice and feminine musculature express themselves in the absence of the male hormone. They do not require the presence of female hormones.Thus, certain sex limited characteristics are expressed in the presence of sex hormones, while certain others are expressed in the absence of certain hormones. The sexual dimorphism in birds is another good example of sex limited inheritance. The bright plumage of the male peacock is a bold contrast to the dull plumage of the female.

Sex Influenced Traits:

The phenotypic expression of a number of autosomal and sex linked genes will be either dominant if the individual is a male or recessive if the individual is a female. These genes are known as sex influenced traits. A classic example is the pattern baldness in man.

A male shows this trait more than a female, because a male is bald if he has only one gene, whereas a woman must receive two genes to be bald. This is because a single gene can operate in the presence of a male hormone.

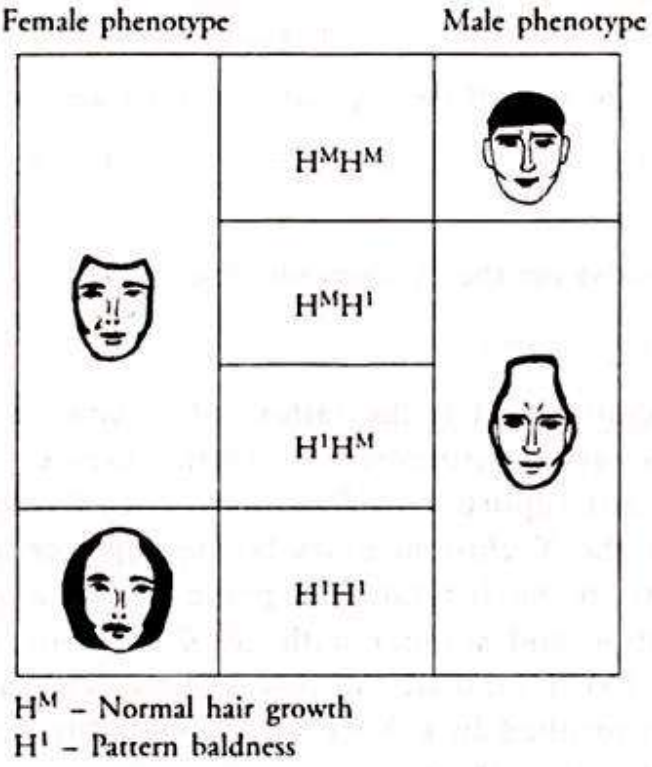


Fig. 16 Pattern baldness in man.

Another example is the length of the index finger. When the hand is placed so that the tip of the fourth finger touches the horizontal line, it will be noted that the index or second finger will not touch this line in many cases (Fig. 17). This short index finger is due to a gene which is dominant in the male and recessive in the female.

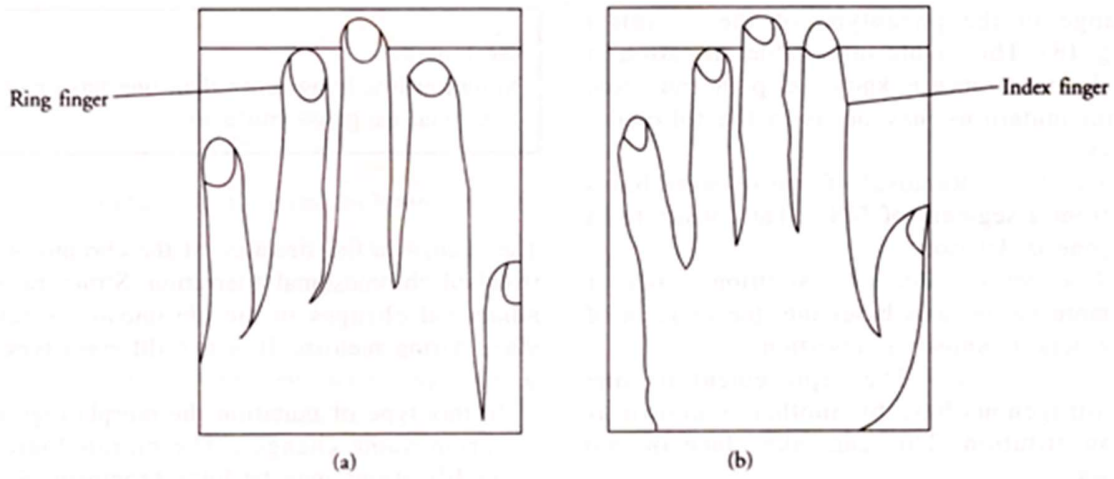


Fig. 17 Sex-influenced inheritance of length of index finger.

Y Chromosome Inheritance:

Y linked genes are genes located in the Y chromosomes. The inheritance of the Y linked genes, also known as holandric genes, is known as Y chromosome inheritance. An example is hypertrichosis, which is the growth of long hair in the ear.

Probable Questions:

1. Briefly describe morphology of a ideal chromosome.

2. Classify chromosome on the basis of position of centromere.

3. What are the differences between primary and secondary constrictions?

4. Define Karyotype and ideogram. What are the differences between asymmetric and symmetric karyotype.

5. Write a short notes on B chromosome.

6.5. Write a short notes on Lampbrush chromosome.

7. Write a short notes on Polytene chromosome.

8. Define mega, micro and limited chromosome.

9. Describe solenoid model of chromosome structure

10. Describe single stranded and double stranded model of chromosome structure.

11. Write down the chemical structure of chromosome.

12. What are the biological importance of chromosome?

13. Describe genic balance theory.

14. Describe the structure of Y chromosome.

15. Describe Dosage compensation.

16. What is Barr body?

17. What are the characteristics of Klinefelter syndrome?

18. What are the characteristics of Turner's syndrome?

19. What are the characteristics of XXX syndrome?

20. What is intersex. State its characteristics.

21. What is sex limited traits. Give examples.

22. what is sex influenced traits. Give examples.

Suggested readings:

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7th Edition.
6. Gene cloning and DNA Analysis by T. A. Brown, Sixth Edition.
7. Genetics . Verma and Agarwal.

Unit-VI

Behavioral genetics influence of single defects on behavior; Genetic analysis of behavior in experimental animals, chromosome anomalies and insight into human behavior

Objective: In this unit you will learn about Behavioral genetics influence of single defects on behavior; Genetic analysis of behavior in experimental animals, chromosome anomalies and insight into human behavior.

BehaviouralGenetics:

Behavioural genetics, also referred to as behaviour genetics, is a field of scientific research that uses genetic methods to investigate the nature and origins of individual differences in behaviour. While the name "behavioural genetics" connotes a focus on genetic influences, the field broadly investigates genetic and environmental influences, using research designs that allow removal of the confounding of genes and environment. Behavioural genetics was founded as a scientific discipline by Francis Galton in the late 19th century, only to be discredited through association with eugenics movements before and during World War II. In the latter half of the 20th century, the field saw renewed prominence with research on inheritance of behaviour and mental illness in humans (typically using twin and family studies), as well as research on genetically informative model organisms through selective breeding and crosses. In the late 20th and early 21st centuries, technological advances in molecular genetics made it possible to measure and modify the genome directly. This led to major advances in model organism research (e.g., knockout mice) and in human studies (e.g., genome-wide association studies), leading to new scientificdiscoveries.

Findings from behavioural genetic research have broadly impacted modern understanding of the role of genetic and environmental influences on behaviour. These include evidence that nearly all researched behaviors are under a significant degree of genetic influence, and that influence tends to increase as individuals develop into adulthood. Further, most researched human behaviours are influenced by a very large number of genes and the individual effects of these genes are very small. Environmental influences also play a strong role, but they tend to make family members more different from one another, not more similar. Despite progress on these findings, the field continues to wrestle with unrealistic statistical and scientificassumptions.

Selective breeding and the domestication of animals is perhaps the earliest evidence that humans considered the idea that individual differences in behaviour could be due to natural causes.[1] Plato and Aristotle each speculated on the basis and mechanisms of inheritance of behavioural characteristics. Plato, for example, argued in The Republic that selective breeding among the citizenry to encourage the development of some traits and discourage others, what today might be called eugenics, was to be encouraged in the pursuit of an ideal society. Behavioural genetic concepts also existed during the English renaissance, where William Shakespeare perhaps first coined the terms "nature" versus "nurture" in The Tempest, where he wrote in Act IV, Scene I, that Caliban was "A devil, a born devil, on whose nature Nurture can neverstick".

Modern-day behavioural genetics began with Sir Francis Galton, a nineteenth-century intellectual and cousin of Charles Darwin. Galton was a polymath who studied many subjects, including the heritability of human abilities and mental characteristics. One of Galton's investigations involved a large pedigree study of social and intellectual achievement in the English upper class. In 1869, 10 years after Darwin's Origin of the species, Galton published his results in Hereditary Genius. In this work, Galton found that the rate of "eminence" was highest among close relatives of eminent individuals, and decreased as the degree of relationship to eminent individuals decreased. While Galton could not rule out the role of environmental

influences on eminence, a fact which he acknowledged, the study served to initiate an important debate about the relative roles of genes and environment on behavioural characteristics. Through his work, Galton also "introduced multivariate analysis and paved the way towards modern Bayesian statistics" that are used throughout the sciences—launching what has been dubbed the "StatisticalEnlightenment".

The field of behavioural genetics, as founded by Galton, was ultimately undermined by another of Galton's intellectual contributions, the founding of the eugenics movement in 20th century society. The primary idea behind eugenics was to use selective breeding combined with knowledge about the inheritance of behaviour to improve the human species.[3] The eugenics movement was subsequently discredited by scientific corruption and genocidal actions in Nazi Germany. Behavioural genetics was thereby discredited through its association to eugenics. The field once again gained status as a distinct scientific discipline through the publication of early texts on behavioural genetics, such as Calvin S. Hall's 1951 book chapter on behavioural genetics, in which he introduced the term "psychogenetics", which enjoyed some limited popularity in the 1960s and 1970s.[8][9] However, it eventually disappeared from usage in favor of "behaviour genetics".

The start of behavior genetics as a well-identified field was marked by the publication in 1960 of the book Behavior Genetics by John L. Fuller and William Robert (Bob) Thompson. It is widely accepted now that many if not most behaviours in animals and humans are under significant genetic influence, although the extent of genetic influence for any particular trait can differ widely. A decade later, in February 1970, the first issue of the journal Behavior Genetics was published and in 1972 the Behavior Genetics Association was formed with Theodosius Dobzhansky elected as the association's first president. The field has since grown and diversified, touching many scientific disciplines.

Genetic analysis of behaviour in experimental animals:

Animal research can serve as models of gene-environment interactions and diseases identified in humans. In the case of social control of disease processes, the choice of species to be studied depends on the level of social interactions that needs to be examined. For example, rodent models can demonstrate how differences in social status, population density, or early experiences interact with genetic makeup to affect susceptibility to disease (e.g., examine effects of social factors in knockout or knock-in animals [or inbred strains] that differ in susceptibility to infection, cancer, autoimmunity). The advantages of rodent models include significant control over genetic, physiological, behavioral, and social factors and relatively short reproductive, developmental, and life cycles. They are amenable to studying a variety of important psychosocial variables, including social isolation, social relationships, attachment, parenting, temperament, and motivationalstates.

However, nonhuman primate models, which offer limited control over genetic factors and have a longer life span, may be best suited to examine the consequences of more complex social factors, such as those involving cooperation or trust. For example, after bouts of aggression, nonhuman primates demonstrate reconciliatory behavior that is thought to be important for maintaining cooperative social hierarchies (de Waal, 2000). Some aspects of human behavior (e.g., optimism,hope, guilt) may be studied in animals only when the investigator can demonstrate a robust animal model with multiple behavioral paradigms as well as shared neural mechanisms.

In addition, animal models developed for traditional biomedical research are also powerful models for studying the psychosocial modulation of known mechanisms of specific human diseases. There are many animal species, strains, and transgenic models developed through biomedical science, that have been well characterized in terms of the genetic, molecular, and cellular processes underlying human disease. Studying these animals in a variety of psychosocial paradigms, based on variables identified through survey, epidemiological, and human experimental research, can test hypothesized causal relations derived from correlational data in humans.

It is essential to study animals as evolved biological systems in which surviving and reproducing in particular social and physical environments have selected a constellation of interactions between social, behavioral, physiological systems, and gene function. Doing so reveals insights and principles that also underlie human health and disease but that are not salient in the modern world or in a typical biomedical approach. Moreover, ethology and evolutionary biology recognize that individual differences are not necessarily just "noise," but represent different evolved strategies for survival in different contexts. Taking an ethological approach to variation in strategies reveals the range of gene-environment interactions that occur within species as they have evolved in their natural ethological and ecological contexts.

Studies of deer mice (*Peromyscus maniculatus*), who live in highly seasonal environments, reveal that function of the immune system requires significant energy, so much so that during winter an animal trades off entering puberty and becoming reproductive in order to sustain the energetic requirements of fighting infectious disease (Prendergast and Nelson, 2001; Nelson, 2004). It is not the demands of the cold weather itself that signals this trade-off, but rather the shortened days that precede seasonal temperature change, allowing the animal to modulate relative balance of immune function and reproduction in anticipation of the energetic demands ofwinter.

In house sparrows, immune activity increases energy expenditure, illustrating the energetic costs of immune function that could otherwise be deployed to growth (Martin et al., 2003). Such animal research, set in an ecological context, provides a powerful animal model for such trade-offs in humans. When social structure restricts resources and results in a population living in an environment with a high pathogen load, slower growth can result, as is the case of children in the lowlands of Bolivia. This presumably happens because the allocation of energetic resources to immune function has been diverted from growth (McDade, 2005). This dynamic interaction between social access to energy stores, pathogen interaction, fat deposition, and growth likely involves leptin, a pleiotropic molecule with cytokine properties that is produced by fat cells during an inflammatory response (Faggioni et al., 2001; Fantuzzi, 2005).

Animal research has clarified concepts that are key to understanding the effects of social environment on health and disease and gene function, extending and moderating the conclusions based on epidemiological studies in humans. These concepts include genetics, immune and neuroendocrine function, causality, pleiotropy, and life-span fitness.

Genetics requires a broad conception that includes both functional genomics (intra-individual changes in gene expression over time) and the more traditional topic of structural polymorphism (interindividual variations in DNA sequence or epigenetic characteristics). This broad conceptualization is essential because social influences on gene transcription are fairly well studied, while few studies have examined the relationships between social factors and genetic polymorphisms. That such effects exist is likely because structural polymorphisms generally exert their effects in the context of expressedgenes.

An essential role of animal research is to test the relationship between presumptive genetic influences (e.g., inferred from studies of heritability) and defined genetic influences (e.g., effects attributable to the expression of specific genes or epigenetic characteristics). The immune system includes classical immune cells (e.g., leukocytes) as well as other cellular contexts relevant to disease pathogenesis or host defense, such as somatic cells responding to pathogens through innate immune responses (e.g., "danger signals" produced by Toll-like receptors, Type I interferon production). The neuroendocrine system also is broadly defined to include not only true neurally driven hormone production (e.g., hypothalamic-pituitary-adrenal [HPA] axis), but also neuroeffector processes that do not necessarily involve systemic hormone distribution (e.g., local effects of neurotransmitter release from autonomic or sensory neurons or neuropeptides such as vasopressin and oxytocin).

Part of the reason so few genetic determinants of immune response currently are presently known may be an overly restrictive focus on "immune system" genes. Polymorphisms in many "nonimmune" genes, which are regulated by the psychosocial environment through physiological systems, may also influence

leukocyte function and/or the pathogenesis of diseases involving immune or inflammatory components. For example, catecholamines are known to influence several aspects of leukocyte function (Sanders and Straub, 2002; Kavelaars, 2002), and polymorphisms in genes encoding their alpha—and beta—adrenergic receptors are associated with differential incidence of asthma, parasitic infections, and cardiovascular disease (Ramsay et al., 1999; Ulbrecht et al., 2000; Ukkola et al., 2001; Weiss, 2005; Thakkinstian et al., 2005; Lanfear et al., 2005). Glucocorticoids, another physiological system exquisitely sensitive to the psychosocial environment, play a key role in regulating inflammatory gene expression (Webster et al., 2002), and polymorphisms in the glucocorticoid receptor gene (NR3C1) have been linked to cardiovascular and autoimmune disease (Lin et al., 1999; Ukkola et al., 2001; Jiang et al., 2001; Dobson et al., 2001; van Rossum et al., 2002).

Chromosomal anomalies and insight into human behaviour:

The belief that demonic and supernatural forces caused deviant behavior was once widely held by human societies. In the western world it was reflected in such practices as witch burning and exorcism. Beginning with the Age of Enlightenment, the concept of the "bad seed," the idea that incorrigible antisocial behavior was inborn, gained acceptance. Many later attempts were made to correlate criminal behavior with certain physical characteristics of theindividual,
and the hypothesis was advanced that criminal behavior stemmed from an atavistic level of biologic organization that was expressed in particular physical characteristics or anthropometric marks of inferiority. Efforts have continued to the present day to relate specific physical characteristics with sociopathic behavior. The studies of Dugdale and Goddard on the Jukes and Kallikak families, respectively, at the beginning of this century identified the role of heredity in the constellation of feeblemindedness, crime, and disease, and since then, much research has focused on heredity. Twins were studied in an attempt to demonstrate that genetic factors were involved in the origins of criminal or antisocial behavior, but the results were inconclusive because of the difficulty inherent in separating genetic from experiential factors.Discovery of the role of chromosome abnormalities in the etiology of human disease gave renewed impetus to the search for a genetic basis underlying behavioral disturbances. The report of Lejeune et al in 1959 linking Down's syndrome with trisomy 21 was quickly followedby discovery of the specific chromosome abnormalities in Turner's syndrome, Klinefelter's syndrome, and other conditions. These abnormalities have been discussed in previous issues. In general, the autosomal defects, when not lethal, confer severe mental and physical handicaps; the effects of the sex chromosome anomalies appear to be more variable.
These are Turner's syndrome (45, X), the triple-X (4 7, XXX) female, Klinefelter's syndrome (4 7, XXY), and the 4 7, XYY male. The most common chromosomal abnormality seen in Turner's syndrome is the absence of the second X chromosome. Affected individuals tend to be short in stature (generally less than 5 feet tall), have webbing of the neck and nonfunctional, "streak" ovaries, and are sterile. Early reports of Turner's syndrome indicated that these individuals were mentally retarded, but most of the studies were carried out on institutionalized patients. What has since been learned about the behavioral effects of Turner's syndrome stems mainly from a series of studies carried out at Johns Hopkins University under the leadership of John Money, who obtained his cases from an outpatient endocrine clinic. Money and his coworkers found that I *Q* was not particularly depressed among Turner patients. In one study of 38 Turner patients, I *Q* was found to range from under 70 to above 130, with a mean of 96, which is close to normal. The most significant finding, however, was a difference of from 10 to 20 points between verbal and performance scores of the Turner's patients on the W eschsler I *Q* test, with the verbal score always higher. This result has been replicated by two other groups, one in Leyden and one in Boston. Methodologic criticism notwithstanding, a verbal-performance difference seems to be substantial. These findings are of interest because a verbal-performance I *Q* difference, favoring verbal, may be a cue to the existence of brain damage in the dominant or involved hemisphere. It is not known, however, whether Turner individuals actually have brain damage. Several investigators have reported an association between

Turner's syndrome and abnormal E E G tracings, but the number of cases is too small to permit firm conclusions. Money and his group attributed the poorer performance I *Q* among Turner cases to what they called "space-form blindness." At least some persons with this syndrome have difficulty in telling left from right in others. Although they have a sense of their own left and right, when tested on a road map and asked whether a turn is being madetothe left or to the right, Turner patients tend to confuse direction, and they display an inability to integrate the fact that objects rotate in space. They also do poorly on the Bender Gestalt test, a test of perceptual motor skills in which the individual is asked to copy designs while looking at them (see opposite page). Presumably, the Turner individuals' lower scores on the performance aspect of I *Q* tests is related to the fact that performance tasks call for motor skills. The deficit in cognitive and perceptual motor functioning is small and could be compensated for even if consistently present. It might not show up as a gross defect in behavioral functioning. The xxx female is usually physically normal and a considerable proportion of those studied have normal gonads. Interest has focused on the x xx abnormality recently because of findings by Arnold Kaplan in Cleveland and, independently, two investigators in the Soviet Union that the incidence of XXX females in mental institutions, particularly among schizophrenic populations, is higher than their incidence in the generalpopulation.

The observation is of interest because it suggests a distinct genotypic basis for a subgroup of the schizophrenias, but the evidence thus far is inconclusive. Many of the women studied have been of advanced age, introducing the possibility that the chromosome defect was not present at birth. It has been reported that a total of 16,000 cases of women institutionalized for mental abnormality or illness. Of this number, 42 were XXX and 46% of thesewereaged 60 or older. The incidence of x xx females in mental institutions is about 2.6 per 1,000, whereas the incidence among newborn females ranges from 0.3 to 1.4 per 1,000.

Thus, it would appear that there is a twofold or greater increase of x xx females in mental institutions over the newborn incidence. The inference is clear that x xx aneuploidy carries a heavy risk of behavioral abnormality, but one important statistic is missing from the calculation-: It is not known how many of the 42 institutionalized women were born with a 47, XXX karyotype. This is an important consideration since two laboratories have independently demonstrated that chromosome abnormalities, particularly those involving the C group of chromosomes, to which the X belongs, tend to increase with advancing age, especially in females. The large increase in x xx females in mental institutions may therefore be an age-related phenomenon. To find out, it would be necessary to compare the institutionalized population with an age-matched sample but, at present, information on the incidence of chromosomal disorders for age groups other than newborns is not availablein sufficient numbers. Almost invariably, it is pointed out that males with an extra X chromosome are mentally subnormal. Most of the evidence comes from anapparentfive- to sixfold increase over the newborn rate of Klinefelter individuals in institutions for the mentally subnormal. The x x \' anomaly is found among newborn males at a rate of about 1.2per I ,000, while the rate among institutionalized mentally subnormal males is 9.4 per 1,000. However, anybody examine the 1 Q scores of Klinefelter individuals detected in endocrine or fertility clinics, he will find that, like those with Turner's syndrome, they show a range from mild retardation to above average. Males with multiple X aneuploidy also show a wide variability in phenotype. Although they all tend to have more or less the same physical stigmata, with hypogonadism the most consistent feature, physical signs vary, depending on where the individual is detected. Those seen in endocrinology clinics tend to have more marked- gynecomastia, female pubic hair distribution, and absence of facial hair, whereas those seen in fertility clinics. Tend to have more normal physical characteristics. Aside from mental subnormality, the outstanding behavioral feature of Klinefelter's syndrome is a reported tendency toward aggressiveness that seems to carry a higher than average risk that the patients will end up in prisons and other penal institutions. A review of the literature that included more than 20 different surveys of prisons, special security institutions, and institutions for the criminally insane revealed that XYYindividuals are to be found in such institutions at a rate of about 8 per 1,000. However, the meaning of the increase over the newborn rate is equivocal. Since other pertinent data on these individuals is missing, such as their ethnic, social, and family background, the conclusions that canbedrawn from the institutional incidence are limited. It has been suggested that the

institutionalization of some Klinefelter individuals may be the result of factors not directly related to the chromosomal disorder. For example, a sentencing judge is more likely to institutionalize someone who does not have family responsibility than someone who is a parent and has dependents. Klinefelter individuals are less likely to be married, and certainly less likely to be parents because they are usually sterile. The decision-making factor involved in how people get into and out of institutions is one of the variables affecting incidence that has not been investigated. Another chromosomal abnormality possibly associated with criminality is the XYYkaryotype. The first case, detected in 1961 in Buffalo, was a 44-year-old man, physically normal, of average intelligence, and the father of seven living children from twomarriages.Most important in this context, he was not a criminal; ·rather, his chromosomal abnormality was discovered because of mongolism andother congenital anomalies among his children. By 1965, a total of 12 cases had been detected, most of them because they had some physical anomaly that brought them to medical attention and karyotyping. The association of an extra Y chromosome with criminality was not made until 1965, when M. D. Casey carried out a sex chromosome survey among individuals in penal institutions in England and discovered 21 with chromosomal abnormalities, of whom one third had an extra Y chromosome. Patricia Jacobs and her colleagues in Edinburgh postulated that the extra Y chromosome might carry the risk of personality defect predisposing the bearer to penal institutionalization. She carried out a chromosome survey of 315 men in a maximum security hospital for criminal psychopaths at Carstairs, Scotland, and found nine with the XYY karyotype, or almost 3% of the inmate population. Jacobs and her colleagues alsoobservedthat, on the average, these individuals were approximately six inches taller than the chromosomally normal inmates. It is from this study that the association of the XYY karyotype with criminality and tallness can be said to have originated. Subsequently, a series of surveys were carried out by different groups of investigators studying populations in penal institutions. In most of the studies, sampling focused on taller than- average individuals and, in fact, many XYY individuals were found. From these surveys, the following characteristics emerged as being potentially associated with the extra Y chromosome: the individual has one or more physical or physiologic abnormalities; he tends to be tall, mentally subnormal or of lower than average intelligence, impulsive and hyper aggressive; his criminal activity usually begins at an early age; often there is no predisposing family history to account for his criminal behavior, and therefore, inferentially, it is the extra Y chromosome that contributes to his 134 criminality; and lastly, his numbers in maximum security institutions exceed by as much as 20-fold the incidence of XYY males in the newborn population. Therefore, he has a considerably higher than average risk of behavioural dysfunction and conflict with authority. With respect to the physical and physiologic abnormalities, a large variety of abnormal characteristics has been found in at least one or more reported XYY cases. These include hypogonadism, webbing of the neck, vascular abnormalities,varicoseulceration,bone and joint abnormalities, and fingerprint and dermatoglyphic alterations. Some investigators have also noted acne and other skin disorders in theseindividuals.WhileallofthesеabnormalitieshavebeenfoundinasmallproportionofXYY cases, it is important to note that the majority have appeared to be physically normal. Studies of urinary and plasma testosterone levels in XYY men are of interest because of the known association between androgens and aggressive behavior. Elevated testosterone levels might be a mechanism through which the extra Ychromosomecould influence or exacerbate behavior, particularly behavior arising at the time of puberty. Although individual cases of XYY men have been shown to have elevated levels of testosterone, when compared with chromosomally normal fellow inmates, no significantly different plasma or urinary testosterone levels have been found. Institutionalized XYY and XY males, however, both appear to have higher testosterone excretion levels than chromosomally normal noninstitutionalized controls. It is possible that institutionalization alone has an effect on testosterone levels. At any rate, the testosterone findings have at this point very dubious significance. Gonadotropin levels have also been studied, and here again, when the XYY males are matched with an adequate control group, no significant differences appear. Height is the one characteristic that has shown a strongly suggestive association with the XYY karyotype. Ewn when sampling is not confined to males 5'11" or taller, thosewiththe XYY karyotype turn out to have an average height greater than males with a single Y chromosome. For example, in Jacobs' survey at Carstairs, in which virtually every inmate was studied, the nine XYY men detected had a mean height of

181.2 cm, whereas the 305 inmates with a single Y chromosome had a mean height of 170.7 cm. Of 23 individuals detected by other investigators since 1961 for reasons other than height, 11 of them were six feet tall or taller, so the data do seem to show that the Y chromosome is related to height. The implication that the XYY karyotype carries a risk for sociopathic behavior, however, remains to be proved. Data imputing a higher risk for criminal behavior, including youthful age at first conviction, come mainly from the study by W. H. Price and P. B. Whatmore of the nine XYY males discovered at Carstairs. They compared these nine individuals, of whom seven were in a wing of the hospital for the mentally subnormal and two were in a wing for the mentally ill, with 18 inmates in the same institution selected at random from the entire chromosomally normal population of the hospital. Their findings indicated XYY's commit fewer crimes of violence against persons than the controls; XYY's manifest disturbed behavior at an earlier age than controls, as measured by age at first conviction - 13.1 years for the XYY's as against 18 years for the controls; siblings of theXYY'scommitsignificantlyfewercrimesthansiblingsofcontrols.ThereforetheXYY's criminal behavior is not influenced by familial environment and begins at an age too early to be influenced by factors outside the home; and XYY's are not amenable to rehabilitation. In other words, the x Y Y's behavior was thought to be incorrigible.

The investigators concluded that the XYY's behavior dysfunction is associated solely with the chromosomal abnormality, but this conclusion is premature for a number of reasons. First, it need not be argued that a familial predisposition to crime, determined by the number of convictions among the siblings, is present in the families of controls; 11 of the 18 controls came from families in which there was no history of criminal conviction among the siblings.The lack of criminal behavior in other family members does not rule out familial encouragement of aggressive behavior, however. Parental disciplinary and socialization practices have an influence on aggressive behaviour that can be seen very early in a child's development. Influences on behavior from peers, school, and other sources outside the home also begin at an early age. Furthermore, it is doubtful whether age at first conviction is any indication that behavioural dysfunction begins at an earlier age in XYY's than in chromosomally normal individuals convicted of crimes. Indeed, the increased height of the XYY boy may be the critical factor. It is certainly conceivable that a court would be more likely to convict and punish a tall than a short adolescent. Subsequent studies have produced contradictory evidence on this point, ome showing no difference in age at first conviction between x \' \' and x Y inmates. As for the behavioral characteristic of aggressiveness that has been linked with the x *Y* \' karyotype, the Price and Whatmore study made the point quite clearly that the x \' Y's at Carstairs were no more aggressive than their chromosomally normal controls. According to their penal records, the nine XYY inmates had a total of 92 convictions and the 18 controls 210. Only 9% of the XYYconvictions were for crimes against persons, however, in contrast to 22% among the controls. On an observational basis, the x Y Y's also seemed to exhibit less aggressive behavior in the institution than the chromosomally normal inmates, who were more openly hostile and more likely to have violently aggressive outbursts. Other studies comparing criminal records have produced varying behavior, however.

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comparing criminal records have produced varying findings. By and large, .there does not seem to be any significant difference in the kind or frequency of crimes committed by XYY individuals as compared with fellow x Y inmates.

Since x Y v's have been identified who do not exhibit any abnormal or criminal behavior, it seems entirely possible that other genetic and/or environmental factors contribute to the antisocial behavior of the XYY's who have been studied in institutions. The earlyfindingsfrom a prospective study of four XYY boys identified at birth are of interest in this regard. At three or four years of age, three of these boys were found to be functioning and developing normally, but the fourth child was described as hard to manage, hostile, and given to climbing to heights and breaking things. He was the one child out of the four who had been placed in a foster home, and by the time he was four years old, had already been in multiple foster homes.

Even if XYY's are more aggressive than normal individuals, which is' unclear from the studies done to date, one could not necessarily attribute it to a causal effect of the extra Y chromosome. One would have to look very carefully at the kinds of extra-institutional environments in which these individuals were reared and, specifically at how the expression of aggression was handled in those environments. If it can be proved that the extra Y chromosome is related to behavioraldysfunction -specifically, aggressiveness - the important issue will be how to treat these individuals to ameliorate the effects. Price and Whatmore concluded that institutionalized XYY's were rehabilitative failures, to be relegated to a life of crime or institutional confinement. As for rehabilitation failure, little is known about the techniques employed to help these individuals. In a maximum security institution, relatively ineffective methods may have been used. There is considerable evidence that the expression of aggression by an individual varies substantially from one environmental setting to another. It should be possible, through behavioural therapy or environmental construction in or out of institutions, to ameliorate or suppress the behavioural effects of an extra Y chromosome, assuming there are any. To summarize, then, the hypothesis that individuals with an extra Y chromosome have an increased risk for aggressive behavior or criminality appears attractive, but at this time there is essentially no good evidence to support it. In 1971, Jacobs carried out a chromosome survey of 2,500 men in penal institutions in Scotland and found no increased incidence of either XXY or XYY males over their respective newborn incidences. Fragmentary as our statistics are for the U.S., it can be assumed that at least 95% of males with the XYY karyotype are functioning at levels of behaviour adequate to keep them from coming into conflict with thelaw.

The available evidence of a risk for behavioral dysfunction must be interpreted very carefully because of the potential implications for individual families. For example, there seems to be no uniformity of opinion among geneticists or pediatricians as to whether to withhold the information from the parents of children detected with the x Y Y karyotype. Some centershave withheld the information from parents, but hiding information raises ethical problems and possibly legal ones as well. Some investigators have immediately told the parents that the child has a chromosomal abnormality; some have told the parents that the child has an increased risk for abnormal behavior. If the information is conveyed and the parents are told that there is a possibility, albeit small, that their child is going to behave abnormally, they are likely to have expectations of abnormal behavior. Where parents have been told, attempts have been made to convey the idea that the evidence for an increased risk of psychopathology is not very strong.

Several considerations need to be kept in mind in assessing the association between chromosomes and behavior. Firstly, each of the chromosomal disorders discussed here shows considerable phenotypic variability. For each, some proportion of cases have E E G abnormalitiesorepilepsy,orboth,butthefindingisnotconsistentanddependstoalargeextenton where the individual is detected. Patients in an institution for the mentally subnormal tend to have a relatively higher rate of E F. Gabnormality, just as do the chromosomally normal individuals in the same institution. Move the sampling to outpatient clinics and the incidence of all types of abnormalities, physical and, presumably, behavioral, as well as FE G, decreases in frequency, which suggests that the findings are not specific for the chromosomal defect in question. There is also a broad range of phenotypic variability in each of the chromosome abnormalities, a case in point being Money's finding that the 1 Q's of Turner's syndrome

patients range from 70 to 130. But while it is probable that persons with Turner's syndrome are not more likely to be retarded than others, the difference in verbal and performance IQ maybespecific for the syndrome.

Secondly, numerous assumptions need to be made when attempting to determine whether a given chromosomal disorder carries a risk for behavioural dysfunction. In general, such attempts are made by comparing the institutional incidence of the abnormality with its incidence among newborn infants. In doing so, it is assumed that the two groups being compared are similar, that is, that they come from the same social class, background, ethnic group, and so on. Another assumption is that there is no differential mortality for the individual carrying a chromosomal defect as compared with chromosomally normal individuals. If there is a differential postnatal mortality for the chromosomal defect, its incidence in the institutionalized population might be an understatement of the true risk for behavioral dysfunction. On theotherhand, if there is an increase of aneuploidy with age, as may be the case with the x xx disorder, the institutional incidence rate may overstate the true risk for behavioral dysfunction. These and other variables have complicated the interpretations that can be drawn from the studies done to date on the association between behavioural dysfunction and chromosomal abnormality.

Thirdly, the conceptualization and assessment of the environments in which individuals with chromosomal disorders have been nurtured and in which they are functioning when detectedhas been inadequate. Environments simply cannot be ordered along a single harmonious- disharmonious or favorable-unfavorable continuum. An environment that may be favorable for the development of one set of behaviors,as, for example, the development of independence and self-reliance, may differ from an environment favorable for the development of other adaptive behaviors. Although techniques to assess social climates and to provide functional analyses of environments have been developed in recent years, these have not yet been applied in humanbehavior genetics research. Lastly, it is almost always assumed that chromosomal disorders must have abnormal consequences for psychologic development and functioning. However, possible adaptive consequences are conceivable and require more attention in future research. For example, there is evidence that taller males are more popular than average and have a greater likelihood of becoming leaders with high self-esteemduring their adolescence. If, as the evidence suggests, some x Y Y males are taller than their peers, they may, on the bases of compatibility or similarity be more likely to be attracted to and imitate other taller males. Such individuals may thus have a higher than average probability of becoming socially assertive and attaining economic success and of making outstanding social adjustments andcontributions.

It is worth noting that about 1 in 200 babies is born with a chromosomal disorder. This means that about 12,000 to 18,000 children are born in the U.S. annually with an abnormality of either the autosomal or sex chromosomes. At any given time, more than a million persons are possibly carriers of chromosomal abnormalities, but relatively few of them are in institutions because of behavioraldysfunction.The majority of individuals with sex chromosome anomalies seem to be functioning in society reasonably well, indicating that the correlation between abnormal behavior and specific chromosomal disorders is not a strong one. To ascribe to a single chromosome particular personality characteristics or patterns of behaviour overlooks the fact that the behavioural phenotype of all human beings results from the interaction of genotype and environment.

Probable Questions:

1. Define behavioral genetics. What is its significance.
2. Describe some experiments on animals which depict behavioural genetics study.
3. How XYY genotype affects one's behaviour?
4. How XXX genotype affects one's behaviour?

Suggested readings:

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7th Edition.
6. Gene cloning and DNA Analysis by T. A. Brown, Sixth Edition.
7. Genetics . Verma and Agarwal

Unit-VII

Environmental effects and gene expression: effects of external and internal environment; phenocopies

Objective: In this unit you will learn about Environmental effects and gene expression and also the effects of external and internal environment including phenocopies

Introduction:

The gene expression is determined by two features called as penetrance and expressivity of the genes. Penetrance is the ratio of individuals exhibiting expected phenotype and expressivity is the extent of gene expression in an individual. The phenotype of an individual is determined by the genotype or the type of gene expressed. In general, phenotypic changes occur in individuals when exposed to various environmental factors. But the query, "Is the genotype of an individual is influenced by external environment?" lead to several researches throwing light on the effect of external or environmental factors like temperature, light, chemicals and nutrition in gene expression. Besides the effect of internal factors like hormones and metabolism on gene expression, external factors were also found to affect the gene expression and ultimately exhibiting phenotypicchanges.

Penetrance and Expressivity:

The presence of a gene does not always bear an absolute relationship with the appearance or absence of a trait. In the ABO blood group system, the genes are expressed in an absolute way. But in many other instances the gene is expressed in a variable manner, i.e. the visible phenotype shows varying intensities.

The terms penetrance and expressivity are used to describe variable gene expression. Penetrance is the proportion of individuals that show an expected phenotype. When a gene is completely penetrant it is always expressed; when incompletely penetrant, the gene is expressed in some individuals, not in others, the proportions depending upon the degree of penetrance.For example in the recessive traits which Mendel studied, the phenotype was expressed fully when the gene was in homozygous condition; this is due to 100 per cent penetrance. Suppose instead that in a hypothetical cross, only 60 per cent of individuals show the expected trait when all 100 are carrying the gene; we say that in this case penetrance is 60 per cent.Expressivity is the degree to which a gene is expressed in the same or in different individuals. Thus the gene for lobe eye in Drosophila may show a complete range of phenotypic expression in different individuals. Some flies may have a normal sized eye, in others the eye is smaller, in still others the eye is absent.

Temperature:

The earliest studies related to the effect of temperature on genetic constitution were done on the Himalayan breed of rabbits and Siamese cats. Coat colour in rabbits is controlled by multiple alleles of a gene.When one of the recessive alleles c^h is present in the homozygous condition (c^hc^h), the Himalayan coat colour results. Such a rabbit is a mosaic with white fur all over the body except the nose, paws, ears and tail which are black (Fig. 5.1).

Fig. 5.1 The Himalayan rabbit.

The black extremities are the portions which have lower temperature (less than 34°C) than the rest of the body. If the extremities are exposed to higher temperature artificially, the new hair which starts growing is white.

Similarly, if some portion of the body bearing albino fur is artificially kept at a lower temperature, the new hair formed is black. These observations explain the temperature sensitive behaviour of the allele (c^h) which controls Himalayan trait in the homozygous state.The allele codes for an enzyme used in pigment formation which is temperature sensitive and is inactivated by temperatures above 34°C resulting in albino phenotype; if temperature is lower the same alleles promote synthesis of pigment and the phenotype is black.When rabbits of this genotype are grown at cold temperatures, they are completely black. The Siamese cat shows the same pigmentation pattern as the Himalayan rabbit due to the presence of similar type of temperature-sensitive allele.In Drosophila temperature changes the penetrance of the gene known as tetraptera which controls wing development. At 25°C the gene has 35 per cent penetrance so that the corresponding number of flies develop wings whereas 65 per cent do not. At 17°C penetrance is much reduced so that only one per cent of flies show the winged phenotype.
The recessive gene vg/vg which produces vestigial wings in Drosophila is also influenced by temperature. At 32°F the wings are feebly developed and extend very little from the body (Fig. 3.1). At 40°F the wings are better developed and have some venation. At 88°F wings are well developed with conspicuous venation.

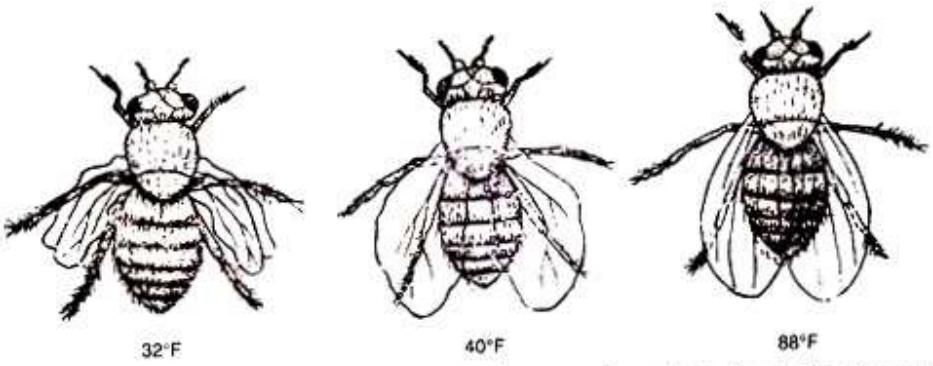


Fig. 3.1 Influence of temperature on the expression of the gene for vestigial wings (*vg*) in *Drosophila*.

Some temperature-sensitive mutations are exhibited in bacteriophages. In general the temperature at which normal phenotypes are produced is referred to as permissive temperature: that which produces mutant phenotypes is called restrictive temperature. Some lethal mutations in viruses and in Drosophila are temperature sensitive. Among plants, colour of flower in primrose changes from red to white when temperature is raised above 86°F.

Light:

There is a gene in maize plants which controls anthocyanin pigment formation. When ears of plants carrying the homozygous gene are exposed to sunlight by removing the green leafy coverings on the young cobs, the kernels become bright red in colour ("sunred").If however, the blue violet rays of the light spectrum are

prevented from reaching ears of maize plants (by wrapping red cellophane paper around them so that only red rays penetrate the cells) the sunred phenotype is not visible.

In this case sunlight interferes with one or more chemical reactions leading to pigment formation. The reddish freckles on the sensitive skin of white skinned human races are also caused by sunlight in a similar way.In human beings a skin cancer known as xeroderma pigmentosum is caused by a homozygous recessive gene. The skin becomes extremely sensitive to sunlight so that even a minor exposure to faint light gives rise to pigmented spots on the facial skin. The spots can become cancerous and if they spread to other parts of the body, death results. If an individual homozygous for the recessive gene is not exposed to light, the gene is not able to express itself.

Environment and Sex Determination:

The marine worm *Bonellia* demonstrates the effect of environment on sex. In this sexually dimorphic organism the female is very large, about 10 cm in length; the male is 3 mm long and lives inside the cloaca of the female.

If the free swimming larvae that have arisen from fertilised eggs remain in the sea bed away from the females, they develop into female worms. But if females are available, the larva settles on the female proboscis, draws nourishment from it, and develops into a male.Of the many experiments performed with Bonellia, one is most interesting and relevant here. If Bonellia are raised in the laboratory in a tank containing artificial sea water, the free-swimming larvae settle down at the bottom of the tank and develop into females. But if the artificial sea water is agitated by some mechanical device, the larvae develop into males.

Phenocopies:

Depending upon the extent to which the environment influences the genotype, the changes in the phenotype may be subtle or dramatic. Sometimes the phenotype becomes altered by the environment in such a way that the new phenotype resembles another phenotype produced by known genes. The induced phenotype is not inherited and is called a phenocopy.

In many instances phenocopies result from application of specific treatments like radiation, chemicals poisons, temperature shocks etc. The Himalayan rabbit described develops a coat that is all black if the rabbit is made to live in a cold environment.The Himalayan rabbit is a phenocopy of the genetically black rabbit. If both rabbits live together at moderately high temperature, the Himalayan rabbit has a phenotype very different from the genetically black rabbit.One of the most striking examples of phenocopies could be observed in what were known as thalidomide babies in the early 1960's. A number of deformed children were born in West Germany and Great Britain to mothers who had taken the tranquilizing drug thalidomide in their sixth week of pregnancy.The abnormal children showed deformities in limbs; some had one, two or three limbs, others had no limbs at all. The abnormalities showed a great resemblance to another phenotype known as phocomelia caused by a recessive gene.Diabetes mellitus is a heritable human trait associated with reduced amounts of the hormone insulin that is secreted by the pancreas. In the presence of insulin glucose is absorbed by the cell membranes. When the hormone is not produced in sufficient quantity, the unabsorbed glucose passes into the blood and urine. The exact mode of inheritance of diabetes is not properly understood.There are different types of diabetes arising from different causes; it therefore seems likely that there are several gene pairs controlling the trait. On the other hand the study of a pair of genetically identical twins, one of whom had diabetes the other not, indicates that the condition is due to a recessive gene with low penetrance.

If proper doses of insulin are administered to a diabetic person, he reverts to the normal phenotype. In other words, control of diabetes produces a phenocopy of the normal individual. There are many other examples in

human beings where, by giving drugs, the mutant genotype produces a phenocopy of the normal phenotype.In haemophiliac patients, a specific protein required for blood clotting is either defective or deficient. If however, an anti-haemophiliac factor isolated from humans is injected into a patient, a phenocopy of the normal individual results. Similarly, if thyroxine is administered to a child whose thyroid gland does not secrete this substance in adequate quantities, the normal phenotype is produced.

The creeper trait in chickens is observed sometimes in domestic fowl when the newly hatched chickens have the legs drawn up under the body. The affected chicken is not able to walk but creeps along the ground.The creeper trait (Fig. 3.2) is expressed by the heterozygous condition of a dominant gene which is lethal when homozygous. Creeper chickens can also be produced if incubating eggs of normal fowls are treated with boric acid or insulin. Such induced creepers are phenocopies of the genetically controlled heterozygous creeper chickens.

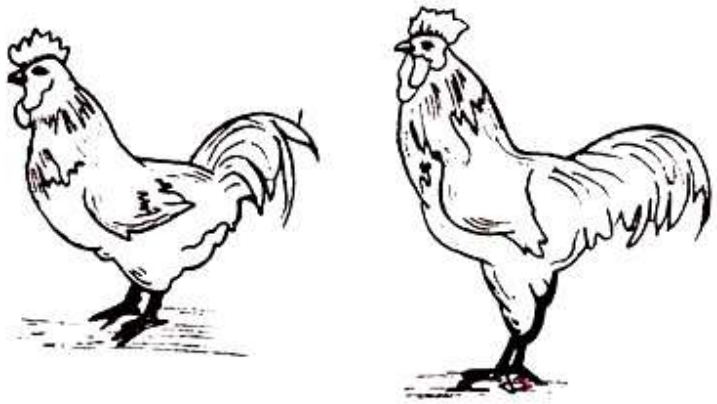


Fig. 3.2 Chicken expressing creeper trait (left) compared with a normal chicken (right).

Due to a recessive gene, maize plants become dwarfed, because they are deficient in the plant growth hormone known as gibberellic acid. But if the hormone is supplied to the dwarf plants they grow to normal height producing phenocopies of normal plants.

Environmental Effects and Twin Studies:

In human beings it is not possible to perform controlled breeding experiments. Twin studies are perhaps the best way of determining as to whether the observed differences between individuals are due to heredity.Twins are of two types—monozygotic or identical twins that arise from a single fertilised egg and have identical genotypes; dizygotic or fraternal twins which arise from two fertilised eggs and are therefore no more genetically alike than siblings (brothers and sisters).

The correct identification of twin types is difficult and unreliable unless done by a physician. For assessing the role of environment in heredity, the percentage of concordance (both twins showing identical phenotype) and discordance (different phenotypes) for a given trait must be determined for twins of both types.In general if concordance percentage for a trait is high in the case of monozygotic twins, and much less in dizygotic twins, one can conclude that heredity has played a role. If the concordance rate is similar in monozygotic and dizygotic twins, it suggests that the environment is determining the phenotype. From studies of a large number of twins it has been found that measles (caused by infection with Rubelia virus in early pregnancy) is largely controlled by the environment.

On the other hand conditions like diabetes, schizophrenia, Rickets and tuberculosis appear to be controlled by the genotype. Another useful aspect of twin studies is to determine the effects of different environments on identical genotypes by analysing those rare cases of monozygotic twins that have been separated from birth and reared apart. However in absence of adequate data it is not possible to conclude much on this aspect as yet.

Human Intelligence:

A number of studies have been done to determine how much of human intelligence and I.Q. are controlled by the genotype and how much by the environment. Both clarifications and complications have been revealed. The differences in intelligence among different racial groups have been extensively studied by Arthur Jensen in 1969. This work is highly controversial and has been much debated.Nevertheless, it is generally agreed that intelligence is under the control of several gene pairs interacting with the environment. From twin studies it has been further estimated that about one-half to three-fourths of human intelligence is determined genetically; the remainder is controlled by the environment.

Drug Resistance:

It is fairly well established that mosquitoes develop resistance to DDT and other insecticides used for eradicating malaria. The resistance develops due to change in the genotype in response to the environment, and is inherited.Similar resistance is reported also in insects which carry the causal agent for some other diseases like dengue fever, yellow fever, filariasis and river blindness. A number of pests which are harmful to major crops such as rice, maize, cotton, wheat and potato are also known to have become resistant to a wide range of insecticides.

Probable Questions:

1. What is penetrance and expressivity?
2. How temperature affect gene expression?
3. How light affect gene expression?
4. How sex is determined by environment?
5. Define and explain phenocopies.
6. How intelligence is controlled by genotype?
7. How drug resistance is controlled by genotype?

8 How twins are effected by environment?

Suggested readings:

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7th Edition.
6. Gene cloning and DNA Analysis by T. A. Brown, Sixth Edition.
7. Genetics . Verma and Agarwal

Unit-VIII

Sex determination in Human and role of Y-chromosome. Twin studies; concordance and discordance; identical and fraternal twins

Objective: In this unit you will learn about sex determination in humans and role of Y chromosome in the process. You will also learn about twin study, concordance and discordance.

Sex Determination:

A sex-determination system is a biological system that determines the development of sexual characteristics in an organism. Most organisms that create their offspring using sexual reproduction have two sexes. Occasionally, there are hermaphrodites in place of one or both sexes. There are also some species that are only one sex due to parthenogenesis, the act of a female reproducing without fertilization.

In many species, sex determination is genetic: males and females have different alleles or even different genes that specify their sexual morphology. In animals this is often accompanied by chromosomal differences, generally through combinations of XY, ZW, XO, ZO chromosomes, or haplodiploidy. The sexual differentiation is generally triggered by a main gene (a "sex locus"), with a multitude of other genes following in a dominoeffect.

In other cases, sex of a fetus is determined by environmental variables (such as temperature). The details of some sex-determination systems are not yet fully understood. Although, they do provide concrete analysis of complete biological sex-determinism. Hopes for future fetal biological system analysis include complete-reproduction-system initialized signals that can be measured during pregnancies to more accurately determine whether a determined sex of a fetus is male, or female. Such analysis of biological systems could also signal whether the fetus is hermaphrodite, which includes total or partial of both male and female reproductionorgans.

Some species such as various flowers and fish do not have a fixed sex, and instead go through life cycles and change sex based on genetic cues during corresponding life stages of their type. This could be due to environmental factors such as seasons and temperature. Human fetus genitals can sometimes develop abnormalities during maternal pregnancies due to mutations in the fetuses sex-determinism system, resulting in the fetus becomingintersex.In nature a large number of diverse mechanisms exist for determination of sex in different species. The fruit fly Drosophila melanogaster and human beings are very important in thedevelopment of genetic concepts because in these two organisms, and in many others, individuals normally occur in one of two sex phenotypes, male orfemale.
In these species males produce male gametes, sperm, pollen or microspores while females produce female gametes namely, eggs, ovules or macrospores. In many species the two sexes are phenotypically indistinguishable except for the reproductive organs. Sex determination is aimed at identifying the factors responsible to make an organism a male or female or in some cases a hermaphrodite. So far the mechanism of sex determination has been related to the presence of sex chromosomes whose composition differs in male and femalesexes.
However, in recent years sex determination has been differentiated from sex differentiation, and sex determination mechanism is explained more on the basis of the specific genes located on sex chromosomes and autosomes. Sex determination is recognized as a process in which signals are initiated for male or female developmental patterns.

During sex differentiation, events occur in definite pathways leading to the development of male and female phenotypes and secondary sexual characters. Significant progress has been made in understanding the mechanism of sex in human beings and other mammals and new genes have been identified.

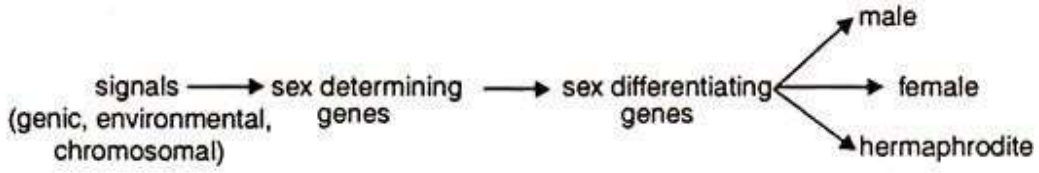


Fig. 1. Sex determination and sex differentiation mechanism involving two sets of genes and the signals.

Chromosome Theory of Sex Determination:

Sex determination in higher animals is controlled by the action of one or more genes. The testis determining factor (TDF) gene is the dominant sex determining factor in human beings. Hemking a German biologist identified a particular nuclear structure throughout the spermatogenesis in some insects. He named it as "X-body" and showed that sperm differed by its presence or absence. The X body was later found to be a chromosome that determined sex. It was identified in several insects and is known as the sex or Xchromosome. Thus, the chromosome theory of sex determination states that female and male individuals differ in their chromosomes. Chromosomes can be differentiated into two types, autosomes and sex chromosomes. Sex chromosomes carry genes for sex. In some animals, females have one more chromosome than males, thus they have two X chromosomes and males have only one.

Females are therefore cytologically XX and males are XO, where 'O' denotes the absence of X chromosome. During meiosis in the female the 2X chromosome pairs and separates producing eggs that contain a single X chromosome. Thus all eggs are of the same type containing only one X chromosome.During meiosis in the male, the single X chromosome moves independently of all the other chromosomes and is incorporated into half of the sperm, the other half do not have any X chromosome. Thus, two types of sperms are produced, one with X chromosome and the other without the X chromosome or designated as 'O'.

When the sperm and eggs unite, two types of zygotes are produced; XX that develop into females and XO that develop into males. Because both of these types are equal in number, the reproductive mechanism preserves a 1:1 ratio of males tofemales.In many animals, including human beings, males and females have the same number of chromosomes. This numerical equality is due to the presence of a chromosome in the male called the 'Y' chromosome, which pairs with the X. During meiosis in the male, the X and Y-chromosomes separate from each other producing two types of sperm, one type with X chromosome and the other type having Y chromosome.

The frequencies of the two types are approximately equal. Females with XX chromosomes produce only one type of eggs, all with X chromosome. In random fertilization, approximately half of the zygotes are with XX chromosomes and the other half with XY chromosomes leading to a sex ratio of 1:1.This mechanism is called XX – XY type of sex determination.The XY mechanism is more prevalent than the XO mechanism. The XY type is considered characteristic in higher animals and occurs in some plants. This mechanism is operative in Drosophila melanogaster and human beings. Both species exhibit the same pattern of transmission of X and Y chromosomes in normal individuals in natural populations. In human beings, the X chromosome is considerably longer than the Y chromosome.The total complement of human chromosomes includes 44 autosomes: XX in the female and XY in the male. Eggs produced by the female in oogenesis have a complement of 22 autosomes plus an X chromosome. Sperm from the male have the same autosomal number and either an X or a Y chromosome. Eggs fertilized with sperm containing a Y chromosome result in zygotes that develop into males; those fertilized with sperm containing an X chromosome develop into females.

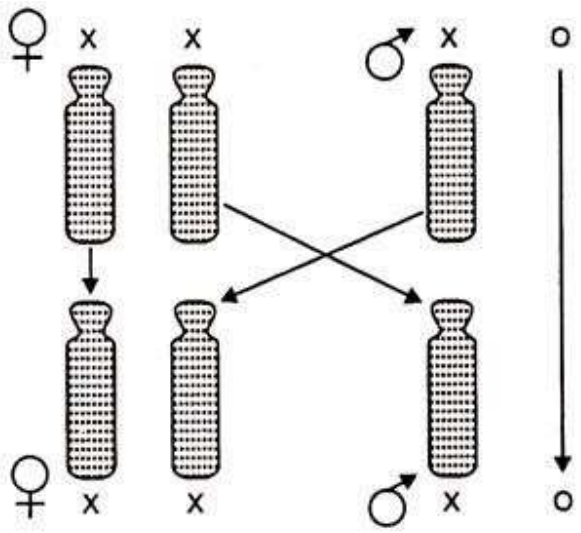


Fig. 2. Inheritance of sex chromosomes in animals with XX-XO mechanism.

In animals with XX-XY mechanism of sex determination, females (XX) produce gametes that have the same chromosome composition (one X plus one set of autosomes). These females are homogametic sex as all the gametes are the same. The males of these animals are heterogametic as they produce two types of gametes, one half containing one X chromosome plus one set of autosomal chromosomes and the other one half contain one Y chromosome plus one set of autosomes.

Animals with Heterogametic Females:

In many birds, moths and some fish, the sex determination mechanism is identical to the XX-XY mechanism but the females are heterogametic (ZW) and males are homogametic (ZZ). This mechanism of sex determination is called ZZ-ZW.

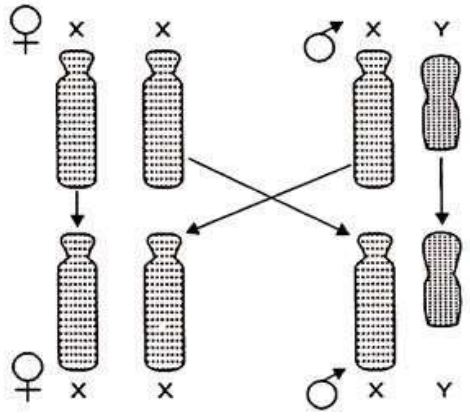


Fig. 3. Inheritance of sex chromosomes in animals with XX-XY mechanism.

n this mechanism the relationship between sex chromosomes and sex phenotypes is reversed. In birds the chromosome composition of the egg determines the sex of the offspring, whereas in humans and fruit flies, the chromosome composition of the sperm determines the sex of the offspring.

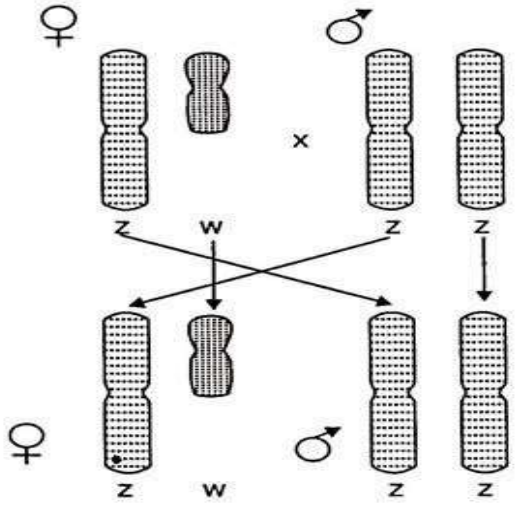


Fig. 4. Sex determination in birds with ZZ-ZW mechanism.

Process of Sex Determination in Human Beings:

In human beings, sex is determined by the number of X chromosomes or by the presence or absence of the Y chromosome. In human beings and other placental mammals, maleness is due to a dominant effect of the Y chromosome. The dominant effect of the Y chromosome is manifested early in development when it directs the primordial gonads to differentiate into testes. Once the testes are formed, they secrete testosterone that stimulates the development of male secondary sexual characteristics. Testis determining factor (TDF) is the product of a gene called SRY (Sex determining Region of Y), which is located in the short arm of the Y chromosome of the mouse. SRY was discovered in unusual individuals whose sex was not consistent with their chromosome constitution – males with XX chromosomes and females with XYchromosomes.

Some of the XX males carried a small piece of the Y chromosome inserted into one of the X chromosomes. It is evident that this small piece carried genes for maleness. Some of the XY females carried an incomplete Y chromosome. The part of the Y chromosome that was missing corresponded to the piece that was present in the XX males.

Its absence in the XY females prevented them from developing testes. These observations show that a particular segment of the Y chromosome was required for the development of the male. Further studies showed that the SRY gene is located in this male determining segment. Like that of the human SRY gene is present in the Y chromosome of the mouse and it specifies male development (Fig below).

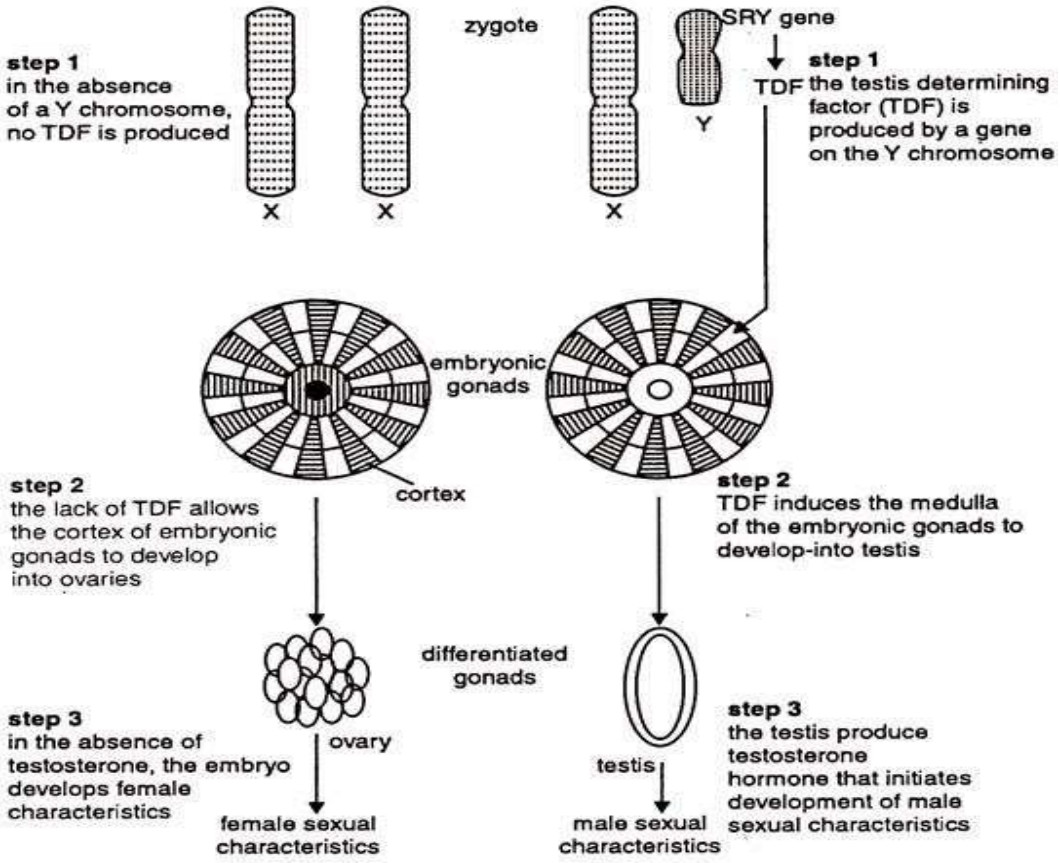
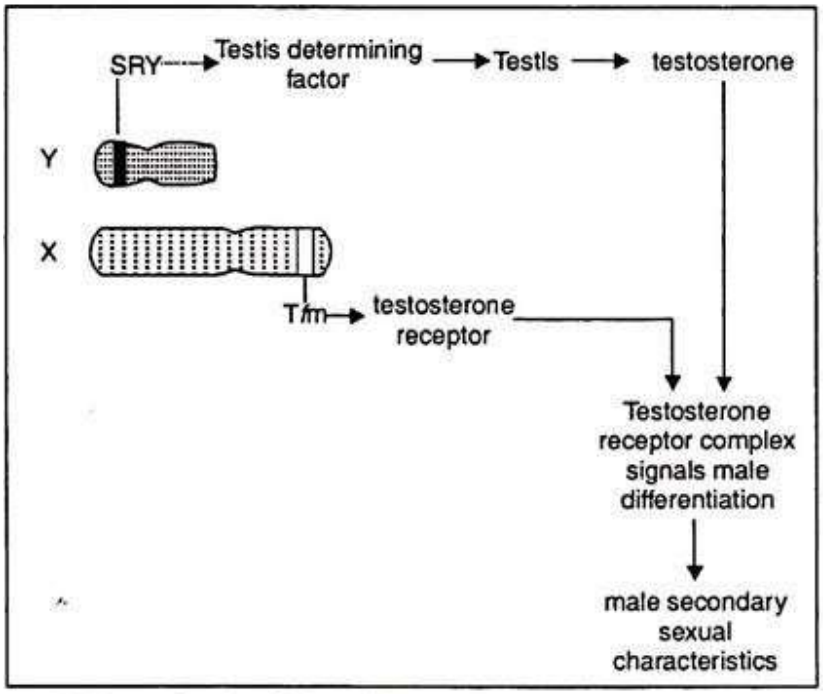


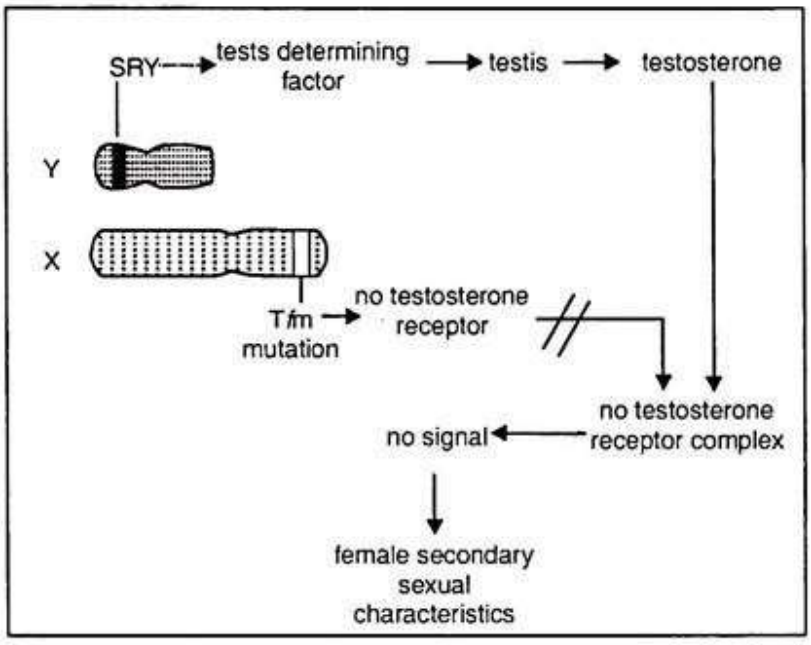
Fig. 5. Sex determination in human beings. Maleness is due to TDF by a gene on the Y chromosome.

After the formation of the testes, testosterone secretion initiates the development of male sexual characteristics. The hormone testosterone binds to receptors of several types of cells. This binding leads to the formation of a hormone – receptor complex that transmits signals to the cell instructing how todifferentiate.

The combined differentiation of many types of cells leads to the development of male characteristic like beard, heavy musculature and deep voice. Failure of the testosterone signaling system leads to nonappearance of the male characters and the individual develops into a female. One of the reasons for failure is an inability to make the testosterone receptor.



(a) Normal male with the wild type Tfm gene.



(b) Male with the tfm mutation and testicular ferminizaton.

Fig. 6. Testicular feminization, a condition caused by an X-linked mutation. tfm that prevents the production of the testosterone receptor.

Individuals with XY chromosomal composition having this biochemical deficiency first develop into males. In such males, although testis is formed and testosterone secreted, it has no effect

because it cannot reach the target cell to transmit the developmental signal. Individuals lacking the testosterone receptor therefore can change sexes during embryological development and acquire female sexual characteristics.However, such individuals do not develop ovaries and remain sterile. This syndrome known as testicular feminization is due to a mutation in an X-linked gene, tfm that codes for the testosterone receptor. The tfm mutation is transmitted from mothers to sons who are actually phenotypically female in a typical X- linked manner.

Master Regulatory Gene:

In human beings irregular sex chromosome constitutions occur occasionally. Any number of X chromosomes (XXX or XXXX), in the absence of a Y chromosome give rise to a female. For maleness, the presence of a Y chromosome is essential and even if several X chromosomes are present (XXXXY), the presence of a single Y chromosome leads to maleness.The Y chromosome induces the development of the undifferentiated gonad medulla into testis, whereas an XX chromosomal set induces the undifferentiated gonadal cortex to develop into ovaries. The gene on the Y chromosome that induces the development of testes is called as Testis Determining Factor (TDF). It has been isolated, characterized and found to encode a protein that regulates the expression of other genes.Thus, the TDF gene is the master regulator gene that triggers the expression of large number of genes that produce male sex phenotype. In the absence of TDF gene, the genes that produce femaleness predominate and express to produce a female phenotype. The TDF exerts a very dominant effect on development of the sex phenotype.

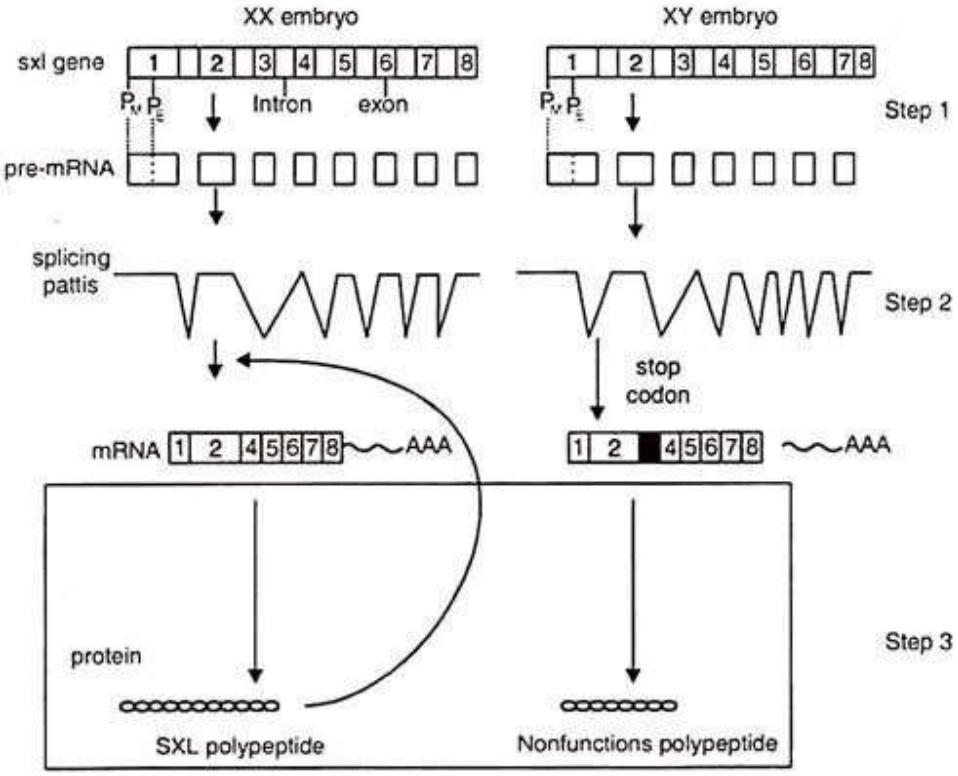
Genic Balance Theory of Sex Determination in Drosophila:

In Drosophila investigations by C.B. Bridges have shown that X chromosomes contain female determining genes and male determining genes are located on the autosomes and many chromosome segments are involved. The genie balance theory of sex determination in Drosophila explains the mechanism involved in sex determination in thisfly.
The Y chromosome in Drosophila does not play any role in sex determination. Sex in this animal is determined by the ratio of X chromosomes to autosomes. Normal diploid insects have a pair of sex chromosomes, either XX or XY, and three pairs of autosomes. These are denoted by AA, each A representing one set of haploid autosomes. Flies with abnormal number of autosomes can be produced by genetic manipulation as shown in Table below.

Table 1. Ratio of X chromosomes to autosomes and the phenotype of *Drosophila.*

X chromosomes (X) and Sets of autosomes (A)	X : A ratio	Phenotype
1X 2A	0.5	Male
2X 2A	1.0	Female
3X 2A	1.5	Metafemale
4X 3A	1.33	Metafemale
4X 4A	1.0	Tetraploid female
3X 3A	1.0	Triploid female
3X 4A	0.75	Intersex
2X 3A	0.67	Intersex
2X 4A	0.5	Tetraploid male
1X 4A	0.33	Metamale

Whenever the ratio of X chromosomes to autosomes is 1.0 or above, the sex of the fly is female, and whenever it is 0.5 or less, the fly is male. If the ratio is between 0.5 and 1.0, it is an intersex with both male and female characters. In all these phenotypes, Y chromosome has no role to play but it is required for the fertility of the male. In Drosophila sex determination mechanism, an X- linked gene called Sex lethal (Sxl) plays an important role (Fig. below).



Step 1. Transcription in XX embryos, a molecular signal based on the X:A ratio initiates transcription of the Sxl gene from promoter P_E. Later transcription is initiated at promoter P_M in both XX and XY embryos.

Step 2. Splicing in XX embryos, the Sxl transcripts are spliced to contain all the exons except exon 3. In Xy embryos, the Sxl transcipts are splicied to contain all the exons including exon 3.

Step 3. Translation in XX embryos, the Sxl mRNA in translated into a polypeptide (SXL) that regulates splicing, including the splicing of SXL transcripts. In XY embyryos, a stop codon in exon 3 prevents the SXL mRNA from being translated into a functional polypeptide.

Fig. 7. Sex specific expression of the sex-lethal (Sxl) gene in *Drosophila*.

A number of X linked genes sets the level of Sxl activity in a zygote. If the ratio between X chromosomes and autosomes is 1.0 or above, the Sxl gene becomes activated and the zygote develops into a female. If the ratio is 0.5 or less, the Sxl gene is inactivated and the zygote develops into a male. A ratio between 0.5 and 1.0 leads to mixing of signals and the zygote develops into an intersex with a mixing of male and female characters.

The sex ratio of X chromosomes to autosomes and the phenotype of Drosophila determination pathway in Drosophila has three components:

(i) A system to ascertain the X : A ratio in the earlyembryo,

(ii) A system to convert this ratio into a developmental signal,and

(iii) A system to respond to this signal by producing either male or femalestructures.

The system to ascertain the X : A ratio involves interactions between maternally synthesized proteins that have been deposited in the eggs cytoplasm and embryologically synthesized proteins that are coded by several X-linked genes. These latter proteins are twice as abundant in XX embryos as in XY embryos and therefore provide a means for counting the number of X chromosomespresent.Because the genes that encode these proteins effect the numerator of the X : A ratio, they are called numerator elements. Other genes located on the autosomes affect the denominator of X : A ratio and are therefore called as denominator elements. These encode proteins that antagonize the products of numerator elements (Fig.below).

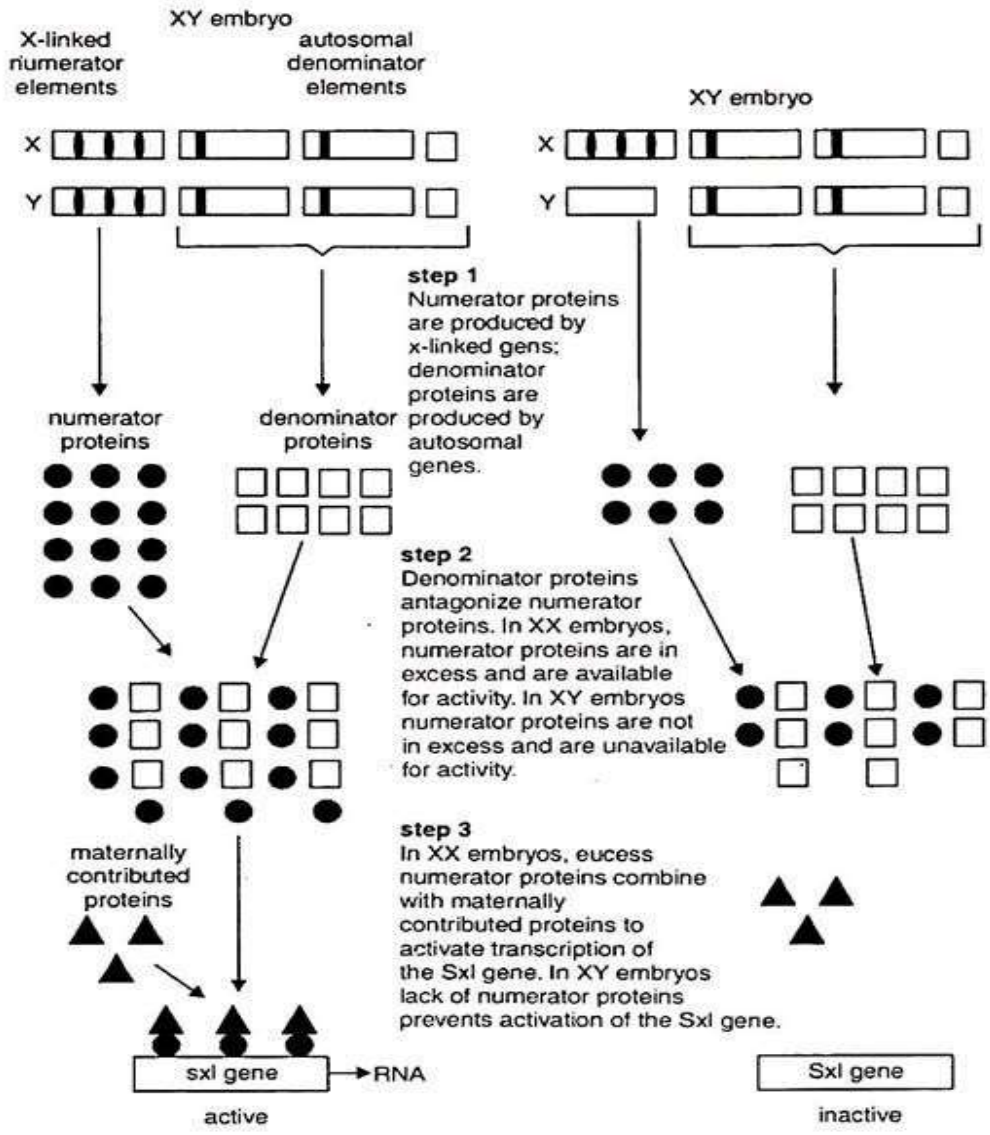


Fig. 8. Ascertainment of the X : A ratio by numerator and denominator elements in *Drosophila*.

The system for ascertainment of the X : A ratio in Drosophila is therefore based on antagonism between X-linked (numerator) and autosomal (denominator) gene products. Once the X : A ratio is ascertained, it is converted into a molecular signal that controls expression of the X-linked sex lethal gene (Sxl), the master regulator of the sex determination pathway.

Early in development, this signal activates transcription of the Sxl gene from PE' the gene's 'early' promoter, but only in XX embryos. The early transcripts from this promoter are processed and translated to produce functional sex-lethal proteins, denoted Sxl. After only a few cell divisions, transcription from the PE promoter is replaced by transcription from another promoter, PM.

The so called maintenance promoter of the Sxl gene. Interestingly, transcription from the PM promoter is also initiated in XY embryo. However, the transcripts from PM are correctly processed only if Sxl protein is present. Consequently, in XY embryos, where this protein is not synthesized, the Sxl transcripts are alternately spliced to include an exon with a stop codon, and when these alternately spliced transcripts are translated, they generate a short polypeptide without regulatoryfunction.

Thus, alternate splicing of the Sxl transcripts in XY embryos does not lead to the production of functional Sxl protein and in the absence of this protein, these embryos develop as males. In XX embryos, where Sxl protein was initially made in response to X : A signal, Sxl transcripts from the PM promoter are spliced to produce more Sxl proteins.

In XX embryos, this protein is therefore, a positive regulator of its own synthesis forming a feedback mechanism that maintains the expression of the Sxl proteins in XX embryos and prevents its expression in XY embryos. The Sxl protein also regulates the splicing of transcription from another gene in the sex determination pathways, transformers (tra). These transcripts can be processed in two differentways.

In chromosomal males, where the Sxl protein is absent, the splicing apparatus always leaves a stop codon in the second exon of the tra RNA. Thus, when spliced tra RNA is translated, it generates a truncated polypeptide. In females, where the Sxl protein is present, this premature stop codon is removed by alternate splicing in at least some of the transcripts. Thus, when they are translated, some functional transformer protein tra is produced. The Sxl protein therefore allows the synthesis of functional tra protein in XX embryos but not in XY embryos (Fig. 9).

The tra protein also turns out to be a regulator of RNA processing. Along with tra 2, a protein encoded by the transformer 2 (tra 2) gene, it encodes the expression of double sex (dsx) an autosomal gene that can produce two different proteins -through alternate splicing of its RNA. In XX embryos, where the tra protein is present, dsx transcripts are processed to encode a DSX protein that represses the genes required for male development.Therefore, such embryos develop into females. In XY embryos, where the TRA protein is absent, dsx transcripts are processes to encode a DSX protein that represses the gene required for female development. Consequently, such embryos develop into males. The dsx gene is therefore, the switch point at which a male or female developmental pathway is chosen. From this point, different sets of genes are specifically expressed in males and females to bring about sexual differentiation.

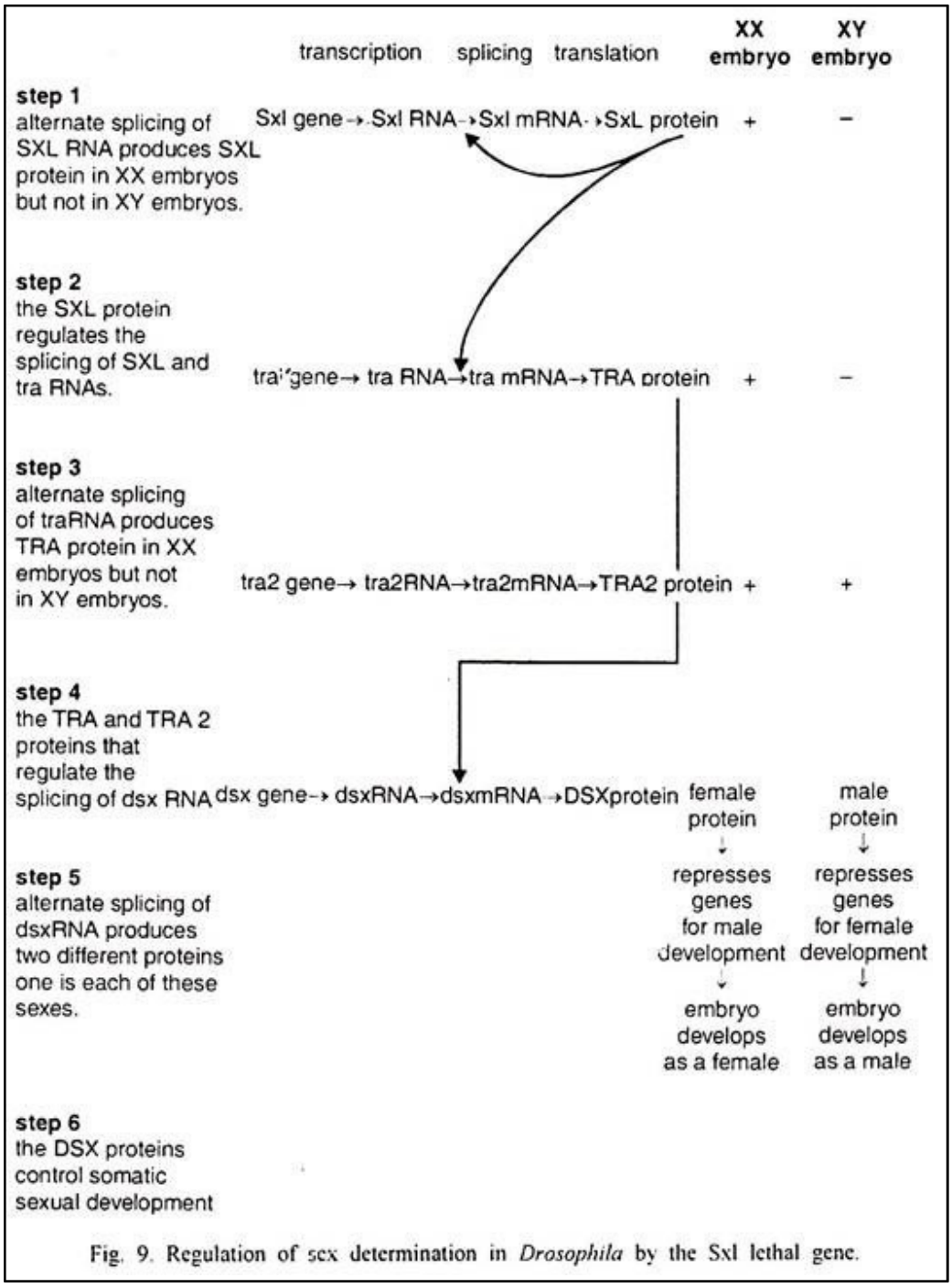


Fig. 9. Regulation of sex determination in *Drosophila* by the Sxl lethal gene.

Twin Studies:

Twins can be either dizygotic (fraternal) or monozygotic (identical). Dizygotic twins are the result of two different ova fertilized by two different sperm. Monozygotic twins are the result of one ovum fertilized by one sperm that divides to form two embryos. In the past, the only way of differentiating between monozygotic and dizygotic twins at birth was their sex and appearance. If the twins were of unlike sex, they are said to be dizygotic and if they were like-sexed and looked identical, they are said to be monozygotic. But this is not reliable. Today sex, placentation cord, blood type, HLA antigens and DNA fingerprinting are all used to differentiate between Monozygotic and Dizygotic twins.

However, DNA fingerprinting has become the only accurate method to differentiate between Monozygotic and Dizygotic twins. The monozygotic twins are genetically identical and any discordance between them is due to environmental influences whereas differences within dizygotic twin pairs are likely to be a combination of genetic and environmental factors.

Both monozygotic and dizygotic twins are known to have an increased risk of structural defects compared to the singletons. Structural defects in monozygotic twins however, are three times more frequent than among dizygotic twins and approximately 2-3 times more frequent than in single-tones.The incidence of monozygotic twins is thought to be constant throughout the world. By contrast, the incidence of dizygotic twins varies from population to population with a higher prevalence in some areas like Nigeria, and lower prevalence in other areas, as in Japan.

The prevalence of monozygotic twins in remarkably constant and has not been observed to be affected by environmental or maternal factors. Ultrasound studies done early in pregnancy have shown that at least 10% of twin pregnancies are either lost early in pregnancy by miscarriage or are reduced to singletons. Several studies have confirmed that the number of twins at delivery is considerably less than the number of twins conceptions seen on ultrasound examinations in early pregnancy. Some of the mechanism that have been suggested for the vanishing twin include vascular compromise, life threatening malformations, or spontaneous mutations incompatible with life.

Dizygotic Twins:

Their genetic contribution is different since it comes from two different ova and two different sperm. Dizygotic twining is a common occurrence in animals. Mammals are known to have sizable litters, generally due to poly-ovulation, making every member of a litter a dizygotic twin. Dizygotic twins produced by the fertilization of multiple ova may be result of superfecundation and it occurs when two different ova are fertilized by two different sperm in more than one act of coitus, either during one ovarian cycle or in subsequent cycles. Dizygotic twins may also arise from superfetation. Superfetation occurs when a second fertilized ovum implants in a uterus already containing a pregnancy of at least one month.

Superfetation has been suggested in some cases in whom the twins are markedly discordant for birth weight supposedly due to different gestational ages. Polar body twins are other types of dizygotic twins, thought to arise from the simultaneous fertilization of the meiotic product of the same primary oocyte—the oocyte and the polar body—by two different spermatozoa.All most all dizygotic twins have two placentas, two chorions and two amnions, i.e., be diamniotic and di- chorionic. However these two may fuse and look like one. The highest dizygotic twining rate is seen in the black populations (Africans) and lowest in Asian populations. But dizygotic twining rate is closely related with maternal age, parity, height, weight and also on gonadotropin levels.
Tall and heavy women are more likely to give birth to dizygotic twins than are short and thin women: There are many reports of familial dizygotic twining and the female members of these families are thought to have an inherited predisposition to multiple ovulation and in turn have a higher number of dizygotic twin pairs when compared to general populations.

Monozygotic Twins:

These are also known as identical twins and are the result of the fertilization of one ovum by one sperm. The single fertilized ovum then divides into two embryos; both embryos are thought to have the same genetic contribution.The major cause for the monozygotic twining in human is still unknown, however several mechanisms have been proposed, which are:

(1) Lack of O_2 prior to implantation which caused developmental arrest and splitting in the zygote

(2) Delayedimplantation

(3) Disturbances in the developmentalclocks

(4) Delayedfertilization

(5) Rupture of zonapellucida

(6) Congenital anomaly or an abnormality indevelopment

(7) Discordance in the expression of genetic information like X-inactivation's, imprinting, uniparental disomy, changes in the chromosome number and also mitochondrialmutations.

The incidence of monozygotic twins is constant throughout the world and it is about 3-4/1000 births. The rate of monozygotic twining appears to be unaffected by maternal age, parity, height or weight. But a few families have been reported in which monozygotic twining occurs more frequently than expected. This has been termed as "Familial monozygotic twining" and it is generally inherited from both the maternal and paternal side of the family.

It has also been suggested that this is due to a single gene effect which is unaffected by the sex of the parent transmitting gene. Monozygotic twins are known to have a higher incidence of all types of congenital anomalies and some of them are very unique to the monozygotic twining process itself.The sex ratio, i.e., the proportion of males to the combination of males and females—among monozygotic twins is lower than among dizygotic twins or singletons. Conjoined twins have an even lower sex ratio than that of monozygotic twins. Female conceptions may be at higher risk of late splitting of theembryo.

Anomaly	Descriptions
Fetus in fetu	Small parasitic dead twin attached to a normal twin which are often confused with a tumor. Generally located at the origin of the superior mesenteric vessels. Others sites have also been reported.
Fetus papyraceous	Mummified dead fetus usually attached to the placenta and present with a normal or more viable twin.
Acardia	Twin with an absent or rudimentary or non-functioning heart and whose circulation has been sustained by a normal twin. Associated with a higher rate of chromosomal anomalies
Conjointed Twins	Incomplete twins resulting from an abnormality of the twining process. They are derived from a single zygote and are always of the same sex. Incidence varies from 1-20,000 to 1-1,00,000. Females makes make up 80% of conjointed twins.

Twin Study and Multifactorial Inheritance:

Most quantitative traits behave in a more complex manner. The trait may be influenced not only by multiple genes but also by environmental factors. There may, in addition, be interactions among genes and between genes and the environment. How, then, can the contribution of genetic factors be dissected out? One of the most powerful tools for doing this is the twin study. Comparison of the concordance of a trait in identical twins with fraternal twins or full siblings is a powerful way to define the degree to which a trait is genetically determined.

For a single gene trait with complete penetrance, identical twins will, of course, be fully concordant. Siblings will be concordant less often, depending on whether they both inherit the mutant gene. For a non-genetic trait, concordance will be the same in identical twins or in fullsiblings and will depend on the degree of similarity of their exposure to environmental or other factors that determine the trait.

For a multifactorial trait concordance in identical twins will be greater than for siblings but not to the extent of a single gene trait with complete penetrance. The degree of concordance in monozygotic twins versus dizygotic twins or full siblings provides a measure of the contribution of genetic factors to the trait. Twin studies have helped to identify genetic contributions not only to congenital anomalies but also to common disorders such as hypertension, asthma and diabetes. Though powerful, twin studies are limited by the relative scarcity of identical twins who have a trait of interest, a problem that has been partly addressed through the development of twin registries. Another limitation is that twins share not only genetic identity but also some common environmental exposures, beginning with having developed in the same of identical twins. These offspring are the uterine environment. Studies of twins separated at birth control for post-natal environmental effects but not for prenatal effects. Another environment, approach has been to compare the

offspring Identical twins. These offspring are the equivalent of half siblings and share half their genes, yet they are born and raised in different environment.

Probable Questions:

1. What do you mean by Sex determination? What is its importance?
2. How sex is determined in XX-XY system?
3. How sex is determined in ZZ-ZW system.
4. What is Genic balance theory?
5. How sex is determined in human being?
6. Describe the role of Y chromosome in human sex determination.
7. Why twin study is important?
8. write down the characteristics of monozygotic twins.
9. 8. write down the characteristics of dizygotic twins.
10. Write a short note on twin study and multifactorial inheritance.

Suggested readings:

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7^{th} Edition.
6. Gene cloning and DNA Analysis by T. A. Brown, Sixth Edition.
7. Genetics . Verma and Agarwal.

Disclaimer:

The study materials of this book have been collected from various books, e-books, journals and other e sources.

Post-Graduate Degree Programme (CBCS)

in

ZOOLOGY

SEMESTER-III

HARD CORE THEORY PAPER

Arthropod of Economic Importance

& Biodiversity and Resource management

ZHT-309

SELF LEARNING MATERIAL

DIRECTORATE OF OPEN AND DISTANCE LEARNING

UNIVERSITY OF KALYANI

KALYANI, NADIA,

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Director's Message

Satisfying the varied needs of distance learners, overcoming the obstacle of distance and reaching the unreached students are the threefold functions catered by Open and Distance Learning (ODL) systems. The onus lies on writers, editors, production professionals and other personnel involved in the process to overcome the challenges inherent to curriculum design and production of relevant Self Learning Materials (SLMs). At the University of Kalyani a dedicated team under the able guidance of the Hon'ble Vice-Chancellor has invested its best efforts, professionally and in keeping with the demands of Post Graduate CBCS Programmes in Distance Mode to devise a self-sufficient curriculum for each course offered by the Directorate of Open and Distance Learning (DODL), University of Kalyani.

Development of printed SLMs for students admitted to the DODL within a limited time to cater to the academic requirements of the Course as per standards set by Distance Education Bureau of the University Grants Commission, New Delhi, India under Open and Distance Mode UGC Regulations, 2017 had been our endeavour. We are happy to have achieved our goal.

Utmost care and precision have been ensured in the development of the SLMs, making them useful to the learners, besides avoiding errors as far as practicable. Further suggestions from the stakeholders in this would be welcome.

During the production-process of the SLMs, the team continuously received positive stimulations and feedback from Professor (Dr.) Sankar Kumar Ghosh, Hon'ble Vice-Chancellor, University of Kalyani, who kindly accorded directions, encouragements and suggestions, offered constructive criticism to develop it within proper requirements. We gracefully, acknowledge his inspiration and guidance.

Sincere gratitude is due to the respective chairpersons as well as each and every member of PGBOS (DODL), University of Kalyani. Heartfelt thanks is also due to the Course Writers-faculty members at the DODL, subject-experts serving at University Post Graduate departments and also to the authors and academicians whose academic contributions have enriched the SLMs. We humbly acknowledge their valuable academic contributions. I would especially like to convey gratitude to all other University dignitaries and personnel involved either at the conceptual or operational level of the DODL of University of Kalyani.

Their persistent and co-ordinated efforts have resulted in the compilation of comprehensive, learner-friendly, flexible texts that meet the curriculum requirements of the Post Graduate Programme through Distance Mode.

All the Self Learning Materials are self writing and collected from e-book, journals and websites.

Prof Manas Mohan Adhikary
Director
Directorate of Open and Distance Learning
University of Kalyani

HARD CORE THEORY PAPER (ZHT- 309)

Arthropod of Economic Importance

& Biodiversity and Resource management

Group A: Arthropod of Economic Importance

Module	Unit	Content	Credit	Class	Time (h)	Page No.
ZHT - 309 (Arthropod of Economic Importance)	**I**	Insect pests: pest fauna (names only) of stored grains; Morphology, bionomics and control of: stored rice grain moth *(Corcyra cephalonica)* and stored pulse beetle *(Callosobruchus chinensis)*	**2.0**	**1**	**1**	
	II	Pest management : Mechanical; Chemical ; Biological; Integrated		**1**	**1**	
	III	Lac culture: Life-history of lac insect, culture method, lac processing, lac products, natural enemies of lac insect and their control.		**1**	**1**	
	IV	Sericulture: Indigenous races, pure races and commercial races of mulberry silk moth; Rearing of mulberry silk moth (moriculture excluded)		**1**	**1**	
	V	Parasitic insects: General remarks on *Phlebotomous, Glossina, Tabanus* and head louse in relation to morphology, habit, habitat, life-cycle and disease caused by them, mode of transmission.		**1**	**1**	
	VI	Parasitic Acarines: General remarks on ticks in relation to morphology, habitat, life-cycle and diseases caused by them.		**1**	**1**	

Unit-I

Insect pests: pest fauna (names only) of stored grains; Morphology, bionomics and control of: stored rice grain moth *(Corcyra cephalonica)* and stored pulse beetle *(Callosobruchus chinensis)*

Objective: In this section you will know about different types of Insect pests. You will also know about morphology, taxonomy and control measures of stored rice grain moth *(Corcyra cephalonica)* and stored pulse beetle *(Callosobruchus chinensis)*

I. Rice grain moth *(Corcyra cephalonica)*

The rice moth can be considered the Indian meal moth of the tropics. It is found in Hawaii and occasionally in some southern U.S. ports. It is much less common on the mainland than the Almond moth, (*Ephestiacautella*), which it is often misidentified. The larvae are general feeders and prefer warm climates and occur commonly in the equatorial regions of Asia, Africa, and Caribbean.

The rice moth is a major pest of flour mills in the tropics. It is a general feeder and can be found infesting stored millet, sorghum, rice, cocoa beans, biscuits, flour, and other seeds. The rice moth populations develop well in hot damp or dry (> 20% rh) areas. These moths can infest mills and storage areas simultaneously with Almond moths.
The moth is usually seen in large numbers on walls, poles, or containers where grains are stored. The larvae are exceptionally good at producing "paper thick" webbing for its cocoons. The caterpillars produce a large amount of frass (in which they hide) compared to other stored food moths. This material can attract other stored food pests such as Flour beetles (*Tribolium*spp).

Taxonomy:

Kingdom: Animalia
Phylum: Arthropoda
Subphylum: Uniramia
Class: Insecta
Order: Lepidoptera
Family: Pyralidae
Genus: Corcyra
Species: cephalonica

Morphology: The adult moth is grey, often with darker spots or venation on the wings; the under wings are off white. The wingspan is 20-23 mm. The tips of the wings are more rounded than those of the *Ephestia* spp. moths. The larva of the rice moth will grow to 15 mm long and are white or cream in colour. The body is covered with fine hairs. A good clue that rice moth larvae are present is the excessively thick layers of webbing.

Life cycle:

Adults light greyish-brown in colour, 12 mm long and with a wing span of about 15 mm, without any markings on the wings but veins are slightly darkened. Head bears a projected tuft of scales. Moths are short lived but realise a fecundity of 150—200 eggs per female within a few days after emergence.

Eggs are laid anywhere, on the grains, among grains, on the containers or on any surface near the grains, either singly or in clusters. Eggs are whitish, oval in shape, 0.5 mm long and having an incubation period of 4-5 days. Tiny larva after hatching is creamy-white, with a prominent head. It moves about actively and feeds on broken grains for sometime and then starts spinning web to join grains.

Full grown larva is pale whitish in colour, 15 mm long with short scattered hairs and no markings on body. Larval period is 25-35 days in summer and may be extended in winter. Pupation takes place inside an extremely tough, opaque whitish cocoon that is surrounded by webbed grains. Pupal period is about 10 days but may extend to 40-50 days to tide over winter moths. Moths commence mating and egg laying immediately after emergence.

Prevention and control:

a. Cultural Control and Sanitary Methods:

Good store hygiene plays an important role in limiting infestation by *C. cephalonica*. The removal of infested residues from last season's harvest is essential as is general hygiene in stores, such as ensuring that all spillage is removed and cracks and crevices filled. Infestations may also be limited by the storage of good-quality grains such as whole cereals with fewer broken grains and foreign matter or milled rice with a high degree of milling (at least 95%) and few broken grains.

b. Plant Extracts:

Vitex negundo leaf powder, neem leaf powder and neem oil were the most effective of a range of plant products tested for efficacy against *C. cephalonica* in stored ground nuts. Four neem products (azadirachtin, azadirachtin-iodine, neem seed kernel extract and neem oil) were tested on the eggs and larvae of *C. cephalonica*for ovicidal, larvicidal, feeding deterrent growth regulatory and antifertility activity. Age, toxicity, mortality, concentration and growth inhibition established a dose-response relationship whereas feeding inhibition and growth disruption were independent events. Petroleum ether extracts (1.5%) of *Azadirachta indica*, *Erythrina indica* and *Piper nigrum* and

Pachyrhizuserosus (at 2%) and methanol extracts (2%) of *A. indica* and *P. nigrum* prevented egg hatching in *C. cephalonica*6 days after treatment. Leaf extracts of *Ricinus communis*, *Lawsoniainermis*, *Acacia nilotica*, *Cassia fistula*, *Eucalyptusrudis, Dalbergia sissoo* and *Parthenium hysterophorus* were tested for ovicidal activity against *C. cephalonica*. Leaf extracts of *Ricinus communis* (100%) produced maximum (89.5%)mortality, with lowest mortality (42.1%) exhibited by *P. hysterophorus*. Oil from sweet flag (*Acorus calamus*) was found to repel 10-day-old larvae of *C. cephalonica* at 0.1-0.5%. Oils from clove, cedar wood, citronella and eucalyptus were also effective.

c. Chemical Control:

Fumigation is one of the most effective ways of killing all stages of this insect in commodities and buildings. Fogging with a 0.5% pyrethrin is recommended only for knocking down exposed adult stages. Use of longer lasting synthetic pyrethroid and insect growth regulators in a fog will give superior results.

II. Stored Pulse Beetle *(Callosobruchus chinensis)*

Callosobruchus chinensis is a common species of beetle found in the bean weevil subfamily, and is known to be a pest to many stored legumes. Although it is commonly known as the adzuki bean weevil it is in fact not a true weevil, belonging instead to the leaf beetle family, Chrysomelidae. Other common names include the pulse beetle, Chinese bruchid and cowpea bruchid. This species has a very similar lifestyle and habitat to *Callosobruchus maculatus* and their identities are often mistaken for each other. This beetle is a common pest targeting many different species of stored legumes and it is distributed across the tropical and subtropical regions of the world. *C. chinensis* is one of the most damaging crop pests to the stored legume industry due to their generalized legume diets and wide distribution

Distribution: Cosmopolitan in the tropics and subtropics of the world. A closely related species, *Callosobruchus maculatus* is found existing along with *chinensis*. Adults of the former species are elongated and darker and posterior part of the abdomen is exposed.

Morphology: The female is about 3-3.5 mm in length; the elytra are red-brown with yellow makings, antennae and legs yellow, female antennae serrate, those of male pectinate. Larva yellowish-white with a brown head and reduced legs, about 5 mm long. Several geographic strains of this species are known whose morphology is slightly different.

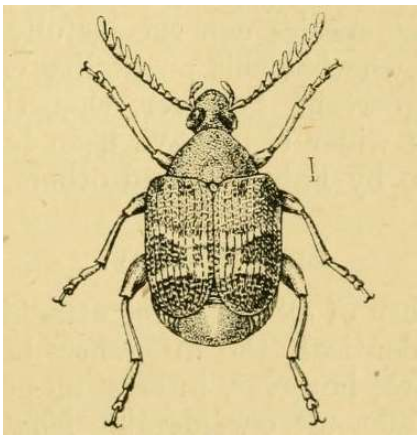


Figure: External morphology of *Callosobruchus chinensis*

Taxonomy:

Kingdom:	Animalia
Phylum:	Arthropoda
Class:	Insecta
Order:	Coleoptera
Family:	Chrysomelidae
Genus:	*Callosobruchus*
Species:	*chinensis*

Host plants: Beans and peas, especially chick-peas with smooth, spherical pods.

Life cycle: Adult beetle is 3-4 mm long, female being larger, brownish in colour, broader at shoulders and rounded posteriorly. There are dark patches on elytra and thorax. Adults show sexual dimorphism. Males possess deeply emarginated or indented eyes and prominently serrate antennae, while in female these characters are not distinctly marked.

In females tip of abdomen is exposed while in males it is covered by elytra. They are active beetles and readily fly when disturbed. Fecundity is about 100 eggs per female. Eggs are whitish, elongated and stuck on the grains or on pods and sometimes on the surface of the container. Incubation period is 3-6 days. Grubs are scarabeiform or eruciform, plump and with short legs and yellowish in colour.

First instar larvae bear functional legs and a pair of thoracic plates to facilitate boring into the seeds. They feed on the inner contents of the grain and may damage several grains during development. Larval period may vary between 12 and 20 days. Pupation takes place inside the grain and pupa is dark brown in colour. Occasionally pupation may take place outside the grain in a cocoon made of excretory matter. Completion of life cycle takes 4-5 weeks and there may be 6-7 overlapping generations in a year.

Economic importance: The Adzuki beetle is a major pest of stored lentils. Pod infestation can start in the field before harvest, the pest thus gaining entrance into storage bins. It may cause substantial damage, coming to over 80% losses in weight and in germination rates. Infested seeds are less nutritious and unfit for humans.

Management:

Horticultural practices: Intercropping with cereals and early harvesting of legumes, before pest attack. Good store hygiene, including the removal of residues from last season's harvest. Heating seeds to 50ºC for one hour kills the eggs and larvae.

Plant resistance: Certain legume varieties with thick, hairy walls are resistant to beetle infestation.

Chemical control: Organophosphates and neem compounds have been used in different countries. Many plant extracts and oils were applied with uneven success, as oviposition deterrents and for beetle control.

Biological control: Several parasitoids of the families Braconidae and Pteromalidae attack *C. chinensis* in various parts of the world. The mite Pyemotes also parasitizes the pest.

Probable Questions:

1. Briefly describe the morphological features of *Corcyra cephalonica.*
2. Describe the life cycle of *Corcyra cephalonica.*
3. How *Corcyra cephalonica* can be controlled?
4. Briefly describe the morphological features of *Callosobruchus chinensis*
5. Describe the life cycle of *Callosobruchus chinensis*
6. How *Callosobruchus chinensis* can be controlled?

Suggested Readings:

1. The Insects by Chapman
2. Modern Entomology by D.B. Thembare
3. Economic Zoology by Shukla and Upadhyay
4. The Insects by Gullan and Carnston
5. Introduction to Economic Zoology by Sarkar, Kundu and Chaki.
6. A textbook of Economic Zoology by Aminul Islam

Unit-II

Pest management : Mechanical; Chemical ; Biological; Integrated

Objective: In this section you will learn about Different mode of Pest management such as Mechanical, Chemical, Biological control of pests. In this section you will also know about Integrated Past management (IPM) system

Introduction: Pest control is the regulation or management of a species defined as a pest, a member of the animal kingdom that impacts adversely on human activities. The human response depends on the importance of the damage done, and will range from tolerance, through deterrence and management, to attempts to completely eradicate the pest. Pest control measures may be performed as part of an integrated pest management strategy.

In agriculture, pests are kept at bay by cultural, chemical and biological means. Ploughing and cultivation of the soil before sowing reduces the pest burden and there is a modern trend to limit the use of pesticides as far as possible. This can be achieved by monitoring the crop, only applying insecticides when necessary, and by growing varieties and crops which are resistant to pests. Where possible, biological means are used, encouraging the natural enemies of the pests and introducing suitable predators or parasites.

In homes and urban environments, the pests are the rodents, birds, insects and other organisms that share the habitat with humans, and that feed on and spoil possessions. Control of these pests is attempted through exclusion, repulsion, physical removal or chemical means. Alternatively, various methods of biological control can be used including sterilisation programmes.

History:

Pest control is at least as old as agriculture, as there has always been a need to keep crops free from pests. As long ago as 3000 BC in Egypt, cats were used to control pests of grain stores such as rodents. Ferrets were domesticated by 500 AD in Europe for use as mousers. Mongooses were introduced into homes to control rodents and snakes, probably by the ancient Egyptians.

The conventional approach was probably the first to be employed, since it is comparatively easy to destroy weeds by burning them or ploughing them under, and to kill larger competing herbivores. Techniques such as crop rotation, companion planting (also known as intercropping or mixed cropping), and the selective breeding of pest-resistant cultivars have a long history.

Chemical pesticides were first used around 2500 BC, when the Sumerians used sulphur compounds as insecticides. Modern pest control was stimulated by the spread across the United States of the Colorado potato beetle. After much discussion, arsenical compounds were used to control the beetle and the predicted poisoning of the human population did not occur. This led the way to a widespread acceptance of insecticides across the continent. With the industrialisation and mechanization of agriculture in the 18th and 19th centuries, and the introduction of the insecticides pyrethrum and derris, chemical pest control became widespread. In the 20th century, the discovery of several synthetic insecticides, such as DDT, and herbicides boosted this development.

Biological control is first recorded around 300 AD in China, when colonies of weaver ants, *Oecophyllas maragdina*, were intentionally placed in citrus plantations to control beetles and caterpillars. Also in China, ducks were used in paddy fields to consume pests, as illustrated in ancient cave art. In 1762, an Indian mynah was brought to Mauritius to control locusts, and about the same time, citrus trees in Burma were connected by bamboos to allow ants to pass between them and help control caterpillars. In the 1880s, ladybirds were used in citrus plantations in California to control scale insects, and other biological control experiments followed. The introduction of DDT, a

cheap and effective compound, put an effective stop to biological control experiments. By the 1960s, problems of resistance to chemicals and damage to the environment began to emerge, and biological control had a renaissance. Chemical pest control is still the predominant type of pest control today, although a renewed interest in traditional and biological pest control developed towards the end of the 20th century and continues to this day.

Different types of Pest Control:

A. Mechanical pest Control:

Mechanical pest control is the management and control of pests using physical means such as fences, barriers or electronic wires. It includes also weeding and change of temperature to control pests. Many farmers at the moment are trying to find sustainable ways to remove pests without harming the ecosystem.

Different methods which are employed in Mechanical control are as follows:

1. Keep insects away from the plants. Many flying insects (aphids, butterflies, flies, capsids, thrips, etc.) can be kept away from the crop with the help of insect netting. By covering the soil or substrate with polythene, cloth or with special collars around the stem base it is possible to protect plants against larvae that eat roots or sub-soil stems. These measures also stop the development of larvae and pupae that need soil for completion of their life cycle, and keep them from further spreading.

2. Trap insects. With the help of sticky traps, insect-o-cutors, trap plants, pheromone traps etc. it is possible to trap winged insects.

3. Use temperature treatments to kill harmful organisms. There are different methods:

a. Hot water Seeds, bulbs, tubers and cuttings can be immersed in hot water to kill potential pests such as insects, mites, nematodes, fungi and bacteria.

b. Hot air A hot air treatment can also kill harmful organisms in plants, bulbs and seeds.

c. Solarisation If the soil is covered with transparent polythene for several weeks in summer, temperatures can rise so high through solar radiation that pest organisms are killed.

d. Steaming Treatments with steam will disinfect soil, substrate, crates, etc.

4. Use flooding as a technique. If a piece of ground can be flooded for a sufficient period of time, most of the harmful organisms will die due to lack of oxygen.

5. Remove infested plant materials. Place them in a bag, and destroy.

B. Chemical Pest control :

Chemical pest control methods have been used for thousands of years by civilizations which had much less knowledge than the current population. Sumerians found out that sulfur gives great results in insect extermination.

However, the actual revolution in chemical pesticides happened during the 18th and 19th century when the industrial revolution required much more efficient pest treatments in terms of scale, effectiveness and speed. To present days, chemical pest control methods are among the major types of vermin extermination practices and despite the fact that pesticides often lead to serious health issues, chemical compounds are vastly produced and sold across the whole world.

Here are the major types of chemical pesticides that are used nowadays in agriculture, domestic and commercial properties for pest control of various insects and rodents:

I. Fungicides

Fungicides are chemical compounds or organic organisms with biocidal properties, which help for the destruction of fungi and fungal spores. Fungi may cause severe disruption of any agricultural process. This leads to losses of yield and lowers the final quality of the production. Apart from using fungicides in agriculture, they give very good results when used to cure fungal infections inside animals. The major active ingredient of almost any fungicide is sulphur, which may turn out to be 0.5% of what is contained inside some of the heavier fungicides. Here is the full list of fungicides used in agriculture. Refer to it whenever you need additional information

Advantages of Fungicides

a. Fungicides control mycotoxin-producing pathogens

b. Great value per cost. An artichoke production company in California earned 27 million pounds of artichokes for 1 000 pounds of chemical fungicide being invested.

c. Kills fungi and fungal spores with great efficiency

List of Natural Fungicides

Fungi can be controlled effectively without the use of chemicals. It's much more sustainable and causes no damage to the soil or pollution of any kind. Some plants do great job in the fight with fungi because of their naturally evolved defensive system. Processing plants into sprays, mixtures and compounds of any other kind, turns out to be a great way to make natural fungicide. You can use any of the following extracts for the preparation of natural fungicides: Nimbin, Tea tree oil ,Citronella oil, Jojoba oil, Rosemary oil, Monocerin, Oregano Oil

II. Insecticides

Insecticides are any chemical substances that are used for insect extermination. They successfully help to eliminate insects and any life stage, including ovicides, larvicides, eggs and larvae. Particular types of insecticides are used for exact purposes in a field like agriculture and medicine. One of the main reasons for the increased productivity of agriculture in the last century is the development of better and more affordable insecticides. On the other hand, insecticides are able to cause damage to the ecosystem and health, that's why recent studies and efforts are made towards increased implementation of organic pest control methods.

There two major classifications of insecticides:

a. With residual effect

b. Without residual effect (contact insecticides)

Another classification of the insecticides may be made based on their repellent qualities, this categorizes them in:

a. Repellent

b. Non-repellent

Repellent insecticides are more suitable when a pest is targeted for extermination. This way it will bring much of the insecticide to the colony and will wipe it out. Repellents are used in agriculture when people want only to keep the pest away from plants. When extermination is not planned, repellents give great efficiency. The drawback is that repellents should be applied more often, especially if water has been applied over the plants after a rain for instance. Repellents have much quicker effect in terms of crops preservation but don't deal with the source of the infestation, while non-repellent insecticides kill the insect but such compounds are often more toxic and contaminate both – the soil and the crops often beyond the suggested requirements.

Synthetic insecticides

a. Organophosphates and carbamates

b. Neonicotinoids

c. Ryanoids

d. Organochlorides

e. Pyrethroids (*should not be mistaken with pyrethrin, which is an organic compound)

III. Nematicides

Nematicide is a chemical pesticide which kills nematodes that parasitize on plants. The use of this chemical pesticide is very important for potato crops because of the soil-borne nematodes. Of course, nematicides may be natural such as extracts of neem oil. Non-fumigant nematicides are among the most popular type. They have low volatility and spread easily after sprayed on soil. If water is sprayed on the soil the spreading of the non-fumigant nematicides becomes much faster.

In case the nematicides are naular, you can enhance their effect by manually inserting them deeper in the soil. A rainfall would also help but if the insecticide has been sprayed as a liquid.

Non-fumigant Nematicides

Non-fumigant nematicides have low volatility and diffuse through the soil (generally for short distances only) dissolved in the soil solution. Their movement may be enhanced by water movement through irrigation or rainfall. If in granular formulations, their distribution may be enhanced by physical incorporation into the soil.

Side Effects of Nematicides

a. Groundwater contamination with toxins

b. Exposure to chemicals

c. People who use machinery for insecticide application are at higher risk.

d. Delayed harvest

e. Specific minimum time for residual effect to fade away is required which may postpone harvest.

f. Pesticide poisoning

g. Higher levels of mortality occur when certain regulations for usage are not followed.

IV. Rodenticides

Rodenticides are chemical pesticides, designed specifically for the extermination of rodents such as rats and mice. Most rodenticides are lethal and do not serve only as repellents. They are produced and applied in the form of food which the rodents consume. It may take several hours to a few days for a rodent to be killed after consuming a rodenticide.

However, rodents often sense the threat and observe the rodenticide for a long time before consuming it. This is known as poison shyness and to reduce this, scientists now develop rodenticides with a very strong residual effect. Instead of killing the rodent instantly, it causes dehydration and haemorrhage which cannot be stopped. This helps for avoiding problems related to rodents dying inside tiny crevices.

Advantages and Disadvantages of Chemical Pest Control:

a. Effectiveness

Chemicals exterminate any pest that hasn't adapted to the deadly substance inside the agent. They eradicate fast and with an efficiency of up to 100%. Most are very easy to apply and can get in the way of pests that hide in small crevices and other hidings.

b. Quickness

Some chemicals for pest control kill slowly because of the active ingredient. However, most pesticides are designed to exterminate the vermin in less than 3-4 days, which is much faster compared to organic methods of pest control such as importation or augmentation.

c. Precise targeting (localized)

Contrary to biological pest control methods, the chemical substances may target a specific area with high precision. On the other hand, if you release pest-destroying animals, there is no control over their behaviour – they may spread wherever they want.

d. Easy application

This one helps the customers a lot in their efforts towards DIY pest control with ready-to-use products. Most pesticides sold on the market are packed inside bottles, designed for easy use and application. They are readily available and spraying them on your crops takes few minutes and a little more time before that to read the instructions, which is something we highly recommend before using pesticides of any type.

e. Improving productivity

Pesticides become more and more effective in time but sometimes at the cost of being more toxic and unsustainable. Efforts of scientists are made towards researching pesticides that cause lower pollution and side effects on human health. However, it's difficult to achieve that because animal species

evolve against the chemicals and more poison is required to exterminate pests that are resistant to the old forms of pesticides.

f. Sports facilities maintenance

Sports facilities such as pitches and football terrains are endangered by pests too. If the turf is not maintained properly, it will be destroyed and the field will become unusable. Pesticides are used even there for the exterminate pests such as white grub worms, chinch bugs, bluegrass weevils, ants and more.

Disadvantages of Chemical Pest Control:

a. Promote evolution

The use of chemical pesticides stimulates the pest to develop resistance to the chemicals used in the pesticides. The resistance is based on alterations in the genetics of the vermin and every future generation becomes increasingly pesticide-proofed. This works very well among rodents which produce several litters per year.

b. Resurgence (non-precise targeting)

Resurgence happens when the use of pesticides affects the environment and disrupt the organic pest control. The most tremendous side effects of all are when significant animal species such as bees get killed by collateral damage after spraying with chemical pesticides.

Another side effect of resurgence is when pest-destroying animals such as parasitoid wasps are killed and they pray, mostly other pest insects, no longer have a natural enemy and start to multiply so quickly that completely overwhelm entire agriculture.

c. Persistent Organic Pollutants

POP, known as persistent organic pollutants, are extremely dangerous to the non-target organism but also affect the health of people by causing cancer, infertility or problems to the endocrine system.

Impact of Chemical Pesticides on the Environment:

a. Surface water contamination

b. Ground water contamination

c. Soil contamination

d. Air contamination

e. Effects on plants

f. Effects on animals

g. Direct impact on humans

Texture of Chemical Pesticides:

There are chemical pesticides in different form. You can find it in liquid or harder form and each has its specifications, advantages and disadvantages. These are the most common forms of chemical pesticides.

a. Granules / pellets:

The insecticides are soaked into coarse particles such as ground corn or nut shells. This way the toxic chemicals come out slowly and have a residual effect. The environment is polluted not at once but instead much slower.

Such type of insecticides is used against soil-dwelling insects for more effective results and penetration into the soil.

b. Dusts chemical pesticides

Inert particles such as ash, chalk and talk are used for making a pesticide in dust form. Their most comprehensive application is to disperse them directly onto the surface. Such a particle is usually so small and thin that they immediately stick to the body parts of insects. This makes it easier for the active chemical ingredient to start taking effect right after the moment of application. Dust chemical pesticides are very unsuitable for application in the open as they are very easily spread by the wind and get into the human body through the respiratory system.

c. Soluble powders / Wet powders

Those are chemical pesticides which are distributed in a powder-like form and require to mix them with water. Such foliar insecticides are described as wettable powders due to the ease of their transportation.

d. Emulsifiable concentrates

Chemical pesticides based on emulsifiable concentrates do not have a residual effect on fruits and vegetable. They are mostly used in the form of sprays to treat pests in urban and industrial areas. In standard emulsifiable pesticides, the emulsifier is usually dissolved in an organic solvent and the chemical concentration is watered with higher amounts of water.

e. Aerosols

Those are insecticides that have been produced and packed inside a spray can and have a solvent inside, added by the manufacturer. Such pesticides have a very long expiration date and are in most cases ready-to-use immediately after purchase. Some aerosols are designed specifically for fogging machines that are used by pest control experts for fumigation services indoors and outdoors.

C. Biological Control of Pests:

Biological control may be defined as the utilization of a pest's natural enemies in order to control that pest. It is the control of pests and parasites by the use of other organisms, e.g., of

mosquitoes by fishes which feed on their larvae. In other words, it is a practice in which an organism is used against another organism.

Under this practice, there are four types of pest control:

(i) Classical biological control or importation, in which a natural enemy from another geographical area, often the area in which the pest originated from, is introduced to contain the pest below the economic injury level, EIL, the definition of EIL is the pest density at which the difference between ne curve showing value of the crop and the curve showing cost of achieving this pest density is neatest;

(ii) Inoculation, in which the periodic release of a control agent is required so that it is available throughout the year. Inoculation is widely practiced in the control of arthropod pests in glasshouses, where crops are removed, along with their pests and their natural enemies at the end of the growing season;

(iii) Augmentation, which involves the release of an indigenous natural enemy in order to supplement an existing population, and is therefore carried out repeatedly usually to coincide with a period of rapid growth of pest population; and

(iv) Inundation, which s the release of large numbers of natural enemy, with the aim of killing those pests present at the time. These are usually termed biological pesticides. However, insects have been main agents of biological control against both insect pests and weeds.

General Theory of Biological Control:

The classical theory of biological control based on the Nicholson-Bailey model is an equilibrium theory (Huffaker and Messenger, 1976). According to this theory, a successful biological control IS produced by the predator imposing low, stable host equilibrium (Fig. 3.9).

But a successful bio-control agent should be host-specific, synchronous with the pest, should have high intrinsic rate of increase (r), should be able to survive with few prey available, and should have high searching ability. All these properties are shown by insect parasitoids than predators. Successful bio-control agents cause density-dependent losses in the host population.

Spatial density dependence occurs when parasitoids or predators cause a higher fraction of losses in dense host patches than in sparse host patches (Hossell, 1977). If predators can aggregate in patches of high host density, then, according to this theory, biological control of the pest is much more likely. The theory has been challenged recently by Murdoch et al. (1985). They have based their view on a non-equilibrium model of predator-prey interaction. The model assumes that a stable equilibrium of predator and prey is not necessary for satisfactory biological control. Pest populations may fluctuate wildly without pest densities exceeding the economic threshold. According to Krebs (1994), the non-equilibrium model is a meta-population model and, as such, emphasizes that population in different patches may fluctuate independently.

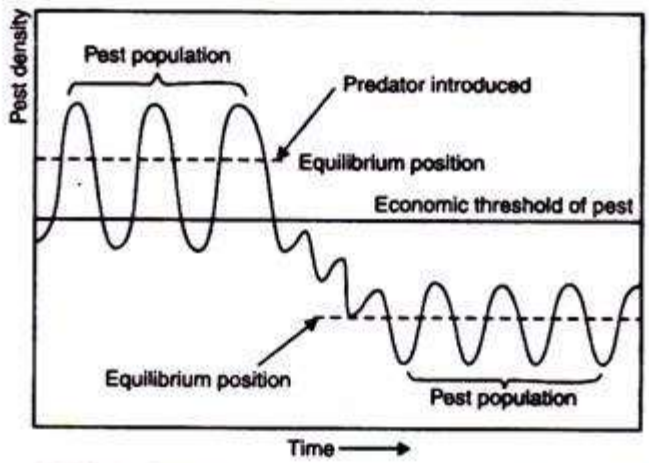


Fig. 3.9. Classic type of biological control in which the average abundance of an insect pest is reduced after the introduction of a predator. The economic threshold is determined by humans' activities, and its position is not changed by biological control programmes. (After van den Bosch *et al.* 1982)

Biological Control by Predators and Parasitoids:

Although predators are considered poor candidates for biological control, they have been used in a number of cases. For example, a small predaceous ladybird beetle, Rodoliacardinalis, commonly called vedalia, has been used to control the cottony- cushion scale insect (Iceryapurchasi), a pest of citrus trees. Adult Parasitoids (Hymenoptera) lay their eggs in or near other insects. The larval parasitoid then develops inside its host and kills it before or during the pupal stage.

Biological Control by Parasites:

Some calcid wasps control a number of major pests. The oriental fruit fly, Dacus dorsalis, a pest of ripe fruits in Hawaii has been controlled by three species of parasitic wasps of the genus Opius (*O. vandenboschi, O. longicaudatus and O oophilus*). This example also illustrates that several parasites of the same pest can be released without having any adverse effect on the overall control. Although the three control agents competed for the same host, the one with superior qualities displaced the others and became dominant.

In this case *O. vadenboschi* derived the advantage from attacking first instar larvae and thereby inhibiting the development of the eggs and larvae of *O. longicaudatus*, which favoured older host larvae for oviposition. Likewise, *O. oophilus*, which oviposits in the eggs of the host, are already present as larvae by the time hosts are suitable for attack by *O. vadenboschi.*

The geometrid moth Operopheterabrumata or winter moth, a pest (defoliator) of hardwood forest and ornamental trees in Canada and Europe, has been controlled by a tachinid fly, *Cyzenis albicans*, and a wasp *Agrypon flaveolatum*. However, in this case there was no displacement. Instead, the two species that are compatible and complimentary to each other were able to bring about control. *C. albicans* was very effective at high host densities, whereas the superior searching ability of *A. flaveolatum* made it effective at low host densities.

(a) Bacteria:

The use of spore-forming bacteria as a means of controlling the larvae of the Japanese beetle (*Popillia japonica*), a serious pest of fruits and vegetables, provided the first encouragement for the application of bacteria in insect control. *Bacillus popilliae* and *Bacillus lentimorbus* that cause types A and B milky disease of Japanese beetle can both be mass produced and are sold as a spore dust for injection into the soil. Infected larvae that die in the soil become 1 source of contamination for other larvae feeding in the vicinity. Larval population can be substantially reduced in this way and the Bacillus spores persist in the soil to infect larvae from generation to generation.

Another spore- forming bacteria Bacillus thuringiensis is a facultative pathogen that infects a variety of insects, including the larvae of lepidopterans, flies, and beetles. The bacteria can be cultured on artificial media and is therefore quite economical to produce. Commercial preparations of *Bacillus thuringiensis* (Biotsol, Dipel, Thuricide) containing both spores and crystals are used is a biological insecticide on a variety of crops. The rather specific nature of *Bacillus thuringiensis* to kill a few groups of foliage feeders and not to harm beneficial species is of great value in management programmes.

(b) Fungi:

Most entomogenous fungi are internal pathogens. They belong to all the four major taxonomic groups of true fungi, but only a few are frequently associated with insect disease outbreaks. The most commonly used in insect control are Beauvariabassiane (white muscardine disease) and Metarrhiziumanisopliae (green muscardine disease), both of which are fungi imperfect. The infective unit of an entomogenous fungi is usually a spore which germinates on the surface of the host's integument. Once the host tissue is invaded, the fungus can complete its life cycle, but the survival and germination of spores is critical to the development of an epidemic. Facultative fungi such as Beauvaria and Metarrhizium can be cultured on artificial media, thereby facilitating the production of spore preparations which may be used in biological control. As with most biological control agents, fungi can be used for either persistent or short-term control. A fungus can be introduced into an area where it becomes established and kills the host year after year. Alternatively, fungal spore preparations can be used as microbial insecticide similar 10 the way *Bacillus thuringiensis* is used. However, few attempts have been made to colonize entomogenous fungi. Most projects have involved the redistribution of indigenous fungi or those associated with introduced pests, rather than the importation of foreign species. The best example of attempts to establish new fungal pathogens in disease free areas involves the introduction of Coelomomyces against mosquito larvae, but so far the success has been limited. The successful use of repeated application of fungal spores as microbial insecticides has been reported for achieving short-term reductions of pest populations. The major limiting factor in initiation of fungal disease in insect populations is the effect of the microclimate on spore survival and germination.

The optimal temperature range for the growth of entomogenous fungi is fairly narrow, and relatively high humidity is needed by most fungi to germinate and successfully penetrate their host before they can produce the new spores required to spread the disease. Sunlight also kills the spores. Consequently, the application of a spore preparation must coincide with both the presence of susceptible hosts and suitable environmental conditions. Best success can usually

be obtained by applying the spores in the absence of sunlight such as on a warm evening after either rain or irrigation which provides the needed humidity.

(c) Viruses:

The insect pathogenic viruses are called inclusion viruses, as opposed to non-inclusion viruses in which the virus particles or viruses are free within the cells of the host. The virus particles first multiply in the nuclei, but later continue to replicate in the cytoplasm. The disease eventually kills the insect, leaving it hanging as a fragile sac of virus like the one which results from nuclear polyhedroses infection. A few non-inclusion viruses also attack insects. But with the exception of Tipula Iridescent Virus (TIV) and Mosquito Iridescent Virus (MIV) that might prove useful in mosquito control, most attention has been given to the inclusion viruses. The very fact that the virus particles enclosed in a protein matrix maintain their infectivity for many years means that the inclusion viruses can be stored as concentrated preparations for later application with conventional pesticide spray equipment.

It has been shown that a nuclear polyhedroses virus is highly effective against a variety of forest sawflies and, as it persists in the environment, it provides continuous regulation of the pest in some areas. Several nuclear polyhedroses virus are being mass produced for possible use against a variety of pests, including cotton bollworm, tobacco budworm, com earworm, cabbage looper, forest tent caterpillar, and alfalfa butterfly. However, one of the problems with viruses is that there are periods when they have little effect on the pest populations. A virus may remain latent in a pest population for several generations and then develop epizootics when the pest population comes under stress. Generally, short-term control can be achieved by frequent applications of virus preparations so that there is an active innoculum in the pest environment for a long period.

Genetic Control:

Genetic control is a type of biological control that uses two strategies to reduce pest problems. First, crop plants can be manipulated to increase their resistance to pests. Second, we can attempt to alter the genome of the pest species so that they become sterile or less harmful. Resistant varieties of many crop plants have been developed by selective breeding (Maxwell and Jennings, 1980). However, resistant plants do not necessarily have chemical defences. Strains of cotton plant produced with low gossypol (a chemical that occurs in green parts and seeds of cotton plant and is toxic to chickens and pigs) content are quite low in resistance to insect pests. Resistant crop plants have also been developed by genetic engineering. Genes that produce resistance in one species can be transferred into a crop plant to make the crop genetically resistant to specific pests. Bacteria may also be used as vehicles to carry bio-pesticide genes. For example, in 1987 the first success was reported of inserting a gene (the toxin gene of *Bacillus thurengiensis*) into tobacco plants, conferring resistance against Lepidoptera. *Bacillus thurengiensis* (Bt) is the main focus at present for developing insect resistant crops (Lambert and Peferoen 1992).

This bacteria normally lives in the soil and carries a gene for a toxic protein that kills the larvae of moths and butterflies. By splicing this gene into bacteria that normally live on crop plants, genetic engineers have produced insect- resistant crops. Insect pests would inject the bacteria while feeding on the plant and thereby is poisoned. Alternatively, the Bt genes that produce the toxins can be transferred directly into the plant's genome, so that the plant would protect itself As of 1992 tobacco, potato, cotton and tomato plants have been genetically

engineered with Bt genes (Lambert and Peferoen, 1992). The development and use of such transgenic plants has immense potential. However, one major problem is that pest insects will become resistant to the bio-pesticide, just as they become resistant to chemical pesticides (Pimentel 1991).

The simplest genetic manipulation that can be carried out on a pest species is sterilization. A large number of pests are sterilized by radiation or by chemicals and released into the wild where they can mate with normal individuals. This technique leads to a decrease in birth rate of the pest and control can be achieved. The most notable success of this technique is the near extinction of the screw-worm fly, *Cochliomyia hominivorax*, which lays its eggs on fresh wounds of livestock and wild animals. Another example of successful use of sterile-insect method was the suppression of mosquito *Culex pipiens quinque fasciatus* on a small island off Florida (Patterson, et.al. 1970). However, the sterile insect method cannot be used for all pest populations because it requires the rearing and sterilizing of a large number of individuals and isolation of target area so that natural males from outside the area may not be able to reach there to undrmine the programme.

Integrated Pest Management:

Integrated Pest Management (IPM) is an effective and environmentally sensitive approach to pest management that relies on a combination of common-sense practices. IPM programs use current, comprehensive information on the life cycles of pests and their interaction with the environment. This information, in combination with available pest control methods, is used to manage pest damage by the most economical means, and with the least possible hazard to people, property, and the environment. The IPM approach can be applied to both agricultural and non-agricultural settings, such as the home, garden, and workplace. IPM takes advantage of all appropriate pest management options including, but not limited to, the judicious use of pesticides. In contrast, *organic* food production applies many of the same concepts as IPM but limits the use of pesticides to those that are produced from natural sources, as opposed to synthetic chemicals. Integrated control (Stem et.al, 1959) or integrated pest management (IPM) uses biological, chemical and cultural methods of control. This system is ecologically sound because it relies on natural biological control as much as possible and depends on chemical treatments only when absolutely necessary. However, to achieve integrated control, we need to understand the population dynamics of the pest species and also of the crop system. Gonzaliz (1970) has compared developing an IPM programme to building a house. Sampling, establishment of economic thresholds, and knowledge of natural mortality in agro-ecosystems form the foundation of the house.
The variety of control methods available, such as, chemical, biological, host resistance and autocidal methods form the studs. The integrated programme forms the roof. How well the roof holds up depends upon the strength of the foundation and the number of studs that support it (Fig. 3.10).

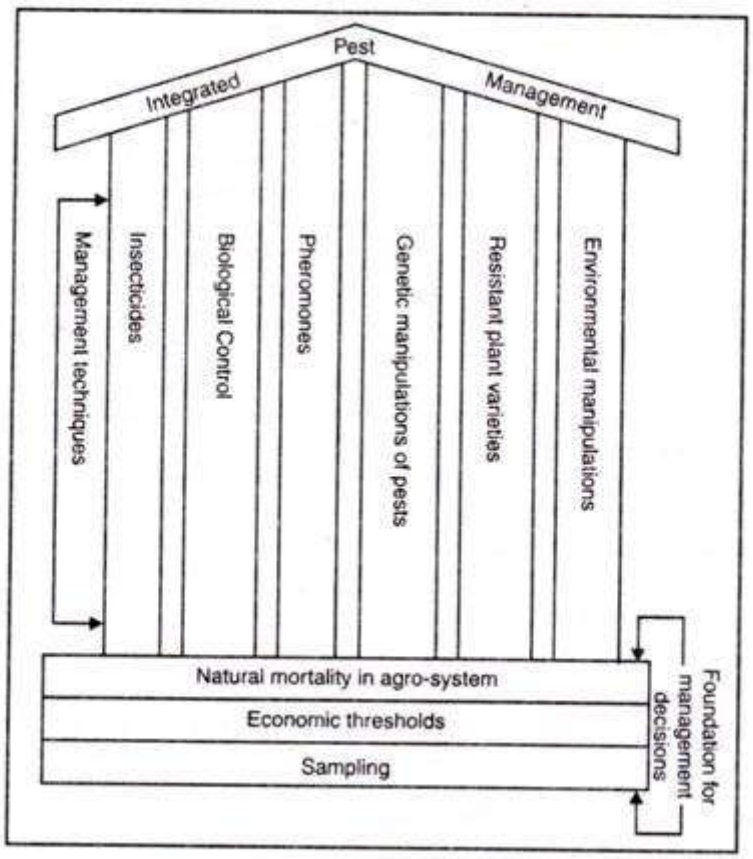


Fig. 3.10. Diagrammatic representation of an integrated pest management programme. (After Gonzaliz, 1970)

Based on the above consideration, the integrated pest management prescribes the use of the following:

(a) Predators: for example, the use of ladybeetles and lacewings against agricultural pests, or beetles to control weeds, or herbivorous fishes to control weeds.

(b) Parasites: For example, the use of calcid wasps against pests.

(c) Pathogens: For example, the use of viruses and bacterial infections those are specific for a pest.

(d) Decoy plant: Cultivation of low-value crops to attract pests away from high-value crops,

(e) Hormonal stimulants: Such as juvenile hormones which prevent insects from completing their cycle.

(f) Pheromones: Sex lures and other bio-chemicals that regulate pest behaviour,

(g) Degradable chemical insecticides: Organic phosphates and others.

(h) Biological insecticides: Such as *Bacillus thuringiensis* because their action is specific.

(i) Slow-release herbicides: Such as copper sulphate is used for algae control.

(j) Artificial selection for disease and pest resistance rather than for short term yield as such.

(k) Rotation and diversification of crops; strip-cropping; the use of frap crops; planting pest- resistant strains of crops; timing crop-planting so as to avoid pest outbreaks; altering the distance between crop rows.

(l) Chemical or radiation sterilization,

(m) Modifications in the use of water and fertilizers.

Thus, the strategies employed in integrated pest management are multiple and diverse. The various strategies may be used singly or in combination, simultaneously or in sequence, depending upon the particular pest problem. As the programme involves lot of time, effort and money, actual examples of IPM are limited. As an example we can take the IPM programme developed to control cotton pests in California. The main pest of cotton was *Lygus hesperus*, which feeds on cotton buds. In addition, the cotton bollworm (Heliothiszea) was a secondary pest. The IPM programme required minimum insecticide use to prevent secondary outbreaks. Subsequent studies showed that the lygus bug could only inflict serious damage during the budding season (June-July).

Therefore, insecticide applications were made during this time only. Cultural control was made by introducing thin strips of alfalfa into the cotton field which diverted the lygus out of the cotton. Each field was monitored twice a week from the beginning of budding to the end of August. Plant development, pest and natural enemy date were all collected, leading to a successful pest control programme. Overall, the programme represents a proper integration of biological controls and carefully timed insecticide treatments. Giese et. al. (1975) have given an example of integrated control in respect of alfalfa pest management. The alfalfa weevil (Hyperapostica) is the most important alfalfa pest affecting the alfalfa crop and an integrated control programme was designed for this weevil (Armbmst and Gyrisco, 1982).

How do IPM programs work?

IPM is not a single pest control method but, rather, a series of pest management evaluations, decisions and controls. In practicing IPM, growers who are aware of the potential for pest infestation follow a four-tiered approach. The four steps include:

a. Set Action Thresholds:

Before taking any pest control action, IPM first sets an action threshold, a point at which pest populations or environmental conditions indicate that pest control action must be taken. Sighting a single pest does not always mean control is needed. The level at which pests will either become an economic threat is critical to guide future pest control decisions.

b. Monitor and Identify Pests:

Not all insects, weeds, and other living organisms require control. Many organisms are innocuous, and some are even beneficial. IPM programs work to monitor for pests and identify them accurately, so that appropriate control decisions can be made in conjunction with action thresholds. This monitoring and identification removes the possibility that pesticides will be used when they are not really needed or that the wrong kind of pesticide will be used.

c. Prevention:

As a first line of pest control, IPM programs work to manage the crop, lawn, or indoor space to prevent pests from becoming a threat. In an agricultural crop, this may mean using cultural

methods, such as rotating between different crops, selecting pest-resistant varieties, and planting pest-free rootstock. These control methods can be very effective and cost-efficient and present little to no risk to people or the environment.

d. Control:

Once monitoring, identification, and action thresholds indicate that pest control is required, and preventive methods are no longer effective or available, IPM programs then evaluate the proper control method both for effectiveness and risk. Effective, less risky pest controls are chosen first, including highly targeted chemicals, such as pheromones to disrupt pest mating, or mechanical control, such as trapping or weeding. If further monitoring, identifications and action thresholds indicate that less *risky* controls are not working, then additional pest control methods would be employed, such as targeted spraying of pesticides. Broadcast spraying of non-specific pesticides is a last resort.

Suggested Readings:

1. The Insects by Chapman

2. Modern Entomology by D.B. Thembare

3. Economic Zoology by Shukla and Upadhyay

4. The Insects by Gullan and Carnston

5. Introduction to Economic Zoology by Sarkar, Kundu and Chaki.

6. A textbook of Economic Zoology by Aminul Islam

Probable questions:

1. What is IPM? How it works?

2. What is Biological Control of pest management? Write down the role of bacteria in pest control.

3. Write down the role of fungi in pest control.

4. Write down the role of viruses in pest control.

5. What is rodenticides? State its application in pest control.

6. What is insecticides? State its application in pest control.

7. What is fungicides? State its application in pest control.

8. What is fungicides? State its application in pest control.

9. Write down the textures of chemical pesticides.

10 Describe the advantages of chemical pesticides.

11. Describe the disadvantages of chemical pesticides.

12. Write down the different mode of Mechanical pest control.

Unit-III

Lac culture: Life-history of lac insect, culture method, lac processing, lac products, natural enemies of lac insect and their control

Objective: In this unit you will learn about life-history of lac insect, culture method, lac processing, lac products, natural enemies of lac insect and their control

Introduction:

Lac culture is the scientific management of lac insects to obtain a high amount of quality lac. This involves selection and maintenance of host plants, inoculation of host plants with healthy lac insects, collection and processing of lac and protection against enemies. Lac is the resinous secretion of lac insects. Two species of lac insects *Tachardialacca* and *T. chinensis* are common, of which the former one is predominant in India. India is the highest lac-producing country. Thailand is next in order.

History:

Lac has been used in India from time immemorial for several purposes, from the epic of Mahabharat it has been recorded that Kauravas built a palace of lac for the destruction of Pandavas. We come across references of lac in the Atharvaveda and Mahabharata, so it can be presumed that ancient Hindus were quite familiar with lac and its uses. Scientific study of lac started much later. In 1709 Father Tachard discovered the insect that produced lac. First of all Kerr (1782) gave the name *Coccus lacca* which was also agreed by Ratzeburi (1833) and Carter (1861). Later Green (1922) and Chatterjee (1915) called the ac- insect as *Tachardialacca* (kerr). Finally, the name was given as *Lacciferlacca*.

Introduction to Lac Insects:

Two strains of the lac insects are recognised in India, RANGEENI and KUSMI. The lac insects that thrive on the host plant Kusum is referred to as Kusmi whereas the Rangeeni strain generally grows on host plants other than Kusum. Each strain completes its life cycle twice a year but the seasons of maturity differ considerably.

There are four lac crops in a year that are named after the Hindi months (Table 4.2). Lac insects under the genus Kerria are generally bi-voltine with two broods in a year. But few species like *K. lacca* mysorensis (host plant-Sal), and *K. sharda* (host plant-Kusum) are tri-voltine having three broods in a year. Again, species belonging to the genus *Paratachardia* (host plants-Tea, Sandal, etc.,) are all univoltine.

Table 4.2 : Strains of lac insect and their characteristics		
Characteristics	**Kusmi**	**Rangeeni**
Host plant	Kusum is main, others include Ber, Siris, Semialata, etc.	Rangeeni strain grows well mainly on Palas and also on a few other trees, but not on Kusum
Types of crop	(a) Jethwi (b) Aghani	(a) Kartiki (b) Baisakhi
Inoculation time	(a) Jethwi : Jan/Feb. (b) Aghani : June/July	(a) Kartiki : June/July (b) Baisakhi : Oct/Nov
Duration of life cycle	(a) Jethwi : Jan/Feb to June/July (b) Aghani : June/July to Jan/Feb	(a) Kartiki : June/July to Oct/Nov. (b) Baisakhi : Oct/Nov to May/June
Harvesting month of crop-yield	(a) Jethwi : June/July (b) Aghani : Jan/Feb	(a) Kartiki : Oct/Nov (b) Baisakhi : May/June
Quality of lac	Superior	Inferior to Kusmi lac

Taxonomy of Lac Insect: (After E.E.Ruppert and R.D. Barnes, 1994)

Phylum: Arthropoda;

Sub-phylum: Uniramia;

Class: Hexapoda/lnsecta;

Subclass: Ectognatha

Order: Hemiptera

Family: Laciferidae

Genus: *Tachardia*

Scientific name: *Kerria (Tachardia) lacca*

The first scientific account about lac insect was given by J. Kerr in 1782, published in Philosophical Transaction of Royal Society of London. The first scientific name given to it was *Tachardialacca* after the name of French Missionary Father Tachardia.

Later it was changed to *Kerria lacca.* Out of nine genera and 87 species of lac insects reported from the world, two genera and 19 species are found in India. Of these 19 species, only *K. lacca* is exploited for commercial production of lac. However, *K. chinensis* in northeastern states and *K. sharcla* in coastal regions of Orissa and West Bengal are also cultivated to certain extent.

Habit and habitat:

The lac insects have a no. of plants as a host plant. About 113 varieties of host plants have been described and 14 are very common in India. Kusum, Khair, Babul, Ber Palas and Ghont plants give better quality of lac.

Distribution:

India has its monopoly on the production of lac. Other countries like Africa, Australia, Brazil, Burma, Sri Lanka, China, France, W. Germany, Japan, Malaya and several other countries.

Food Plants:

The insects live as a parasite, feeding on the sap of certain trees and shrubs. The important trees on which the lac insects breed and thrive well are –

Kusum (*Schleicheratrijuga*)

Palas (*Butea frondosa*)

Ber (*Zizyphusjujuba*)

Babul (*Acacia arabica*)

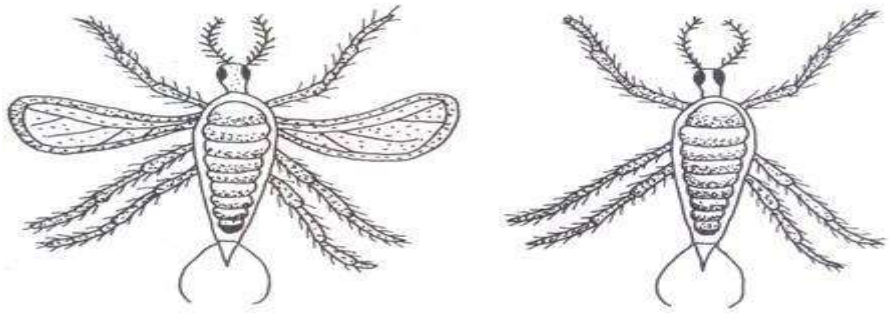
Khair (*Acacia catcchu*)

Arhar (*Calanus indicus*)

Before coming to the actual mechanism of lac secretion and its processing, it is advisable for a lac-culturist to have detailed knowledge of lac insect and its life cycle. The adult lac insect shows a marked phenomenon of sexual dimorphism. The male and female insect varies in shape, size and also in presence or absence of certain body parts.

Structure of Male Lac-insect:

It is larger in size and red in colour. The body is typically divided into head, thorax and abdomen. The head bears a pair of antennae and a pair of eyes. Mouth parts are absent so a male adult insect is unable to feed. Thorax bears three pairs of legs. Wings may or may not be found. (Fig. 33 a, b).Abdomen is the largest part of the body bearing a pair of caudal setae and sheath containing penis at the posterior end.



(a) Male with wings (b) Male without wings

Fig. 33. Adult male lac-insect.

Structure of Female lac-insect:

It is smaller in size. Head bears a pair of antennae and a single proboscis. Eyes are absent. Thorax is devoid of wings and legs. The loss of eyes, wings, and legs are due to the fact that the female larvae after settling down once never move again and thus these parts become useless and ultimately atrophy. Abdomen bears a pair of caudal setae. It is female lac insect which secretes the bulk of lac for commerce.

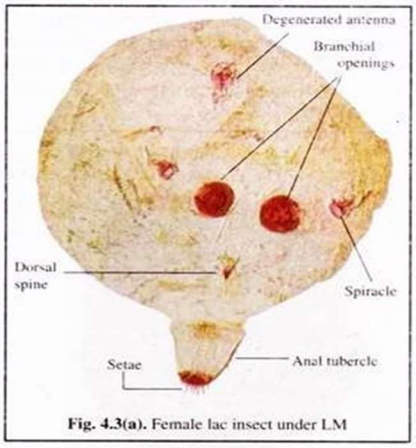


Fig. 4.3(a). Female lac insect under LM

Fertilization: After attaining the maturity, males emerge out from their cells and walk over the lac incrustations. The male enters the female cell through anal tubular opening and inside female cell it fertilizes the female. After copulation, the male dies. One male is capable of fertilizing several females. Females develop very rapidly after fertilization. They take more sap from plants and exude more resin and wax.

Life Cycle:

Egg: The female lac insect is ovoviviparous in nature. So the laid eggs contain fully developed embryos within it. About 300-1000 such eggs are laid in the chambers (cell) in which the female remains encased. The egg laying period may last from 7 to 10 days. The eggs hatch within few hours of laying. But egg laying ceases if the temperature falls below 17°C in summer and 15°C in winter.

Nymphs: Following hatching, the first instar nymph stays within the cell for a brief period. Then the crimson red coloured nymphs, referred to as 'crawlers', come out of the cell in search of suitable host plant branch for settlement. The emergence of lac insect nymphs in huge number is commonly called swarming that continues for several weeks. Boat-shaped nymphs are very small in size (0.5 mm) and divisible into head, thorax and abdomen. Head bears antennae, ocelli and mouth. Thorax has 3 segments, each with one pair of leg and caudal setae are found at the end of abdomen.

On reaching soft succulent twigs, the nymphs settle down close together and start to suck phloem sap through their suctorial proboscis. After one day or so of settling, the nymphs start secreting lac from the hypodermal glands lying under their cuticle keeping open their mouthparts, breathing spiracles and anus. The secreted semisolid lac hardens on exposure to air and the nymph gets fully covered by the lac encasement, called as lac cell.

Metamorphosis:

Within the cell, the nymphs moult thrice before reaching maturity. During first moult both male and female nymphs lose their appendages, legs and eyes. Following this moult, dimorphism appears in their cells. Inside the male cells, the male nymph casts off their second and third moults and matures into adults.

On maturity, the males lose their proboscis and develop antennae, legs and a pair of wings. The male brood cell is slipper- shaped. It bears a pair of branchial pores on the anterior side and a single large circular pore on the posterior side. The posterior hole remains covered by a round trap door or operculum through which adult males emerge. The female brood cell is larger, globular in shape that remains fixed to the twig. The female cell also has a pair of branchial pore and a single round anal tubular opening through which protrudes waxy white filaments (it indicates that the insect inside the cell is alive and healthy).

These filaments also prevent the blocking of the pore during excess secretion of lac. Following second and third moulting, the females retain only mouthparts but fail to develop any wings, eyes or appendages. While developing into adult, the female becomes immobile and large in size to accommodate huge number of eggs. During development, the females continuously secrete resin at a faster rate that coalesces around its body. After 14 weeks, the females shrink in size allowing light to pass into the cell.

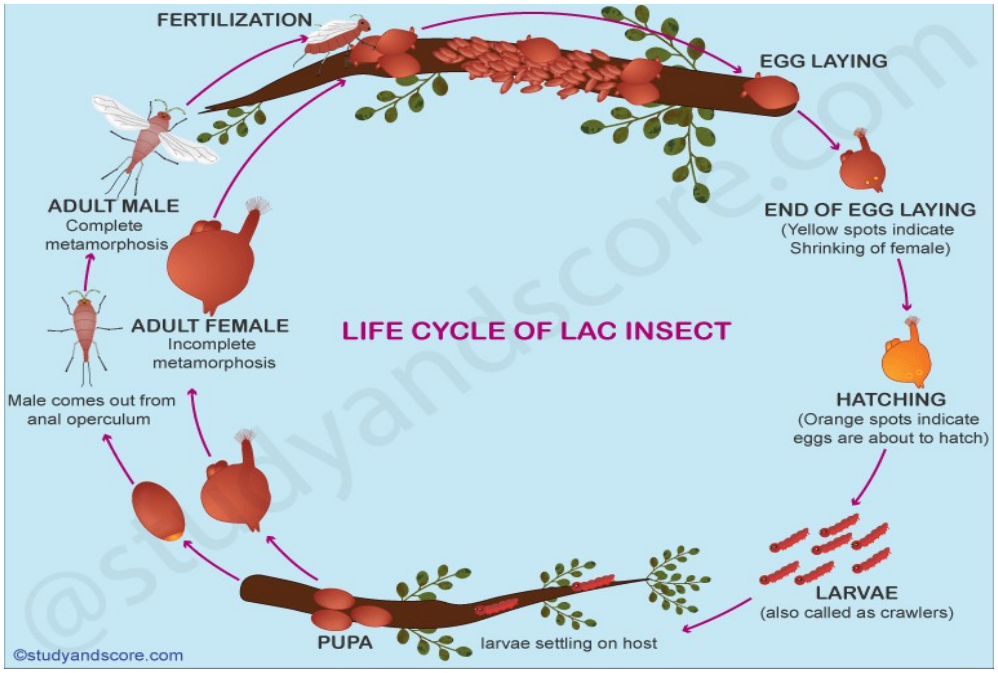
Mating:

Following emergence, the winged males walk over the immobile females and fertilize their eggs. The males die soon after copulation. A male has life of 62-92 hours.

Egg laying:

About the time of egg laying, two yellow spots appear at the rear end of the female cell which gradually enlarge and become orange in colour. At this time, the females contract at one end of the cell and thus vacates a space, called 'ovisac', inside the cell. Then the anal tubercle is drawn in the ovisac and the females start oviposit large number of eggs in the ovisac. The ovisac looks like orange due to presence of crimson lac dye which indicates the time of egg hatching.

Following hatching the nymphs emerge and the whole process begins all over again. After one cycle has been completed, and around the time when the next generation begins to emerge, the resin encrusted branches are harvested. From each crop, some encrusted twigs are retained for inoculation to the new host plants. Parthenogenesis: In the life history of lac insect, parthenogenesis is known to occur when unfertilised eggs are directly hatched into nymphs. It is common in Kartiki crop of Rangeeni Strain.



Lac Secretion:

Lac is a resinous substance secreted by certain glands present in the abdomen of the lac insects. The secretion of lac begins immediately after the larval settlement on the new and tender shoots. This secretion appears first as a shining layer which soon gets hardened after coming in contact with air. This makes a coating around the insect and the twig on which it is residing. As the secretion continues the coating around one insect meet and fuses completely with the coating of another insect. In this way a continuous or semi-continuous incrustation of lac is formed on the tender shoots.

Fig. 36. Lac incrustation.

Use of Lac:

Lac has been used for the welfare of human beings from the great olden days No doubt the development of many synthetic products have made its importance to a little lesser degree, but still it can be included in the list of necessary articles. Lac is used in making toys, bracelets, sealing wax, gramophone records etc.

It is also used in making grinding stones, for filling ornaments, for manufacturing of varnishes and paints, for silvering the back of mirror, for encasing cable wires etc., Waste materials produced during the process of stick lac is used for dying purpose. Nail polish is a good example of the by-product of lac.

Composition of Lac:

Lac is a mixture of several substances, of which resin is the main constituent. The approximate percentage of different constituents of lac is given below:

Resin – 68 to 90%

Dye – 2 to 10%

Wax – 5 to 6%

Mineral matter – 3 to 7%

Albuminous matter – 5 to 10%

Water – 2 to 3%

Cultivation of Lac:

Cultivation of lac involves proper care of host plants, regular pruning of host plant, infection or inoculation, crop-reaping, control of insect pests, and forecast of swarming, collection and processing of lac. The first and perhaps the most important prerequisite for cultivation of lac is the proper care of the host plant. It is the host plants on which lac insects depend for their food, shelter and for completion of their life cycle. There are two ways for the cultivation of host plants. One is that plants should be allowed to grow in their natural way and the function of lac-culturist is only to protect and care for the proper growth of plants.

Another way is that a particular piece of land is taken for the purpose and systematic plantation of host plant is made there. Regular watch is necessary in this case by providing artificial manures, irrigation facilities, ploughing and protecting the plants from cattle and human beings for which the land should be fenced. The larvae of lac insects are inoculated on host plants only after the host plants have reached a proper height.

The lac larvae feed on the cell sap by inserting their proboscis in the tender twigs. The proboscis can only be inserted in the tender young off-shoots. For this before inoculation, pruning of lac host plants is necessary. The branches less than an inch in diameter are selected for pruning. Branches half inch of less in diameter should be cut from the very base of their origin. But the branches more than half inch diameter should be cut at a distance of 1 ½ inch from the base.

Inoculation:

The method by which the lac insects are introduced to the new lac host plant is known as inoculation. This may be of two types, namely "Natural infection" and "Artificial infection". When infection from one plant to other occurs by natural movements of insect, it is called natural infection. This may be due to overcrowding of insect population and non-availability of tender shoots on a particular tree.
Artificial infection takes places through the agencies other than those of nature. Prior to about two weeks of hatching, lac bearing sticks are cut to the size of six inches. They are called"Brood lac". Brood lacs are then kept for about two weeks in some cool place.

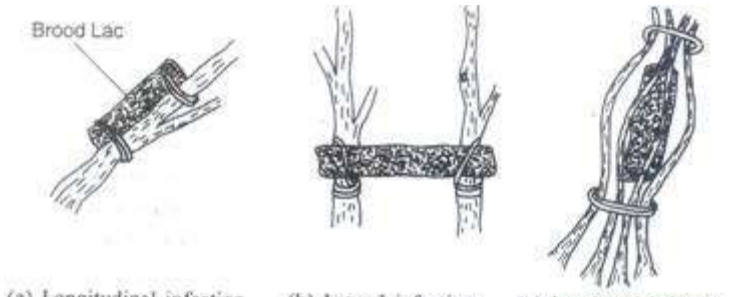


Fig. 37. Three different ways of artificial inoculation of lac.

When the larvae start emerging from this brood lac, they are supposed to be ready for inoculation. Strings can be used for tiding the brood lac with the host plant may be of different types in longitude infection the brood lac is tied in close contact with host branches. In lateral infection the brood lac is tied across the gaps between two branches. In interlaced method, brood lac is tied among the branches of several new shoots.

Lac Crop:

The lac insects repeat its life cycle twice in a year. There are actually four lac crops since the lac insects behave in two ways either they develop on Kusum plants or develop on plants other than Kusum. The lac which grows on Non-Kusum plants is called as "Ranjeem lac," and which grows on Kusum plant is called as "Kusumi lac. Four lac crops have been named after four Hindi months in which they are cut from the tree. They are as follows:

Ranjeeni Crop:

(i) Katki:

Lac larvae are inoculated in June-July. Male insect emerges m August-September. Female give rise to swarming larvae in October-November and the crop is reaped in Kartik (October and November).

(ii) Baisakhi:

Larvae produced by Katki crop are inoculated in October-November, male insects emerges in February-March, females give rise to swarming larvae m June-July, the crop is reaped in Baisakh (April-May).

Kusumi Crop:

(i) Aghani:

Lac larvae are inoculated in June-July, male insect emerges m September, female give rise to swarming larvae in January-February and crop is reaped in Aghan (December-January).

(ii) Jethoi:

The larvae produced by Aghani crop is inoculated in the month of January- February, male emerges in March-April, female give rise to swarming larvae in June- July and the crop is reaped in the month of Jeath (June-July).The time of infection with swarming larvae, the time of emergence of male insects, the time of reaping the crop, and the time of producing swarming larvae by female etc., are shown m tabular form below

Infection with swarming larvae	*Emergence of male insect*	*Crop reaped*	*Female give rise to swarming larvae*
Ranjeeni or Nankusumi Crop			
Katki (June-July)	August	Oct.-Nov.	Oct.-Nov.
Baisakhi (Oct.-Nov.)	Feb.-March	April-May	June-July
Kusumi Crop			
Aghani (June-July)	September	Dec.-Jan.	Jan.-Feb.
Jethoi (January)	March-April	June-July	June-July

Scraping and Processing of lac:

Lac cut from the host plant is called as “stick lac”. Lac can be scraped from the twigs before or after the emergence of larvae. If it is used for manufacturing before the emergence of larvae, the type of lac produced is called as “Ari lac” and if it is used for manufacturing purpose after swarming of larvae has occurred, the lac is said to be Phunkilac”.The scraping of lac from twig is done by knife, after which they should not be exposed to sun. The scraped lac is grinded in hard stone mills. The unnecessary materials are sorted out In order to remove the finer particles of dirt and colour, this lac is washed repeatedly with cold water. Now at this stage it is called as “Seed lac” and is exposed to sun for drying. Seed lac is now subjected to the melting process. The melted lac is sieved through cloth and is given the final shape by moulding. The final form of lac is called “Shellac”. Colour or different chemicals may be mixed during melting process for particular need.

Preparation of Feeding Ground for Lac Insects:

To get good quality lac through cultivation, it is necessary to ensure proper type of feeding ground to the lac insects. The insects need to be provided with succulent shoots, as it cannot drive its slender proboscis through thick bark. For getting a good number of requisite succulent shoots, the most important method is pruning.

Pruning:

Pruning means cutting away old, weak and diseased twigs from the host plants. It is done in January or June. It is very important for cultivation as it induces the host plants to throw out new succulent twigs. Pruning should be done with a sharp instrument to give a short and neat cut. If trees are old and have lost their capacity to produce vigorous shoots of new flush, heavier pruning is carried out to produce the new wood at the expense of the old. Such operation will bring the tree to a better shape, so that subsequent pruning will give the desired flush. Proper pruning should result in a good shape and give plenty of chances for the development of new shoots.

Objectives of Pruning:

(1) To ensure new, good, healthy and succulent shoots.

(2) To ensure availability of large number of shoots (larger area for lac insect settlement).

(3) To provide rest to host plant for maintaining its vigour.

(4) To remove dead, diseased and broken branches.

Types of Pruning in Lac Host Plants:

Two types of pruning have been recommended for lac culture.

(i) Apical/ light pruning:

Branches less than 2.5 cm diameter should be cut from base and branches more than 2.5 cm diameter should be sharply cut leaving a stump of 30-45 cm from the base. Diseased and dead portion of branches should be removed completely. Light pruning is recommended for slow growing conventional host tree species like Palas, Kusum and Ber.

(ii) Basal / heavy pruning:

Branches having less than 7cm thickness should be removed from the base, whereas thicker branches should be cut at a place where it has a diameter of 7 cm. In quick growing bushy host, pruning should be done at a height of 10-15 cm from the ground level, e.g., *Flemingia macrophylla, F. semialata*.

Pruning time:

After several years of experiment done at Indian Lac Research Institute (presently I.I.N.R.G.). Ranchi, Jharkhand, it has been found that the best results are obtained by pruning in February for raising the Kartiki crop and in April for raising the Baisakhi crop of Rangeeni for host plants Ber and Palas. Pruning in these months will give shoots of four and six months old respectively, for the lac larvae to feed on.

In case of Kusum, pruning is best done in the month of June-July and January- February. These months coincide with those in which the crops mature, and so, harvesting of the mature crop serves the purpose of pruning also. Pruning time will, however, need to be adjusted to suit local conditions.

Enemies of Lac Insects and their Control:

There are many natural enemies of lac insects which include vertebrates, invertebrates (insect predators and parasites) and microbial flora.

(1) Vertebrate enemies of lac insects:

The important vertebrate enemies are squirrels and rats. In worst conditions, the damage caused by these enemies can be as serious as 50% of brood sticks. Squirrels are active during the daytime and

the damage by them is more common under forest conditions. Rats are active at night-time and the damage usually occurs near about the villages.

Towards the crop maturity, these pests gnaw the mature lac encrustation on the tree, damage the brood lac tied to trees for inoculation and consume the full grown gravid female lac insects. The damage to brood lac tied to trees interferes with the inoculation, as the brood bundles and the lac encrustations drop to the ground where the larval emergence is taking place. Besides squirrels and rats, monkeys also cause some damage to lac encrustations and to the newly developing shoots from pruned host trees by breaking them.

Control:

It is difficult to control the squirrels and rats under the open field conditions where lac is cultivated. However, scaring away of these animals or poisoning them may be adopted to keep the rodents under attack.

(2) Insect enemies of lac insect:

It has been estimated that on an average, up to 30-40% of the lac cells are destroyed by insect enemies of lac crop. At times, the enemy attack can be so serious as to result in total crop failure.

There are two kinds of enemy insects:

(i) Parasites, and

(ii) Predators.

(i) Parasites:

All parasites causing damage to lac insect belong to the Order Hymenoptera of class Insecta. A list of parasites associated with lac insect, *Kerria lacca* is presented in Table 4.4.

Table 4.4 : Some important Parasites and Predators of lac insect

Parasite of lac insects	Family	Predators of lac insects	Family
Anicetus dodonia	Encyrtidae	*Eublemma amabilis* (Fig. 4.19)	Noctuidae
Atropates hautefeuilli	Encyrtidae	*E. coccidiphaga*	Noctuidae
Aphrastobracon flavipennis	Encyrtidae	*E. cretacea*	Noctuidae
Bracon greeni (Fig. 4.20)	Encyrtidae	*E. scitula*	Noctuidae
Campyloneurus indicus	Encyrtidae	*Pseudohypatopa pulverea*	Blastobasidae
Coccophagus tchirchii (Fig. 4.21)	Aphelinidae	*Catablemma sumbavensis* (Fig. 4.22)	Blastobasidae
Erencyrtus dewitzi	Encyrtidae	*Cryptoblabes ephestialis*	Blastobasidae
Eupelmus tachardiae (Fig. 4.23)	Eupelmidae	*Phroderces falcatella*	Cosmopterygidae
Eurymyiocnema aphelinoides	Aphelinidae	*Lacciferophaga yunnanea*	Momphidae
Lyka lacca	Encyrtidae	*Chrysopa madestes*	Chrysopidae
Marietta javensis	Aphelinidae	*C. lacciperda*	Chrysopidae
Parageniaspis indicus	Encyrtidae	*Berginus maindroni*	Mycetophagidae
Parechthrodryinus clavicornis	Encyrtidae	*Silvanus iyeri*	Cucujidae
Protyndarichus submettalicus	Encyrtidae	*Tribolium ferrugineum*	Tenebrionidae
Tachardiaephagus tachardiae	Encyrtidae	*Phyllodromia humbertiana*	Blattellidae
Teachardiobius nigricans	Encyrtidae	*Ishonoptera fulvastrata*	Blattellidae
Aprostocetus (Tetrastichus) purpureus (Fig. 4.24)	Eulophidae	*Dolichoderus thoracicus*	Formicidae

Among the parasites listed, *Tachardiaephagus tachardiae* and *Tetrastichus purpureus* are the most abundant lac associated parasites. They lay their eggs in the lac cells and the grubs (larvae) after hatching start to feed on the lac insect within its cell.

(ii) Predators:

The predators, on the other hand, are more serious and may cause damage up to 30-35 per cent to the cells in a crop. The important predators of lac insects are listed in Table 4.4. Of the predators, *Eublemmaamabilis* and *Pseudohypato papulverea* are the most destructive to lac insects and are in regular occurrence but their incidence may vary from season to season, place to place and crop to crop.

Prevention and Control of Insect Enemies:

Preventive measures:

(a) Parasite- and predator- free brood lac should be used for inoculation.

(b) Self-inoculation of lac crops should be avoided as far as possible.

(c) Inoculated brood bundles should be kept on the host tree for a minimum period only.

(d) Phunki (empty brood lac sticks) should be removed from the inoculated trees in 2-3 weeks time.

(e) All lac cut from the tree and all phunki brood lac (after use as brood lac) not required for brood purpose should be scraped or fumigated at once.

(f) Cultivation of Kusmi strain of lac should be avoided in predominantly Rangeeni area and vice versa.

Mechanical control:

Use of 60 mesh synthetic netting (brood bag) to enclose brood lac for inoculation purposes can reduce infestation of enemy insects of lac.

The emerging lac larvae easily crawl out from the minute pores of the net and settle on the twigs of the lac host plants, whereas the emerging adult predator enemies cannot move out of the brood bags and get entrapped within the net. This can check the egg laying by the predator moths on the new crop.

Chemical control:

Application of 0.05% endosulphan at 30-35 days stage of crop has been identified as the most effective dose of insecticide without any adverse effect on the economic attributes of the lac insect.

Microbial control:

Use of bio-pesticide, Thuricide (*Bacillus thuringiensis*) at 30-35 days stage of crop is the effective microbial control measure for important enemy insects of lac in field condition.

Biological control:

Two ant predators, viz. *Camponotus compresus* and *solenopsis geminate*, are the most important and promising for biological control of predator enemies of lac in field condition. Egg parasitoids, viz. *Trichogramma pretiosum, T. chilonis, T. poliae, Trichogrammatoideabactrae* and *Telenomus remits*, have been found to be effective in management of many lac predators like *P. pulverea*.

Again, hyperparasitism is found to happen in some lac cultivation areas where parasites of lac insects could also be controlled biologically by hyperparasitic insects, viz., *Aprostocetus*

(Tetrastichus) purpureus (Fig. 4.24) is secondary parasite of *Coccophaqustchirchii* (Fig. 4.21), and *Eupelmustachardiae* (Fig. 4.23) is a secondary parasite of *Eublemmaamabilis*.

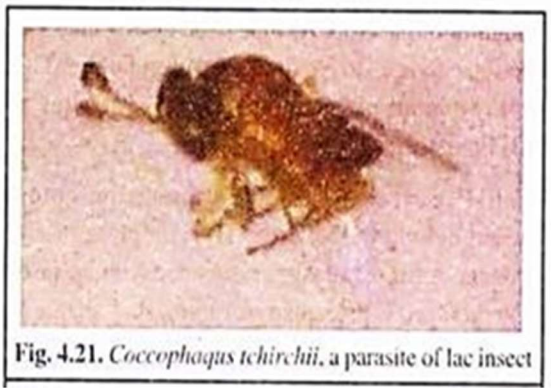


Fig. 4.21. *Coccophaqus tchirchii*, a parasite of lac insect

Fig. 4.22. *Catablemma sunbavensis*, a parasite of lac insect

Fig. 4.23. *Euplemus tachardiae* a parasite of lac insect

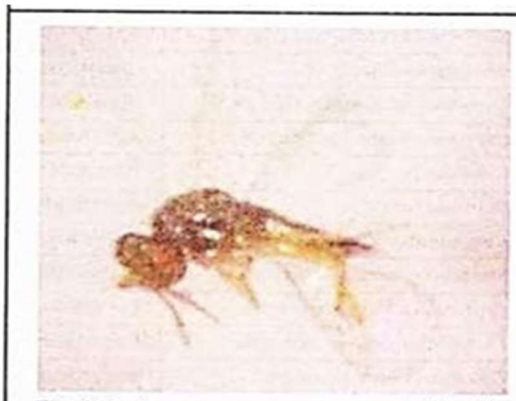


Fig. 4.24. *Aprostocetus purpureus*, a parasite of lac insect

Microbial Flora Associated with Lac Insects:

Two types of microflora, viz. bacteria and fungi, are associated with the lac insects. Bacteria could be symbiotic or pathogenic. Microbial studies revealed that four species, viz. *Micrococcus varians. M. conglomerates, Clostridium sp.* and *Bacillus subtilis*, are found in permanent association with various stages of lac insects. Presence of various symbiotic microflora is considered beneficial for good yield of lac, particularly during rainy season crop. However, on the other hand, association of fungi with lac insect is not always beneficial.

Fungal infection in lac culture causes severe losses of lac yield by:

(1) Killing the lac insects by inhibiting respiration.

(2) Hindering mating process.

(3) Blocking larval emergence.

(4) Affecting lac host efficiency.

Lac culture during rainy season is prone to fungal attack particularly when grown on Ber and Kusum trees due to their steady and spreading crown. Three species of fungi belonging to family Eurotiaceae

and Aspergillaceae, viz. *Aspergillus awamori, Aspergillus terricola* and *Penicillium citrinum,* are reported to cause maximum loss in lac crop. *Aspergillus awamori* (Fig. 4.25) and *Penicillium citrinum* are black and greenish in colour respectively, and make a continuous cover on lac insect cells and thereby blocking their breathing pores which ultimately lead to mortality of lac insects. A pathogenic fungus, *Pythium* sp. in female tests, causes a heavy mortality of the larvae which fail to enclose satisfactorily and lie dead in clusters within the female resinous cells.

Fig. 4.25: Fungal-cover on lac encrustation

Prevention and control:

Application of fungicides, Bavistin (carbendazim 0.05%) and Dithane M-45 (mancozeb, 0.18%) by both dipping of brood lac before inoculation and spraying on standing crop gives significantly better yield of lac. Significant reduction (84% to 75%) in mortality of 2nd instar lac nymphs/larvae can be done by the application of different concentrations of carbendazim and aureofungin on Kusumi stain of lac insects.

Present Position of this Industry in India:

Lac is produced in a number of countries including India, Thailand, Mayanmar, China, Indonesia, Vietnam and Laos. India and Thailand are the major producers, producing on the average 1700 tonnes of lac annually, followed by China. India alone, accounts for about 70/o of global lac production.

Former Bihar is the most important lac producing state of India. The Indian council of Agriculture Research has established Indian Lac Research Institute at Namkum in Ranchi district of Jharkhand.The average of different states in the total quantity of stic lac produced in this country is given below:

Bihar – 55.5%, Madhya Pradesh – 22% ,West Bengal – 10%, Maharashtra – 7.1%, Gujrat – 2.7% , Uttar Pradesh – 1.8%, Assam – 0.6% and Orissa – 0.1%

Total annual global production of pure lac is estimated to be 20,000 tonnes. The average total production of stick lac in India is about 24,000 tonnes, while the annual average pure lac produced in the country is 11,890 tonnes. About 6000 tonnes of pure lac produced in India is exported to different countries of the world, with an average earning of Rs. 202.38 million in term of foreign exchange. It has been estimated that 3-4 million people mostly tribals are engaged in the cultivation and several thousands in addition are engaged in the trade and manufacture of lac. Two main competitors of Indian lac are (i) Thailac, which accounts 50% of the total lac exported, and (ii) Synthetic resion, which have replaced lac in certain field. Shellac being a versatile resion, there is immense scope of increasing its utilisation in various fields and there is also scope to modify it to meet particular need.

Suggested Readings:

1. The Insects by Chapman
2. Modern Entomology by D.B. Thembare
3. Economic Zoology by Shukla and Upadhyay
4. The Insects by Gullan and Carnston
5. Introduction to Economic Zoology by Sarkar, Kundu and Chaki.
6. A textbook of Economic Zoology by Aminul Islam

Probable questions:

1. What is lac? Describe its composition and uses.
2. Describe structure of a male and female lac insect.
3. Write down the life cycle of lac insect with suitable diagram.
4. What are different type of Ranjeeni crops?
5. What are different type of Kusumi crops?
6. What is prunning? What are the objectives of prunning?
7. Describe types of prunning in lac host plant.
8. What is the present status of lac culture in India.
9. Describe different parasites in lac culture. Also state their control measures.
10. Describe different predatores in lac culture. Also state their control measures.

Unit-IV

Sericulture: Indigenous races, pure races and commercial races of mulberry silk moth; Rearing of mulberry silk moth (moriculture excluded)

Objective: In this unit you will know about sericulture: Indigenous races, pure races and commercial races of mulberry silk moth; Rearing of mulberry silk moth.

Introduction:

Silk production has a long history. Silk was discovered by Xilingji (Hsi-ling-chi), wife of China's 3rd Emperor, Huangdi (Hoang-Ti), in 2640 B.C. While making tea, Xilingji accidentally dropped a silkworm cocoon into a cup of hot water and found that the silk fiber could be loosened and unwound. Fibers from several cocoons could be twisted together to make a thread that was strong enough to be woven into cloth. Thereafter, Hsiling chi discovered not only the means of raising silk worms, but also the manners of reeling silk and of employing it to make garments. Later sericulture spread throughout China, and silk became a precious commodity, highly sought after by other countries. Demand for this exotic fabric eventually created the lucrative trade route, the historically famous Silk Road or Silk Route named after its most important commodity. This road helped in taking silk westward and bringing gold, silver and wool to the East. With the mulberry silk moth native to China, the Chinese had a monopoly on the world's silk production. After 1200B.C. Chinese immigrants who had settled in Korea helped in the emergence of silk industry in Korea. During the third century B.C. Semiramus, a general of the army of Empress Singu-Kongo, invaded and conquered Korea. Among his prisoners were some Sericulturists whom he brought back to Japan. They helped in the establishment and growth of sericulture industry in Japan. Another story is that a Chinese princess married an Indian prince. She carried silkworm eggs/mulberry cocoons in her elaborate head dress. She disclosed the secret of raising silkworms thus, silk production spread in India. In 550A.D. moth eggs and mulberry seeds were smuggled from China by two Nestorian monks, sent by Emperor Justinian-I and silk production began in Byzantium. The technique of sericulture spread throughout the Mediterranean countries during the 7th century AD and then to Africa, Spain and Sicily. During latter part of the 19th century, modern machinery, improved techniques and intensive research helped the growth of sericulture industry in Japan. At present, Japan, China, Korea, Italy, Soviet Union, France, Brazil and India are the chief silk producing countries in the World.

Sericulture and its components:

Commercial rearing of silk producing silkworm is called sericulture. It is an agrobased industry comprising three main components:

i) cultivation of food plants of the worms,

ii) rearing of silk worms, and

iii) reeling and spinning of silk.

The first two are agricultural and the last one is an industrial component. There are four varieties of silkworms in India, accordingly sericulture is classified into Mulberry Culture, Tasar Culture, Muga Culture and Eri Culture, and each one is described separately in the following text.

Taxonomy:

Silk producing insects are commonly referred to as serigenous insects. Silkworm is a common name for the silk-producing caterpillar larvae of silk moths. Silk moths belong to Phylum - Arthropoda, Class - Insecta, Order - Lepidoptera, Super family - Bombycoidea. Bombycoidea comprises eight families of which only Bombycidae and Saturnidae are the two important families the members of which produce natural silk. There are several species of silkworm that are used in commercial silk production .These are:

(i) Mulberry silk worm

- *Bombyx mori* (Bombycidae)
- *Bombyx mandarina* (Bombycidae)

(ii) Tasar silk worm

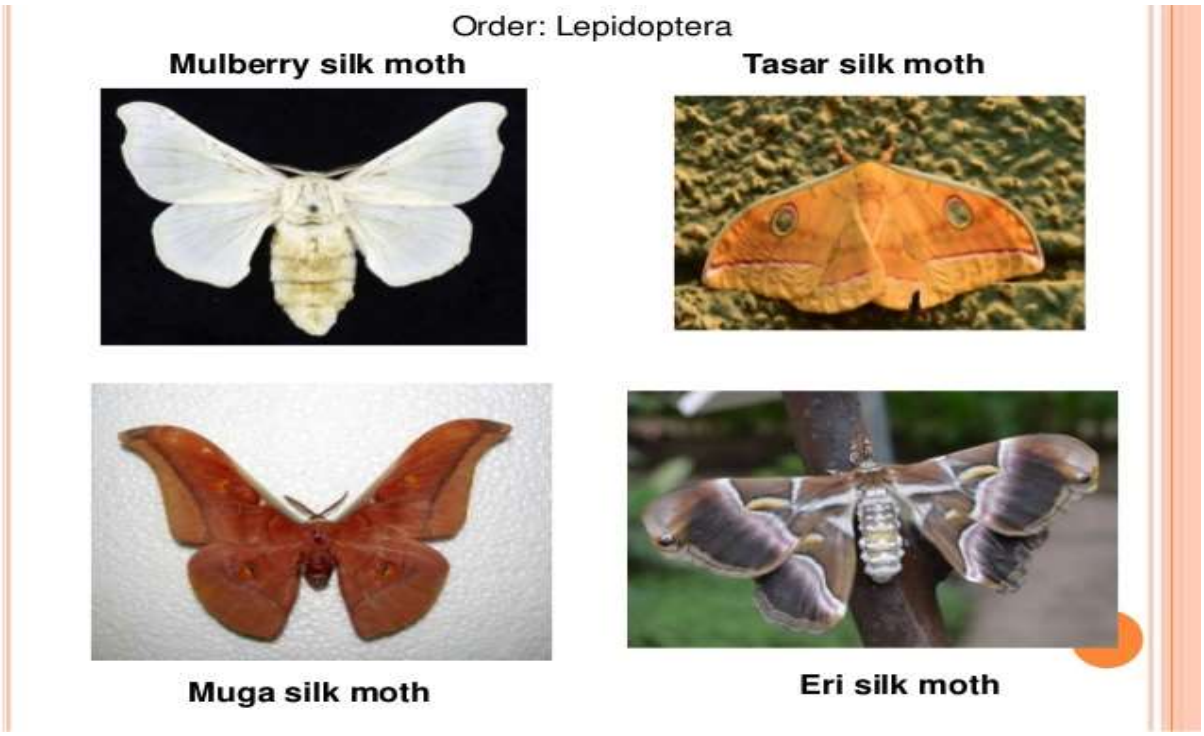
- *Antheraeamylitta* (Saturnidae)
- *Antheraeapernyi* (Saturnidae)
- *Antheraeayamamai* (Saturnidae)
- *Antheraeapaphia* (Saturnidae)
- *Antheraearoyeli*(Saturnidae)

(iii) Muga Silkworm

- *Antheraeaassama* (Saturnidae)

(iv) Eri silk worm

- *Philosamiaricini* (Saturnidae)



Mulberry Culture :

Biology of Mulberry Silkworm:

The insect producing mulberry silk is a domesticated variety of silkworms, which has been exploited for over 4000 years. All the strains reared at present belong to the species B*ombyx mori* that in believed to be derived from the original Mandarina silkworm, *Bombyx mandarina* Moore. China in the native place of this silk worm, but now it has been introduced in all the silk producing countries like Japan, India, Korea, Italy, France and Russia.

The races of mulberry silk worm may be identified on the basis of geographical distribution as Japanese, Chinese, European or Indian origin; or as Uni-, Bi- or Multivoltine depending upon the number of generations produced in a year under natural conditions; or as Tri-, Tetra- and Penta-moulters according to the number of moults that occur during larval growth; or as pure strain and hybrid variety according to genetic recombination.

Life Cycle :

Life cycle of the silkworm consists of four stages i.e. adult, egg, larva, and pupa. The duration of life cycle is six to eight weeks depending upon racial characteristics and climatic conditions. Multi-voltine races found in tropical areas have the shortest life cycle with the egg, larval, pupal and adult stages lasting for 9-12 days, 20-24 days, 10-12 days and 3-6 days, respectively. Seven to eight generations are produced in multi-voltine races. In uni-voltineraces, the egg period of activated egg may last for 11-14 days; the larval period, 24-28 days; the pupal period, 12-15 days and the adult stage, 6-10 days. In nature, uni-voltine races produce only one generation during the spring and the second generation of eggs goes through a period of rest or hibernation till the next spring. In case of bivoltine races, however, the second generation eggs do not hibernate and hatch within 11- 12 days and produce second generation normally during summer and it is the third generation eggs which undergo hibernation and hatches in the next spring, and thus producing two generations in one year.

Egg:

Egg is round and white. The weight of newly laid 2,000 eggs is about 1.0 g. It measures 1-1.3 mm in length and 0.9-1.2 mm in width. With time, eggs become darker and darker. Races producing white cocoons lay pale yellow eggs; while races producing yellow cocoons lay deep yellow eggs. In case of hibernating eggs laid by bivoltine and univoltine races, the egg colour changes to dark brown or purple with the deepening of colour of the serosal pigments.

The eggs may be of diapause or non-diapause type. The diapause type of eggs are laid by the silkworms inhabiting in temperate regions; whereas silkworms belonging to subtropical regions like India lay non-diapause type of eggs. During diapause all vital activities of the eggs cease.

Larva:

After 10 days of incubation, the eggs hatch into larva called caterpillar. After hatching caterpillars need continuous supply of food, because they are voracious feeders. Newly hatched caterpillar is about 0.3 cm in length and pale yellowish white. The larval body in densely covered with bristles. As the larva grows, it becomes smoother and lighter in colour due to rapid stretching of the cuticular skin during different instars of the larval stage. The skin consists of cuticle and hypodermis. Cuticle is made up of chitin as well as protein and is covered with a thin layer of wax, which is capable of being extended considerably to permit rapid growth of the larva during each instar. Nodules are found all over the surface of the body, and the distribution pattern differs according to the variety of silkworm. Larva bears four pairs of tubercles: sub-dorsal, supra-spiracular, infraspiracular and basal tubercle. Each tubercle carries 3-6 setae.

The larval body is composed of head, thorax and abdomen. The head consist of six fused segments. It carries the appendages: antennae, mandibles, maxillae and labium. Median epicranial suture, clypeus and labrum are well developed and prominent. Six pairs of larval eyes or ocelli are located a little above the base of antennae. Five segmented antennae are used as sensory organs. The mandibles are well developed, powerful and adapted for mastication. The maxillary lobe and palpi help in discriminating the taste of food. The prementum is also chitinized, and its distal part carries a median process known as spinneret through which silk is extruded out from the silk gland. The sensory labial palpi are found on both sides of the spinneret.

The thorax has three segments: prothorax, mesothorax and metathroax. Each of the thoracic segments carries ventrally one pair of true legs, which are conical in shape and carry sharp distal claws. These claws are not used for crawling but they help in holding the leaves while feeding.

Abdomen consists of eleven segments, though only nine can be distinguished, as the last three are fused together to from the apparent ninth segment. Third to sixth and last abdominal segment bear a pair of abdominal legs, which are fleshy, unjointed muscular protuberance. Eighth abdominal segment bears caudal horn on the dorsal side.

The abdominal segments carry the sexual markings on ventral side, which are developed distinctly during fourth and fifth instars in the eighth and ninth segments. In females, the sexual marking appear as a pair of milky white spot in each of the eighth and ninth segments and are referred to as Ishiwata's Fore Gland and Ishiwata's Hind Gland respectively. In males a small milky white body known as Herold's Gland appears ventrally in the centre between eighth and ninth segments. Nine pairs of spiracles are present: one pair on the first thoracic segment and eight pairs one on each side of the first to eighth abdominal segments, respectively

The larval growth is marked by four moultings and five instar stages. The full-grown caterpillar develops a pair of sericteries or silk glands. Sericteries or silk glands are modified labial glands. These glands are cylindrical and divided into three segments: Anterior-, middle- and posterior-segments. The inner lining cells are characterized by the presence of large and branched nucleus. These glands secrete silk which consists of an inner tough protein, fibroin, enclosed by a water soluble gelatinous protein, sericin. In Bombyx, the fibrinogen which on extrusion is denatured to fibroin is secreted in the posterior segment of the gland and form the core of the silk filament in the form of two very thin fibres called brins. The sericin, a hot water soluble protein, secreted by middle segment of the gland, holds the brins together and covers them. The duct from another small gland called Lyonnet's gland, that lubricates the tube through which the silk passes, joins the ducts of the silk glands. Finally, the silk is moulded to a thread as it passes through the silk press or spinneret.

Pupa:

Pupa is the inactive resting stage of silkworm. It is a transitional period during which definite changes take place. During this period, biological activity of larval body and its internal organs undergo a complete change and assume the new form of adult moth. The mature silkworm passes through a short transitory stage of pre- pupa before becoming a pupa. During the pre-pupal stage, dissolution of the larval organs takes place which is followed by formation of adult organs. Soon after pupation the pupa is white and soft but gradually turns brown to dark brown, and the pupal skin becomes harder.

A pair of large compound eyes, a pair of antennae, fore and hind-wings, and the legs are visible. Ten segments can be seen on the ventral side, but only nine are visible on the dorsal side. Seven pairs of spiracles are present in abdominal region, the last pair being non-functional. Sex markings are prominent and it is much easier to determine the sex of pupa. The female has a fine longitudinal line on the eighth abdominal segment, whereas such marking is absent in case of male. The pupa is covered within a thick, oval, white or yellow silken case called cocoon. The pupal period may last for 8-14 days after which the adult moth emerges slitting through the pupal skin and piercing the fibrous cocoon shell with the aid of the alkaline salivary secretion that softens the tough cocoon shell.

Adult:

The adult of *Bombyx mori* is about 2.5 cm in length and pale creamy white. After emergence the adult is incapable of flight because of its feeble wings and heavy body. It does not feed during its short adult life. The body of moth has general plan of insect body organization .The ocelli are absent. The antennae are conspicuous, large and bipectinate. The meso- and meta-thorax bear a pair of wings. The front pair overlap the hind pair when the moth is at rest.

The moth is unisexual and shows sexual dimorphism. In male eight abdominal segments are visible; while in female, seven. The female has comparatively smaller antennae. Its body and the abdomen are stouter and larger, and it is generally less active than male. The male moth possesses a pair of hooks known as harpes at its caudal end; while the female has a knob like projection with sensory hair. Just after emergence, male moths copulate with female for about 2-3 hours, and die after that. The female starts laying eggs just after copulation, which is completed within 24 hours. A female lays 400-500 eggs. The eggs are laid in clusters and are covered with gelatinous secretion of the female moth.

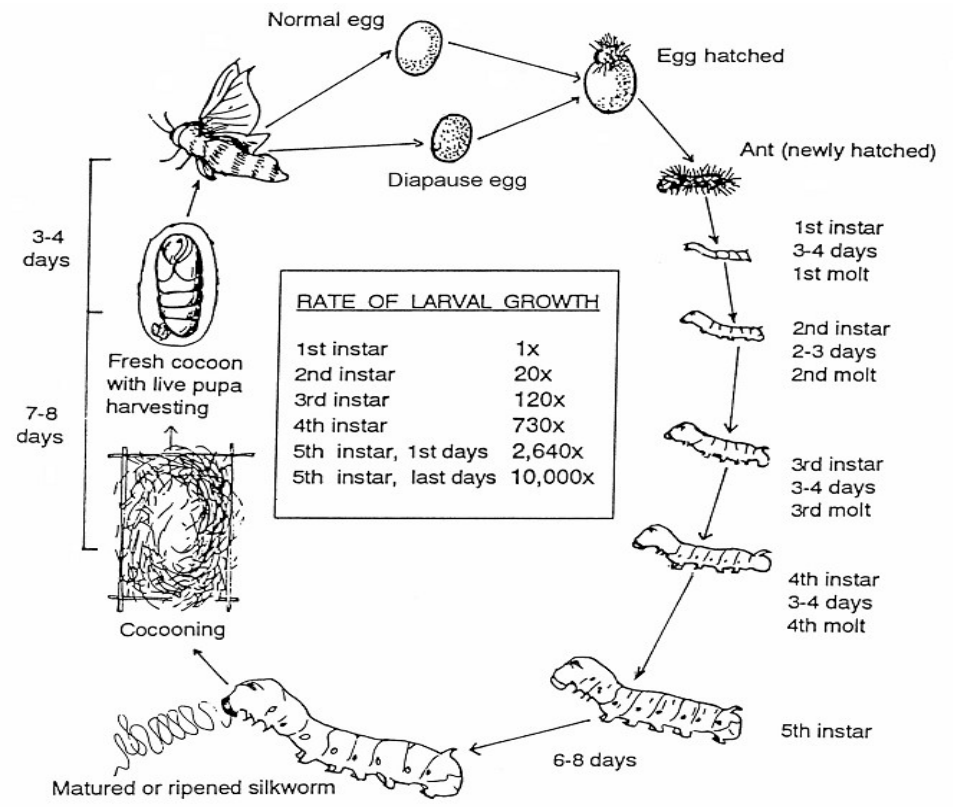


Figure: Life cycle of *Bombyx mori*

Rearing of Mulberry Silkworm

Mulberry Cultivation:

Cultivation of mulberry plants is called moriculture. There are over 20 species of mulberry, of which four are common: *Morus alba, M. indica, M. serrata* and *M latifolia*. Mulberry is propagated either by seeds, root- grafts or stem cuttings, the last one being most common. Cuttings, 22-23 cm long with 3-4 buds each and pencil thick, are obtained from mature stem. These are planted directly in the field or first in nurseries to be transplanted later. After the plants have grown, pruning is carried out routinely which serves two purposes, induction of growth and sprouting of new shoots.

Harvesting of leaves for feeding larva is done in three ways: leaf picking, branch cutting and top shoot harvesting. In leaf picking, individual leaves are handpicked. In branch cutting method, entire branch with leaves are cut and offered to 3rd instar larva. In top shoot harvesting, the tops of shoots are clipped and given to the 4th & 5th instars. The yield and quality of leaf depend upon the agronomic practices for cultivation of mulberry. trees, namely irrigation, application of fertilizers etc. It is estimated that 20,000 to 25,000 kg of leaves can be harvested per hectare per year under optimum conditions. It has also been estimated that to rear one box of 20,000 eggs, 600-650 kg of leaves are required for spring rearing and 500-550 kg for autumn rearing in Japan. In India, to rear 20,000 eggs the quantity of leaves required is about 350-400 kg.

Rearing Equipment:

i) Rearing house: The rearing house should meet certain specification, as the silk worms are very sensitive to weather conditions like humidity and temperature. The rearing room should have proper ventilation optimum temperature and proper humidity. It should be ensured that dampness, stagnation of air, exposure to bright sunlight and strong wind should be avoided.

ii) Rearing stand: Rearing stands are made up of wood or bamboo and are portable. These are the frames at which rearing trays are kept. A rearing stand should be 2.5 m high, 1.5 m long and 1.0 m wide and should have 10 shelves with a space of 20 cm between the shelves. The trays are arranged on the shelves, and each stand can accommodate 10 rearing trays.

iii) Ant well: Ant wells are provided to stop ants from crawling on to trays, as ants are serious menace to silk worms. They are made of concrete or stone blocks 20 cm square and 7.5 cm high with a deep groove of 2.5 cm running all round the top. The legs of the rearing stands rest on the centre of well filled with water.

iv) Rearing tray: These are made of bamboo or wood so that they are light and easy to handle. These are either round or rectangular.

v) Paraffin paper: This is a thick craft paper coated with paraffin wax with a melting point of 55o C. It is used for rearing early stages of silk worms and prevents withering of the chopped leaves and also help to maintain proper humidity in the rearing bed.

vi) Foam rubber strips: Long foam rubber strips 2.5 cm wide and 2.5 cm thick dipped in water are kept around the silkworm rearing bed during first two instar stages to maintain optimum humidity. Newspaper strips may also be used as a substitute.

vii) Chopsticks: These are tapering bamboo rods (1cm in diameter) and meant for picking younger stages of larvae to ensure the hygienic handling.

viii) Feathers: Bird feathers preferably white and large are important items of silkworm rearing room. These are used for brushing newly hatched worms to prevent injuries.

ix) Chopping board and Knife: The chopping board is made up of soft wood it is used as a base for cutting leaves with knife to the suitable size required for feeding the worms in different instar stages.

x) Leaf chambers: These are used for storing harvested leaves. The sidewalls and bottom are made of wooden strips. The chamber is covered on all sides with a wet gunny cloth.

xi) Cleaning net: These are cotton or nylon nets of different mesh size to suit the size variations of different instars of the silk worm. These are used for cleaning the rearing beds, and at least two nets are required for each rearing tray.

xii) Mountages: These are used to support silkworm for spinning cocoons. These are made up of bamboo, usually 1.8 m long and 1.2 m wide. Over a mat base, tapes (woven out of bamboo and 5-6 cm wide) are fixed in the form of spirals leaving a gap of 5-6 cm. They are also called chandrikes. Other types of mountage such as centipede rope mountage, straw cocooning frames etc. are also used.

xiii) Hygrometers and Thermometers: These are used to record humidity and temperature of the rearing room.

xiv) Feeding stands: These are small wooden stands (0.9 m height) used for holding the trays during feeding and bed cleaning.

Other equipment like feeding basins, sprayer, and leaf baskets may also be required.

Rearing Practices:

Silkworms must be reared with utmost care since they are susceptible to diseases. Therefore, to prevent diseases, good sanitation methods and hygienic rearing techniques must be followed. The appliances and the rearing room should be thoroughly cleaned and disinfected with 2-4% formaldehyde solution. Room temperature should be maintained around 25^0 C.

a. Procurement of quality seeds:

The most important step in silkworm rearing is the procurement of quality seeds free from diseases. Seeds are obtained from grainages, which are the centers for production of disease free seeds of pure and hybrid races in large quantities. These centers purchase cocoons from the certified seed cocoon producers. These cocoons are placed in well ventilated rooms with proper temperature (23-25^0 C) and humidity (70-80 %), and emergence of moth is allowed. Grainage rooms may be kept dark, and light may be supplied suddenly on the expected day of emergence to bring uniform emergence. Emerging moths are sexed and used for breeding purposes to produce seed eggs. Three hours of mating secures maximum fertilized eggs. The females are then made to lay eggs on paper sheets or cardboard coated with a gummy substance. Egg sheets are disinfected with 2% formalin, and then washed with water to remove traces of formalin and then dried up in shades. The eggs are transported in the form of egg sheet. However, it is easy to transport loose eggs. To loosen the eggs, the sheets are soaked in water. The loose eggs are washed in salt solution of 1.06-1.10 specific gravity to separate out unfertilized eggs and dead eggs floating on surface. Prior to the final washing, the eggs are disinfected with 2% formalin solution. Eggs are dried, weighed to the required standard and packed in small flat boxes with muslin covers and dispatched to buyers.

b. Brushing :

The process of transferring the silkworm to rearing trays is called brushing. Suitable time for brushing is about 10.00 am. Eggs at the blue egg stage are kept in black boxes on the days prior to hatching. The next day they are exposed to diffused light so that the larvae hatch uniformly in response to photic stimuli. About 90% hatching can be obtained in one day by this method. In case of eggs prepared on egg cards, the cards with the newly hatched worms are placed in the rearing trays or boxes and tender mulberry leaves are chopped into pieces and sprinkled over egg cards. In case of loose eggs a net with small holes is spread over the box containing the hatched larvae and mulberry leaves cut into small pieces are scattered over the net. Worms start crawling over the leaves on the net; the net with worms is transferred to rearing tray.

c. Preparation of feed bed and feeding :

After brushing, the bed is prepared by collecting the worms and the mulberry leaves together by using a feather. The bed is spread uniformly using chopsticks. The first feeding is given after two hours of brushing. Feed bed is a layer of chopped leaves spread on a tray or over a large area. The first and second instar larvae are commonly known as chawki worms. For chawki worms, paraffin paper sheet

is spread on the rearing tray. Chopped mulberry leaves are sprinkled on the sheet and hatched larvae are brushed on to the leaves. A second paraffin paper sheet is spread over the first bed. In between two sheets water soaked foam rubber strips are placed to maintain humidity.

The 4th and 5th instars are reared in wooden or bamboo trays by any of the three methods: viz., shelf-rearing, floor-rearing and shoot-rearing. In shelf rearing, the rearing trays are arranged one above the other in tiers on a rearing stand which can accommodate 10 -11 trays. This method provides enough space for rearing, but it is uneconomical as it requires large number of laborers to handle the trays. Chopped leaves are given as feed in this method. In floor rearing, fixed rearing sheets of 5-7x1-1.5m size are constructed out of wooden or bamboo strips in two tiers one meter apart. These sheets are used for rearing. Chopped leaves are given as feed. This method is economical than the first one because it does not involve much labour in handling of trays. Shoot-rearing is most economical of the three methods. The rearing sheet used is one meter wide and any length long in single tier and the larvae are offered fresh shoot or twigs bearing leaves. This method can be practiced both outdoors and indoors depending upon the weather. Each age of the silk worms could be conveniently divided into seven stages. First feeding stage, sparse eating stage, moderate eating stage, active eating stage, premoulting stage, last feeding stage, moulting stage. The larvae have good appetite at first feeding stage and comparatively little appetite at sparse and moderate eating stages. They eat voraciously during active stage to last feeding stage after which they stop feeding.

d. Bed Cleaning :

Periodical removal of left over leaves and worms' excreta may be undertaken and is referred to as bed cleaning. It is necessary for proper growth and proper hygiene. Four methods are adopted: conventional method, husk method, net method, and combined husk and net method.

e. Spacing :

Provision of adequate space is of great importance for vigorous growth of silkworms. As the worms grow in size, the density in the rearing bed increases and conditions of over crowding are faced. Normally it is necessary to double or triple the space by the time of moult from one to other instar stage, with the result that from the first to third instar the rearing space increases eight fold. In 4th instar, it is necessary to increase the space by two to three times and in 5th instar again twice. Thus, the rearing space increases up to hundred folds from the time of brushing till the time of maturation of worms.

f. Mounting

Transferring mature fifth instar larvae to mountages is called mounting. When larvae are fully mature, they become translucent, their body shrinks, and they stop feeding and start searching for suitable place to attach themselves for cocoon spinning and pupation. They are picked up and put on mountages. The worms attach themselves to the spirals of the mountages and start spinning the cocoon. By continuous movement of head, silk fluid is released in minute quantity which hardens to form a long continuous filament. The silkworm at first lays the foundation for the cocoon structure by weaving a preliminary web providing the necessary foot hold for the larva to spin the compact shell of cocoon. Owing to characteristic movements of the head, the silk filament is deposited in a series of short waves forming the figure of eight. This way layers are built and added to form the compact cocoon shell. After the compact shell of the cocoon is formed, the shrinking larva wraps itself and detaches from the shell and becomes pupa or chrysalis. The spinning completes within 2-3 days in multi-voltine varieties and 3-4 days in uni- and bivoltine.

g. Harvesting of Cocoons:

The larva undergoes metamorphosis inside the cocoon and becomes pupa. In early days, pupal skin is tender and ruptures easily. Thus, early harvest may result in injury of pupa, and this may damage the silk thread. Late harvest has a risk of threads being broken by the emerging moth. It is, therefore, crucial to harvest cocoons at proper time. Cocoons are harvested by hand. After harvesting the

cocoons are sorted out. The good cocoons are cleaned by removing silk wool and faecal matter and are then marketed. The cocoons are sold by farmers to filature units through Cooperative or State Govt. Agencies. The cocoons are priced on the basis Rendita and reeling parameters. Rendita may be defined as number of kg of cocoon producing 1 kg of raw silk.

Post Cocoon Processing :

It includes all processes to obtain silk thread from cocoon.

a. Stifling

The process of killing pupa inside cocoon is termed as stifling. Good-sized cocoon 8-10 days old are selected for further processing. Stifling is done by subjecting cocoon to hot water, steam, dry heat, sun exposure or fumigation.

b. Reeling

The process of removing the threads from killed cocoon is called reeling. The cocoons are cooked first in hot water at 95-97o C for 10-15 minutes to soften the adhesion of silk threads among themselves, loosening of the threads to separate freely, and to facilitate the unbinding of silk threads. This process is called cooking. Cooking enables the sericin protein to get softened and make unwinding easy without breaks. The cocoons are then reeled in hot water with the help of a suitable machine. Four or five free ends of the threads of cocoon are passed through eyelets and guides to twist into one thread and wound round a large wheel. The twisting is done with the help of croissure. The silk is transferred finally to spools, and silk obtained on the spool is called the Raw Silk or Reeled Silk. The Raw silk is further boiled, stretched and purified by acid or by fermentation and is carefully washed again and again to bring the luster. Raw Silk or Reeled Silk is finished in the form of skein and book for trading. The waste outer layer or damaged cocoons and threads are separated, teased and then the filaments are spun. This is called Spun Silk

Diseases and Pests of Silkworms:

I. Diseases:

a. Pebrine:

Pebrine is also known as pepper disease or corpuscle disease. The disease is caused by a sporozoan, *Nosema bombycis* (family Nosematidae). The main source of infection is food contaminated with spores. Infection can be carried from one larva to another by the spores contained in faeces or liberated in other ways by the moths carrying infection. Pebrinized eggs easily get detached from the egg cards. They may be laid in lumps. The eggs may die before hatching. The larva shows black spots. They may become sluggish and dull, and the cuticle gets wrinkled. Pupa may show dark spots. Moths emerging from pebrinized cocoons have deformed wings and distorted antennae. The egg laying capacityof the moth becomes poor

b. Flacherie:

Flacherie is a common term to denote bacterial and viral diseases. It has been classified into following types:-

i) Bacterial diseases of digestive organs: Due to the poor supply of quality mulberry leaves, the digestive physiology of the silkworm is disturbed, and multiplication of bacteria occurs in the gastric cavity. Bacteria like Streptococci, Coli, etc. have been found associated with this disease. Symptoms, like diarrhoea, vomiting, shrinkage of larval body may be seen.

ii) Septicaemia: Penetration and multiplication of certain kinds of bacteria in haemolymph cause septicaemia. The principal pathogenic bacteria are large and small Bacilli, Streptococci, and Staphylococci etc. Symptoms like diarrhoea, vomiting, shrinkage of larval body may be seen. Appearance of foul odour is also a common symptom.

iii) Sotto disease: It is caused by toxin of *Bacillus thuringensis*. The larvae become unconscious, soft, and darkish and rot off.

iv) Infectious Flacherie: It is caused by a virus called Morator Virus which does not form polyhedra in the body of silkworm larvae. The infection occurs mainly through oral cavity. The virus multiplies in the midgut and is released into the gastric juice and is excreted in faeces.

v) Cytoplasmic polyhedrosis: It is caused by a virus called Smithia which form Polyhedra are formed in the cytoplasm of the cylindrical cells of the midgut. The larva loses appetite. The head may become disproportionately large. Infection occurs through the oral cavity.

c. Grasserie:

The disease is also known as Jaundice or Nuclear Polyhedrosis It is caused by a virus called Borrelina, which form polyhedra in the nuclei of the cells of fatty tissues , dermal tissues, muscles, tracheal membrane, basement membrane, epithelial cells of midgut and blood corpuscles. The infected larvae lose appetite, become inactive, membranes become swollen, skin becomes tender and pus leaks out from skin. The larvae finally die.

Muscardine or Calcino: It is of 3 types

i) White Muscardine: It is caused by the fungus, *Beuveriabassiana*. The larva loses appetite, body loses elasticity and they cease to move and finally die.

ii) Green Muscardine: It is caused by *Metarrhiziumanisopliae*. The larva loses appetite, appears yellowish, becomes feeble and dies.

iii) Yellow Muscardine: It is caused by *Isaria farinosa*. Many small black specks appear on the skin. Larvae lose appetite and dies.

II. Pests

***Tricholyga bombycis*:** It is a dipteran fly of the family tachinidae, commonly known as Uzi fly. It is a serious pest of silkworm larvae and pupae. It parasitizes Mulberry and Tasar silkworm.

Dermestid beetles: These insects belong to the order Coleoptera, family dermestidae. This family contains many genera and a large number of destructive species. Some of them are: *Dermestes cadverinus, D. valpinus, D. vorax, D. frishchi,* and *Trogoderma versicolor*. The larvae bore inside the cocoon and eat the pupa. These pests cause great damage and economical loss, as the damaged cocoons cannot be reeled.

Mites: *Pediculoides ventricosus* (order Acarina, class Arachnida) damage the larvae. The toxic substance produced by the mite kills the silkworms. In addition, ants, lizards, birds, rats and squirrel also cause considerable damage to silkworm larvae as well as the cocoons.

Silk and Its Use:

Properties of the silk:

Silk contains 70-75% fibroin and 25-30% sericin protein. The biochemical composition of fibroin can be represented by the formula $C_{15}H_{23}N_5O_6$. It has the characteristic appearance of pure silk with pearly lustre. It is insoluble in water, ether or alcohol, but dissolves in concentrated alkaline solutions, mineral acids, and glacial acetic acid and in ammoniacal solution of oxides of copper. Sericin, a gummy covering of the fiber is a gelatinous body which dissolves readily in warm soapy solutions and in hot water, which on cooling forms a jelly with even as little as 1% of the substance. It is precipitated as a white powder from hot solutions by alcohol. Its chemical formula is $C_{15}H_{25}N_5O_8$. It can be dyed before or after it has been woven into a cloth. The weight in gram of 900m long silk filaments is called a denier which represents size of silk filament.

Silk has following peculiar properties:

1. Natural colour of Mulberry silk is white , yellow or yellowish green; that of Tasar brown; of Muga, light brown or golden; and of Eri, brick red or creamy white or light brown.

2. Silk has all desirable qualities of textile fibres, viz. strength, elasticity, softness, coolness, and affinity to dyes. The silk fibre is exceptionally strong having a breaking strength of 65,000-lbs/sq. inch.

3. Silk fibre can elongate 20% of original length before breaking.

4. Density is 1.3-1.37g/cm3 .

5. Natural silk is hygroscopic and gains moisture up to 11%.

6. Silk is poor conductor of heat and electricity. However, under friction, it produces static electricity. Silk is sensitive to light and UV- rays.

7. Silk fibre can be heated to higher temperature without damage. It becomes pale yellow at 110o C in 15 minutes and disintegrates at 165o C.

8. On burning it produces a deadly hydrocyanic gas.

Use of silk:

Silk is used in the manufacture of following articles:

a. Garments in various weaves like plain, crepe, georgette and velvet.

b. Knitted goods such as vests, gloves, socks, stockings.

c. Silk is dyed and printed to prepare ornamented fabrics for saries, ghagras, lehengas.

d. Jackets, shawls and wrappers.

e. Caps, handkerchiefs, scarves, dhotis, turbans.

f. Quilts, bedcovers, cushions, table-cloths and curtains generally from Erisilk or spun silk.

Parachutes and parachute cords.

g. Fishing lines.

h. Sieve for flour mills.

i. Insulation coil for electric and telephone wire.

j. Tyres of racing cars.

k. Artillery gunpowder.

l. Surgical sutures.

Indian Scenario:

Silk is Nature's gift to mankind and a commercial fibre of animal origin other than wool. Being an eco-friendly, biodegradable and self-sustaining material; silk has assumed special relevance in present age. Promotion of sericulture can help in ecosystem development as well as high economic returns. Sericulture is practiced in India and India is the 5th largest producer of silk in the World. It has been identified as employment oriented industry. All the sections of sericulture industry, viz. mulberry cultivation, silkworm seed production, silkworm rearing, reeling and weaving of silk and collection of byproducts and its processing provide a large scale employment, thereby a source of livelihood for the rural and tribal people. Sericulture industry is rated as the second largest employer in India.
Owing to this peculiar nature, the Indian planners have identified sericulture as one of the best suited occupations for ideal growth and development of rural India. Mulberry sericulture has been traditional occupation in Karnataka, Tamil Nadu, A.P. and Kashmir; Tasar one, in M.P., Chota Nagpur Division and Orissa; Muga one, in Assam, Nagaland, Tripura and Eri one in Assam and West Bengal. North-eastern part of India is the only region in the world where all four varieties of silk are produced. Central and State level Government Silk Departments are actively engaged in addressing the objective of promotion of sericulture in traditional as well as non-traditional regions. With the launching of massive developmental schemes, it is expected to gain an accelerated tempo of sericultural activities in the country, paving way for doubling the employment opportunities in phased manner, and thereby, it may set to bring a soothing touch to the burning problem of acute unemployment in rural India and thus can check the rural migration to urban areas to a certain extent. Sericulture is an agro-based cottage industry involving interdependent rural, semi-urban and urban-based activities in which estimated participation of women is about 60%. Thus, in contrast to any other agro-based profession the role of women in sericulture industry is dominating which will be helpful for improving the status of women in family enterprises. In the light of women welfare through Sericulture industry, the Central Silk Board, a statutory organization, under the Ministry of Textiles, Government of India has established a special component of assistance to Women and NGO's' into the National Sericulture Project.

There are four major research centres for Sericulture in India:

1. Central Sericulture Research and Training Institute, Behrampur (Orissa).
2. Central Sericulture Research and Training Institute, Mysore (Karnataka).
3. Central Tasar Research and Training Institute, Ranchi (Jharkhand).
4. Central Silk Technological Research Institute, Bangalore (Karnataka).

Suggested Readings:

1. The Insects by Chapman
2. Modern Entomology by D.B. Thembare
3. Economic Zoology by Shukla and Upadhyay
4. The Insects by Gullan and Carnston
5. Introduction to Economic Zoology by Sarkar, Kundu and Chaki.
6. A textbook of Economic Zoology by Aminul Islam

Probable questions:

1. Write down scientific names of Mulberry, Tasar , Muga and Eri silkmoth.
2. What is Voltinism? Name one of each type.
3. Describe life cycle of Bombyx mori with suitable diagram.
4. How mounting ids done in sericulture?
5. How cocoons are harvested?
6. Write down post cocoon processing.
7. Describe any two diseases of silkworm.
8. What is the present scenario of sericulture in India.
9. Describe the properties of silk.
10. What are the uses of silk?

Unit-V

Parasitic insects: General remarks on *Phlebotomous, Glossina, Tabanus* and head louse in relation to morphology, habit, habitat, life-cycle and disease caused by them, mode of transmission

Objectives: In this unit you will know about morphology, habit, habitat, life-cycle of some parasitic insects such as *Phlebotomous, Glossina, Tabanus*and head louse. You will also know about disease caused by them and mode of transmission.

Phlebotomous
Common Name : Sand Fly

Introduction:

Sand flies are of great importance as the transmitters of various kinds of leishmaniasis, of a filtrable virus disease called three-day fever or more commonly sand-fly or Papatasi fever and of Oroya fever. These are blood sucking flies and they belong to the genus *Phlebotomous*. These are small, moth like flies, rarely over 5 mm long. Their bodies and wings are black and hairy.

The legs are long and the wings are held roof-like over the abdomen during rest. The antennae are long, consisting of 16 segments which often have a beaded appearance and they are thickly covered with hairs. They live in moist dark place and lay eggs in sandy soil so they are called sandflies. Several species of Sandflies are recognised. These are *P. papatasii, P. minutus, P. argentipes, P. orientalis, P. sergenti, P. nogouchi* etc. Among these species—*P. papatasii* is found in North India, *P. argentipes* in Assam and West Bengal, and *P. minutus* in other states of India

Taxonomy:

P. argentipes, the vector of Visceral Leishmaniasis (VL) in the northeast Indian Sub Continent (ISC), is also present in many Asian countries where leishmaniasis is absent. Indeed, it has been suggested that this species is composed of two or more populations, possibly sibling species, with different vectorial capacities.

In southern Asia, the taxonomic status of the sand fly *P. argentipes*, which transmits *L. donovani,* was reassessed because the variation in morphology, behaviour, and distribution suggested that this was a complex of sibling species. The putative complex is composed of the nomino-typical members *P. argentipessensustricto*, *P. annandalei* (status revived), and *Phlebotomous glaucus* (new status). An allolectotype (the type specimen of the opposite sex of the lectotype [syn. Lectoallotype) is designated for the female of *P. argentipes,* as well as neotypes (a replacement syntype) of the males of *P. annandalei* and *P. glaucus*. Morphological descriptions, illustrations, and keys are available for the identification of adult males and females. Based on female morphological characteristics, *P. argentipes*, *P. annandalei,* and *P. glaucus* can be distinguished from each other using principal component analysis.

P. glaucus is widespread in India, occurring sympatrically (biological species or speciation occurring in the same or overlapping geographical areas without interbreeding) with *P. argentipes* in *L. donovani* endemic foci, whereas *P. annandeli* is peripatric (formation of a new species through evolution) to the type species in Chennai, southern India. 'In copula' is a process of post-copulatory sexual selection occurring as correlated coevolution of male and female reproductive traits, which

drives species isolation. This pattern has been implicated in reproductive isolation among the members of the *P. argentipes* complex.

Systematic Position:

Kingdom: Animalia

Phylum: Arthropoda

Class: Insecta

Order: Diptera

Family: Psychodidae

Subfamily: Phlebotominae

Genus: Phlebotomous

Morphology:

Larva: Larvae are caterpillar-shaped with head capsules and small leaf-like antennae. Distinctive caudal setae can help identify the larvae as sand flies, but larvae are rarely used in taxonomy because very few are ever collected in nature. There are four larval instars ranging in size from0.55 mm long in the 1st to about 3.2 mm long in the 4th. The 1st instar larvae usually have two long caudal setae, but the 2nd instar larvae have 4 caudal setae upon moulting.

Pupae: Pupae resemble a small butterfly chrysalis except that the 4th-stage larval exuvium (cast off exoskeleton) is attached at one end. The exuvium acts as a glue which is attached to a solid substrate and holds the pupa upright.

Adults:. Adult sand flies are about one-third the length of a small mosquito, usually less than 3.5mm in length. They are covered with dense hairs and hold their wings in a characteristic "V" shape over their backs when at rest. The wing veins are parallel to each other and have numerous small "hairs." The eyes are large and dark. The antennae are long and filiform, with 16 segments. Mouth parts are short, dagger-shaped and oriented downward. The thorax is distinctively humped, pushing the head below the upper surface of the thorax. The legs are very long and delicate. Both female and male sand fly adults obtain carbohydrate nutrition from plant juices; however, most females also require at least one blood meal in order to complete development of egg batches. Some are autogenous (able to produce viable eggs without a blood meal). Acquisition of disease agents is therefore incidental to blood meals

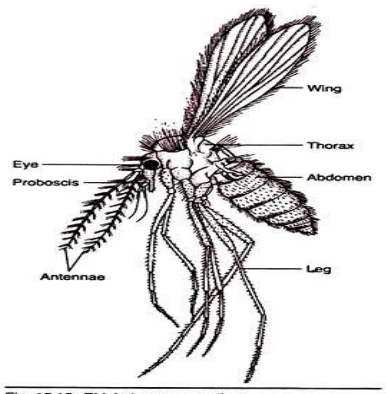
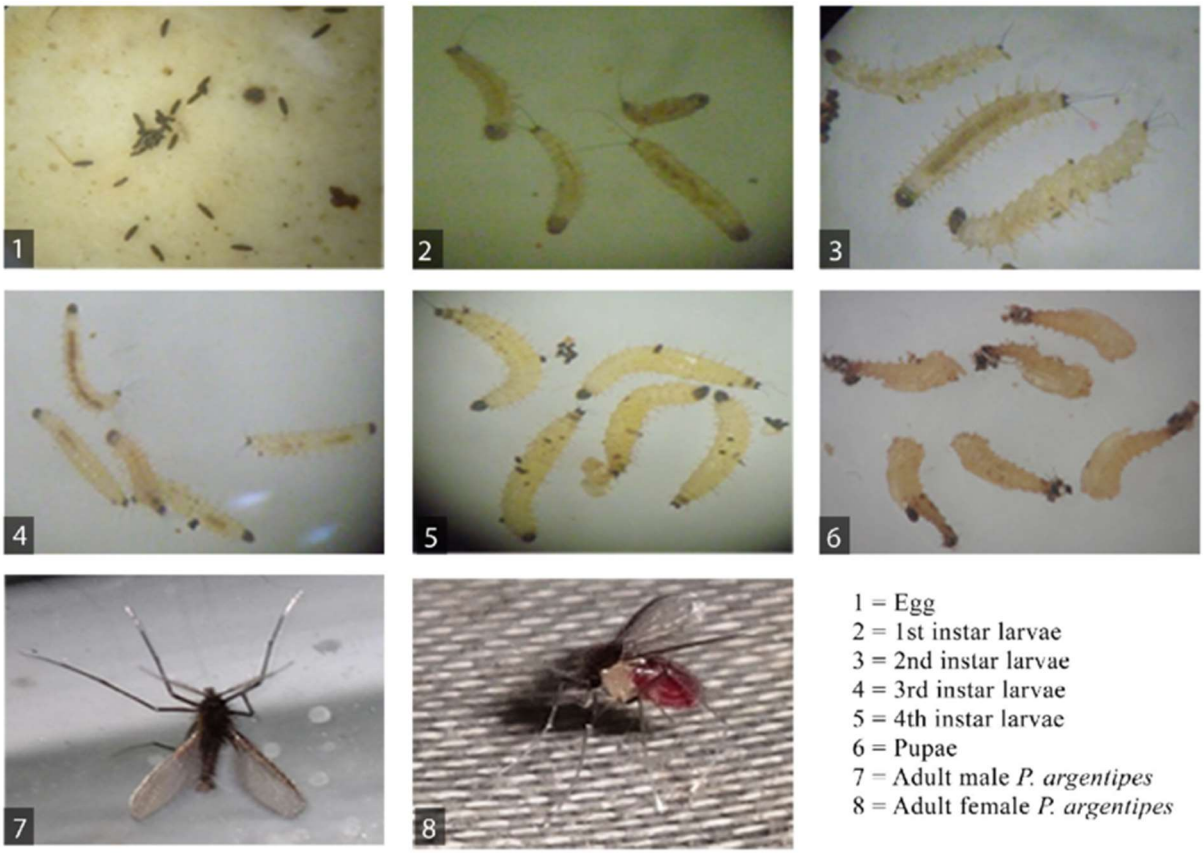


Fig. 15.13: *Phlebotomus argentipes*



Habit:

Both male and female sand flies feed on plant juices and sugary secretions. Females also blood feed to produce eggs. Sand flies use their mouthparts to probe exposed skin, leading to the formation of a pool of blood from which they feed. Sand fly saliva contains pharmacologically active components that aid in the feeding process. Feeding activity is influenced by temperature, humidity and air movement (sand flies are weak fliers so even light wind can inhibit flight and reduce biting). Most species feed at dusk and during the night, when temperature falls and humidity rises. The majority of species feed outdoors, although daytime biting can occur indoors in darkened rooms or among shaded vegetation/trees, especially if disturbed by human activity.

Habitat:

Phlebotomous spp. occur predominantly in warm, humid and tropical climates, usually in savannah and semi-desert vegetation habitats, although a few species occur in temperate zones. They are able to colonise rural, peri-urban and urban areas. Sand flies require a humid microclimate in order for their eggs to develop and larvae need a cool, moist habitat with decaying debris. Adult sand flies often inhabit rock crevices, caves, and rodent burrows, and inperidomestic settings rest in cool, dark and humid corners of animal shelters or human dwellings. Both rodent burrows and peridomestic areas provide ready access to bloodmeals in addition to shelter from the elements.

Host preference:

Female sand flies feed on a wide variety of vertebrate hosts, including humans, livestock, dogs, urban and wild rodents, reptiles, amphibians, and birds. Each species of sand fly may have its own specific host preferences, although host availability is an important factor in determining blood feeding behaviour. It is likely that many species of sand fly are opportunistic and feed on animals to which they have easiest access, as the same species collected from different biotopes often display different feeding patterns. A study of sand fly species from farms and kennels in Italy found that *Phlebotomous*

papatasi, Phlebotomous perniciosus and Phlebotomous perfiliewi fed primarily on the host species (livestock and humans) that were present at the collection site. If many different animals were present, both *Phlebotomous perfiliewi* and *Phlebotomous perniciosus* were found to feed on all of them, suggesting that choice of host is influenced by the presence and proportion of each host. As a result, it is likely that in urban and peri-urban settings humans and domestic dogs are the main targets for sand flies.

Life cycle of sand fly:

Reproduction and oviposition:

Adult sand flies mate soon after emergence; males locate females at resting sites or on vertebrate hosts, with the aid of pheromones. Female sand flies usually lay 30_70 eggs during a single gonotrophic cycle, which are deposited in cracks and holes in the ground or in buildings, animal burrows and among tree roots. The eggs require a microhabitat with high humidity in order to survive, but are not laid in water. Generally, one blood meal results in the production of a single batch of eggs.

The life cycle of the phlebotomine sand fly (*P. argentipes*) is divided into four stages.

Eggs:

The female sand fly (*P. argentipes*) requires blood meals to lay an average of 32.66 eggs. The time lag between the engorgement to oviposition is not less than six days. The eggs are elongated oval-shaped, pale at first and darkening following exposure to air with a single black 'eye spot'. Within one to two weeks of oviposition (unless weather conditions become too cold), eggs hatch. The average incubation period for hatching lasts three–six days, with a mean of 3.96 days at a temperature of 25 ± 2 °C; the hatching rate under these conditions is 67.8%. If weather conditions become too cold, the eggs enter diapause during which they do not develop further. The development process restarts once temperature reaches sufficient levels (i.e. around 25 °C).

Larvae:

Originating from aquatic larvae, the larval stages have adapted to live in moist soil, where development takes on average 15.5 days (range 11–29 days). The larvae emerge through a J-shaped fissure and are legless, whitish, and with a dark head capsule. This usually occurs when humidity levels approach 100%, leading to soil moisture of ~12%.

The larvae possess a cylindrical, elongated, and segmented body. The first instar can be distinguished by the presence of two caudal bristles, while all subsequent instars have four caudal bristles. All larvae feed on dead organic matter and are often found in cracks of walls or rocks, animal burrows, caves, or below decaying leaves. The larvae cannot exist without water in the fluid form, but water may be bound by capillary action. The larvae, particularly younger instars, absorb water with their food and through the skin. In the urban areas, the larvae are found in the floors, loose bricks, and rat holes. Fourth instars can be distinguished by the presence of a prominent sclerite on the dorsum of the penultimate segment.

Pupae:

Following the larval stage, the developing sand fly (*P. argentipes*) enters the pupal stage on floating debris or near the water's edge. The pupae are golden brown and are affixed to the surface of the substrate on which they develop. Shortly before emergence, the wings and eyes turn black. The developmental period for pupal stage ranges from 6 to 10 days, with a mean of 7.65. Adults emerge just before dawn.

Adults:

Male sand flies emerge about 24 h before females, allowing time to rotate their external genitalia 180° to the correct position for mating. Although there have been no field studies of sand fly development time, the time from oviposition to adult emergence at ambient temperatures is around four–six weeks. Adult emergence takes place at a temperature of 25 ± 2 °C. During winter months (December–February), the development period decreases with the duration of each life cycle, from 25 to 40 days per generation.

Only the adult female sand fly (*P. argentipes*) sucks vertebrate host's blood, a requirement for egg production. Both males and females feed on plant sugars. The adults of *P. argentipes* are 2–3 mm long and because sand flies are so small, they have nicknames such as 'no see ums' and 'punkies'. They cannot fly hence display a characteristic 'hopping movement' and consequently cannot move more than 106 meters from their breeding place, though they have been recorded from canopy of trees. Under favorable conditions, development from egg to adult takes approximately one month.

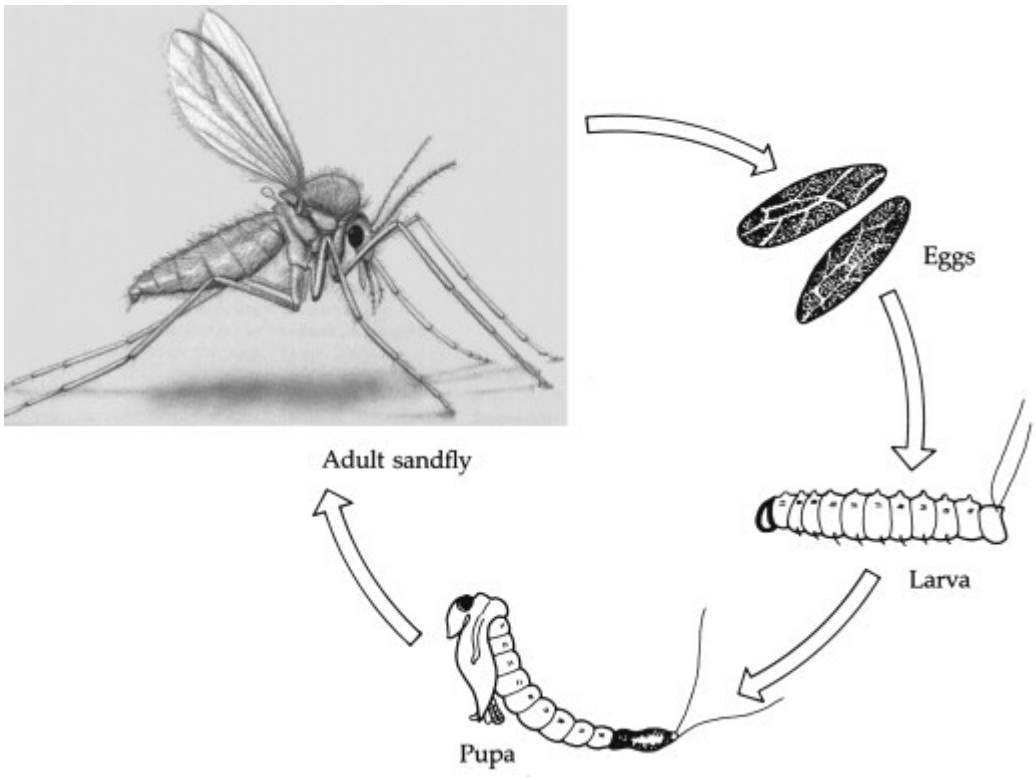


Figure: **Life Cycle of *Phlebotomous argentipes***

Pathogenicity and role of *Phlebotomous* as vector:

In the Old World, *Phlebotomous* sand flies are primarily responsible for the transmission of leishmaniasis, an important parasitic disease, while transmission in the New World, is generally via sand flies of the genus *Lutzomyia*. The protozoan parasite itself is a species of the genus *Leishmania*. Leishmaniasis normally finds a mammalian reservoir in rodents and other small animals such as canids (canine leishmaniasis) and hyraxes. The female sand fly carries the *Leishmania* protozoa from infected animals after feeding, thus transmitting the disease, while the male feeds on plant nectar.

The parasite *Leishmania donovani* is the main causative agent of visceral leishmaniasis (VL) in India, Nepal, and Bangladesh, where it is transmitted by the sand flies of the species *Phlebotomous argentipes*. This species of sand flies was on the brink of elimination in India during the 1960s

following the widespread use of DDT for malaria control. However, there was a resurgence in their population a decade later.

Phlebotomous species are also vectors for bartonellosis, verruga peruana, and pappataci fever, an arboviral disease caused by sandfly fever viruses such as the Naples and Sicilian strains of the genus *Phlebovirus* (family *Bunyaviridae*), which also includes the closely related Toscana virus. In Egypt, two species of medical importance are *Phlebotomous papatasii* and *P. langerni*. These flies are short-lived. Females are bloodsuckers at night; males feed on plant juices. Adults are poor fliers, they usually hop for short distances.

Glossinia sp
Common name – Tsetse fly

Introduction: **Tsetse** sometimes spelled **tzetze** and also known as **tik-tik** flies, are large biting flies that inhabit much of tropical Africa. Tsetse flies include all the species in the genus ***Glossina***, which are placed in their own family, **Glossinidae**. The tsetse are obligate parasites that live by feeding on the blood of vertebrate animals. Tsetse have been extensively studied because of their role in transmitting disease. They have a prominent economic impact in sub-Saharan Africa as the biological vectors of trypanosomes, which cause human sleeping sickness and animal trypanosomiasis. Tsetse are multivoltine and long-lived, typically producing about four broods per year, and up to 31 broods over their lifespans.

Tsetse can be distinguished from other large flies by two easily observed features. Tsetse fold their wings completely when they are resting so that one wing rests directly on top of the other over their abdomens. Tsetse also have a long proboscis, which extends directly forward and is attached by a distinct bulb to the bottom of their heads.

Tsetse were absent from much of southern and eastern Africa until colonial times. The accidental introduction of rinderpest in 1887 killed most of the cattle in these parts of Africa and the resulting famine removed much of the human population. Thorny bush ideal for tsetse quickly grew up where there had been pasture, and was repopulated by wild mammals. Tsetse and sleeping sickness soon colonised the whole region, effectively excluding the reintroduction of farming and animal husbandry. Sleeping sickness has been described by some conservationists as "the best game warden in Africa".

Morphology

Tsetse flies can be seen as independent individuals in two forms: as third-instar larvae, and as adults. Tsetse first become separate from their mothers during the third larval instar, during which they have the typical appearance of maggots. However, this life stage is short, lasting at most a few hours, and is almost never observed outside of the laboratory.

Tsetse next develop a hard external case, the puparium, and become pupae—small, hard-shelled, oblongs with two distinctive, small, dark lobes at the tail (breathing) end. Tsetse pupae are under 1 cm long.[9] Within the puparial shell, tsetse complete the last two larval instars and the pupal stage.

At the end of the pupal stage, tsetse emerge as adult flies. The adults are relatively large flies, with lengths of 0.5-1.5 cm,[9] and have a recognizable shape or bauplan which makes them easy to distinguish from other flies. Tsetse have large heads, distinctly separated eyes, and unusual antennae. The thorax is quite large, while the abdomen is wide rather than elongated and shorter than the wings.

Four characteristics definitively separate adult tsetse from other kinds of flies:

a. Proboscis: Tsetse have a distinct proboscis, a long thin structure attached to the bottom of the head and pointing forward.

b. Folded wings: When at rest, tsetse fold their wings completely one on top of the other.

c. Hatchet cell: The discal medial ("middle") cell of the wing has a characteristic hatchet shape resembling a meat cleaver or a hatchet.

d. Branched arista hairs: The antennae have arista with hairs which are themselves branched.

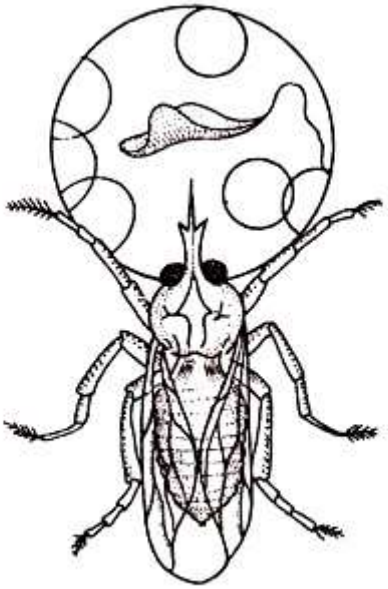


Fig. 15.17: Tsetse fly (*Glossina*)

Life Cycle:

Tsetse have an unusual lifecycle which may be due to the richness of their food source. A female fertilizes only one egg at a time and retains each egg within her uterus to have the offspring develop internally during the first three larval stages, a method called adenotrophic viviparity. During this time, the female feeds the developing offspring with a milky substance secreted by a modified gland in the uterus.[10] In the third larval stage, the tsetse larva leaves the uterus and begins its independent life. The newly independent tsetse larva crawls into the ground, and develops a hard outer shell called the puparial case, in which it completes its morphological transformation into an adult fly.

This life stage has a variable duration, generally 20 to 30 days, and the larva must rely on stored resources during this time. The importance of the richness of blood to this development can be seen, since all tsetse development before it emerges from the puparial case as a full adult occurs without feeding, based only on nutritional resources provided by the female parent. The female must get enough energy for her needs, for the needs of her developing offspring, and for the stored resources which her offspring will require until it emerges as an adult.

Technically, these insects undergo the standard development process of insects, which consists of oocyte formation, ovulation, fertilization, development of the egg, three larval stages, a pupal stage, and the emergence and maturation of the adult.

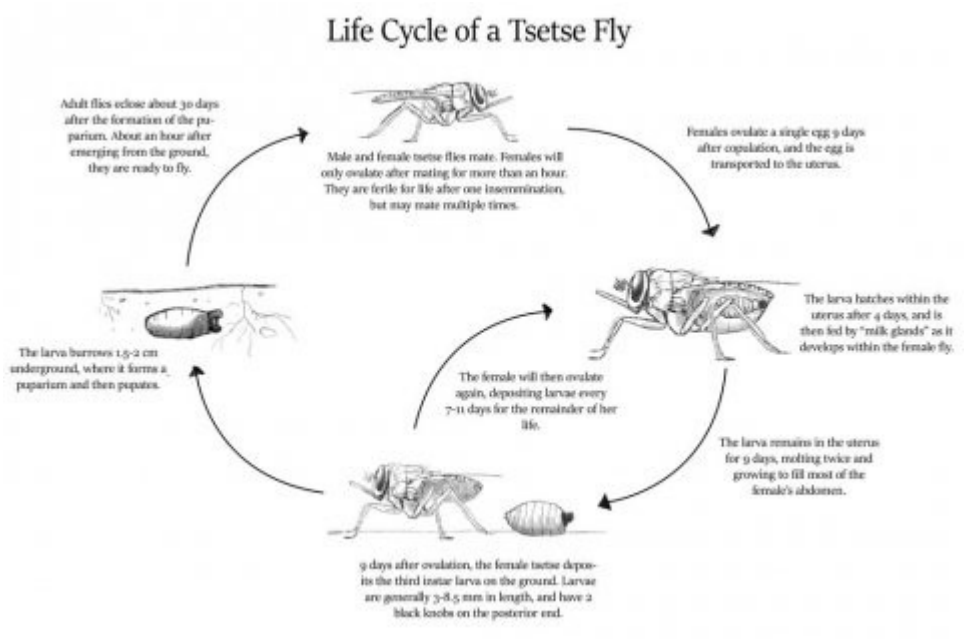


Figure: Life cycle of *Glossinia* sp

Role of *Glossinia* in disease transmission:

Tsetse are biological vectors of trypanosomes, meaning that in the process of feeding, they acquire and then transmit small, single-celled trypanosomes from infected vertebrate hosts to uninfected animals. Some tsetse-transmitted trypanosome species cause trypanosomiasis, an infectious disease. In humans, tsetse transmitted trypanosomiasis is called sleeping sickness. In animals, tsetse-vectored trypanosomiases include *nagana*, *souma*, and *surra* according to the animal infected and the trypanosome species involved. The usage is not strict and while *nagana* generally refers to the disease in cattle and horses it is commonly used for any of animal trypanosomiasis.

Trypanosomes are animal parasites, specifically protozoans of the genus *Trypanosoma*. These organisms are about the size of red blood cells. Different species of trypanosomes infect different hosts. They range widely in their effects on the vertebrate hosts. Some species, such as *T. theileri*, do not seem to cause any health problems except perhaps in animals that are already sick.

Some strains are much more virulent. Infected flies have an altered salivary composition which lowers feeding efficiency and consequently increases the feeding time, promoting trypanosome transmission to the vertebrate host. These trypanosomes are highly evolved and have developed a lifecycle that requires periods in both the vertebrate and tsetse hosts.

Tsetse transmit trypanosomes in two ways, mechanical and biological transmission.

- **Mechanical transmission** involves the direct transmission of the same individual trypanosomes taken from an infected host into an uninfected host. The name 'mechanical' reflects the similarity of this mode of transmission to mechanical injection with a syringe. Mechanical transmission requires the tsetse to feed on an infected host and acquire trypanosomes in the blood meal, and then, within a relatively short period, to feed on an uninfected host and regurgitate some of the infected blood from the first blood meal into the tissue of the uninfected animal. This type of transmission occurs most frequently when tsetse are interrupted during a blood meal and attempt to satiate themselves with another meal. Other flies, such as horse-flies, can also cause mechanical transmission of trypanosomes.

- **Biological transmission** requires a period of incubation of the trypanosomes within the tsetse host. The term 'biological' is used because trypanosomes must reproduce through several generations inside the tsetse host during the period of incubation, which requires extreme adaptation of the trypanosomes to their tsetse host. In this mode of transmission, trypanosomes reproduce through several generations, changing in morphology at certain periods. This mode of transmission also includes the sexual phase of the trypanosomes. Tsetse are believed to be more likely to become infected by trypanosomes during their first few blood meals. Tsetse infected by trypanosomes are thought to remain infected for the remainder of their lives. Because of the adaptations required for biological transmission, trypanosomes transmitted biologically by tsetse cannot be transmitted in this manner by other insects.

The relative importance of these two modes of transmission for the propagation of tsetse-vectored trypanosomiases is not yet well understood. However, since the sexual phase of the trypanosome lifecycle occurs within the tsetse host, biological transmission is a required step in the lifecycle of the tsetse-vectored trypanosomes.

The cycle of biological transmission of trypanosomiasis involves two phases, one inside the tsetse host and the other inside the vertebrate host. Trypanosomes are not passed between a pregnant tsetse and her offspring, so all newly emerged tsetse adults are free of infection. An uninfected fly that feeds on an infected vertebrate animal may acquire trypanosomes in its proboscis or gut. These trypanosomes, depending on the species, may remain in place, move to a different part of the digestive tract, or migrate through the tsetse body into the salivary glands. When an infected tsetse bites a susceptible host, the fly may regurgitate part of a previous blood meal that contains trypanosomes, or may inject trypanosomes in its saliva. Inoculation must contain a minimum of 300 to 450 individual trypanosomes to be successful, and may contain up to 40,000 cells.

The trypanosomes are injected into vertebrate muscle tissue but make their way, first into the lymphatic system, then into the bloodstream, and eventually into the brain. The disease causes the swelling of the lymph glands, emaciation of the body, and eventually leads to death. Uninfected tsetse may bite the infected animal prior to its death and acquire the disease, thereby closing the transmission cycle.

Disease hosts and vectors:

The tsetse-vectored trypanosomiases affect various vertebrate species including humans, antelopes, bovine cattle, camels, horses, sheep, goats, and pigs. These diseases are caused by several different trypanosome species that may also survive in wild animals such as crocodiles and monitor lizards. The diseases have different distributions across the African continent, so are transmitted by different species.

In humans:

Human African trypanosomiasis, also called sleeping sickness, is caused by trypanosomes of the species *Trypanosoma brucei*. This disease is invariably fatal unless treated but can almost always be cured with current medicines, if the disease is diagnosed early enough.

Sleeping sickness begins with a tsetse bite leading to an inoculation in the subcutaneous tissue. The infection moves into the lymphatic system, leading to a characteristic swelling of the lymph glands called *Winterbottom's sign*.[23] The infection progresses into the blood stream and eventually crosses into the central nervous system and invades the brain leading to extreme lethargy and eventually to death.

The species *Trypanosoma brucei*, which causes the disease, has often been subdivided into three subspecies that were identified based either on the vertebrate hosts which the strain could infect or on

the virulence of the disease in humans. The trypanosomes infectious to animals and not to humans were named *Trypanosoma brucei brucei*. Strains that infected humans were divided into two subspecies based on their different virulences: *Trypanosoma brucei gambiense* was thought to have a slower onset and *Trypanosoma brucei rhodesiense* refers to strains with a more rapid, virulent onset. This characterization has always been problematic but was the best that could be done given the knowledge of the time and the tools available for identification. A recent molecular study using restriction fragment length polymorphism analysis suggests that the three subspecies are polyphyletic, so the elucidation of the strains of *T. brucei* infective to humans requires a more complex explanation. Procyclins are proteins developed in the surface coating of trypanosomes whilst in their tsetse fly vector.

Other forms of human trypanosomiasis also exist but are not transmitted by tsetse. The most notable is American trypanosomiasis, known as Chagas disease, which occurs in South America, caused by *Trypanosoma cruzi*, and transmitted by certain insects of the Reduviidae, members of the Hemiptera.

In domestic animals:

Animal trypanosomiasis, also called *nagana* when it occurs in bovine cattle or horses or *sura* when it occurs in domestic pigs, is caused by several trypanosome species. These diseases reduce the growth rate, milk productivity, and strength of farm animals, generally leading to the eventual death of the infected animals. Certain species of cattle are called *trypanotolerant* because they can survive and grow even when infected with trypanosomes although they also have lower productivity rates when infected.

The course of the disease in animals is similar to the course of sleeping sickness in humans. *Trypanosoma congolense* and *Trypanosoma vivax* are the two most important species infecting bovine cattle in sub-Saharan Africa. *Trypanosoma simiae* causes a virulent disease in swine. Other forms of animal trypanosomiasis are also known from other areas of the globe, caused by different species of trypanosomes and transmitted without the intervention of the tsetse fly.

The tsetse fly vector ranges mostly in the central part of Africa. Trypanosomiasis poses a considerable constraint on livestock agricultural development in Tsetse fly infested areas of sub Saharan Africa, especially in west and central Africa. International research conducted by ILRI in Nigeria, the Democratic Republic of the Congo and Kenya has shown that the N'Dama is the most resistant breed.

Control:

The conquest of sleeping sickness and nagana would be of immense benefit to rural development and contribute to poverty alleviation and improved food security in sub-Saharan Africa. Human African trypanosomosis (HAT) and animal African trypanosomosis (AAT) are sufficiently important to make virtually any intervention against these diseases beneficial.

The disease can be managed by controlling the vector and thus reducing the incidence of the disease by disrupting the transmission cycle. Another tactic to manage the disease is to target the disease directly using surveillance and curative or prophylactic treatments to reduce the number of hosts that carry the disease.

Economic analysis indicates that the cost of managing trypanosomosis through the elimination of important populations of major tsetse vectors will be covered several times by the benefits of tsetse-free status. Area-wide interventions against the tsetse and trypanosomosis problem appear more efficient and profitable if sufficiently large areas, with high numbers of cattle, can be covered.

Vector control strategies can aim at either continuous suppression or eradication of target populations. Tsetse fly eradication programmes are complex and logistically demanding activities and usually involve the integration of different control tactics, such as trypanocidal drugs, impregnated treated

targets (ITT), insecticide-treated cattle (ITC), aerial spraying (Sequential Aerosol Technique - SAT) and in some situations the release of sterile males (sterile insect technique – SIT). To ensure sustainability of the results, it is critical to apply the control tactics on an area-wide basis, i.e. targeting an entire tsetse population that is preferably genetically isolated.

Control techniques:

Many techniques have reduced tsetse populations, with earlier, crude methods recently replaced by methods that are cheaper, more directed, and ecologically better.

Slaughter of wild animals:

One early technique involved slaughtering all the wild animals tsetse fed on. For example, the island of Principe off the west coast of Africa was entirely cleared of feral pigs in the 1930s, which led to the extirpation of the fly. While the fly eventually re-invaded in the 1950s, the new population of tsetse was free from the disease.

Land clearing:

Another early technique involved complete removal of brush and woody vegetation from an area. Tsetse tend to rest on the trunks of trees so removing woody vegetation made the area inhospitable to the flies. However, the technique was not widely used and has been abandoned. Preventing regrowth of woody vegetation requires continuous clearing efforts, which is only practical where large human populations are present. The clearing of woody vegetation has come to be seen as an environmental problem more than a benefit.

Pesticide campaigns:

Pesticides have been used to control tsetse starting initially during the early part of the twentieth century in localized efforts using the inorganic metal-based pesticides, expanding after the Second World War into massive aerial- and ground-based campaigns with organochlorine pesticides such as DDT applied as aerosol sprays at Ultra-Low Volume rates. Later, more targeted techniques used *pour-on* formulations in which advanced organic pesticides were applied directly to the backs of cattle. Tsetse populations can be monitored and effectively controlled using simple, inexpensive traps. These often use electric blue cloth, since this colour attracts the flies. Early traps mimicked the form of cattle but this seems unnecessary and recent traps are simple sheets or have a biconical form. The traps can kill by channelling the flies into a collection chamber or by exposing the flies to insecticide sprayed on the cloth. Tsetse are also attracted to large dark colours like the hides of cow and buffaloes. Some scientists put forward the idea that zebra have stripes, not as a camouflage in long grass, but because the black and white bands tend to confuse tsetse and prevent attack.

The use of chemicals as attractants to lure tsetse to the traps has been studied extensively in the late 20th century, but this has mostly been of interest to scientists rather than as an economically reasonable solution. Attractants studied have been those tsetse might use to find food, like carbon dioxide, octenol, and acetone—which are given off in animals' breath and distributed downwind in an *odor plume.* Synthetic versions of these chemicals can create artificial odour plumes. A cheaper approach is to place cattle urine in a half gourd near the trap. For large trapping efforts, additional traps are generally cheaper than expensive artificial attractants.

A special trapping method is applied in Ethiopia, where the BioFarm Consortium (ICIPE, BioVision Foundation, BEA, Helvetas, DLCO-EA, Praxis Ethiopia) applies the traps in a sustainable agriculture and rural development context (SARD). The traps are just the entry point, followed by improved farming, human health and marketing inputs. This method is in the final stage of testing (as per 2006).

In the late 18th century, the Kotokoli Muslims of Togo held a special ritual in order for their child to have a prosperous life. This ritual consisted of mothers killing the tsetse flies and sprinkling them on horned melon. They would feed their children this delicacy. This ritual is still practiced today in some sub-Saharan tribes.

Sterile insect technique:

The sterile insect technique (**SIT**) is a form of pest control that uses ionizing radiation (gamma ray or X ray) to sterilize male flies that are mass-produced in special rearing facilities. The sterile males are released systematically from the ground or by air in tsetse-infested areas, where they mate with wild females, which do not produce offspring. As a result, this technique can eventually eradicate populations of wild flies. SIT is among the most environmentally friendly control tactics available, and is usually applied as the final component of an integrated campaign.

The sustainable removal of the tsetse fly is in many cases the most cost-effective way of dealing with the T&T problem resulting in major economic benefits for subsistence farmers in rural areas. Insecticide-based methods are normally very ineffective in removing the last remnants of tsetse populations, while, on the contrary, sterile males are very effective in finding and mating the last remaining females. Therefore, the integration of the SIT as the last component of an area-wide integrated approach is essential in many situations to achieve complete eradication of the different tsetse populations, particularly in areas of more dense vegetation.

Tabanus spp
Common name – Horse fly

Introduction:

Horse-flies or **horseflies** are true flies in the family **Tabanidae** in the insect order Diptera. They are often large and agile in flight, and the females bite animals, including humans, to obtain blood. They prefer to fly in sunlight, avoiding dark and shady areas, and are inactive at night. They are found all over the world except for some islands and the Polar Regions.

Morphology:

Adults: Tabanidae flies are large flies, up to 2.5cm long with bodies that are usually dark incolour. The dark bodies may have stripes or patches of colour down them or be entirely coloured in some cases. They have broad heads with biting mouth parts and bulging eyes that are often brightly coloured. The mouthparts are adapted to biting a sucking blood, and always pointed ownwards. They consist of paired mandibles and maxillae which are used for cutting and rasping to create a feeding hole. Blood is then sucked using a protruding hypopharynx. The mouthparts are short and deal roughly with the host, this is often the cause of pain for the host. Only females take a blood meal, males lack mandibles and may only feed on honeydew and nectar. The wings of Tabanidae flies have a distinctive venation.

Larvae: Tabanidae larvae are large, 1.5 - 3cm in length with large biting mandibles. They are off-white in colour with longitudinal striations on the cuticle. There are paired unsegmented appendages, psuedopods, along the body to assist in movement. The posterior of the larvae usually has a respiratory siphon present.

Eggs: Eggs are an off white colour and cigar shaped. They can be between 1 - 3 mm long.

Figure: External features of Horse fly

Habit and Habitat

Horse fly females are aggressive blood feeders, while males do not consume blood but feed on pollen and plant nectars. Female horse flies usually bite large, non-moving mammals on the legs or body. Deer flies, in contrast, attack moving hosts and typically target high on the body, like the head or neck. They rarely bite near the head. Horse flies have a range of hosts that include mammals of almost all sizes, livestock, humans, pets and birds. Should a female horse fly be interrupted when attempting to feed, they will fly off but quickly return to bite again, or go to another host to consume a complete blood meal. Horse fly larvae studied by field researchers feed on midges, crane flies and even other horse fly larvae. Because of their cannibalistic behaviours, horse fly larvae are usually found living alone. Deer fly larvae, on the other hand, usually live in groups. Pupae do not feed. Similar to other blood-sucking insects such as mosquitoes for example, female horse flies use both chemical and visual cues to locate hosts. Carbon dioxide expelled by warm-blooded animals provides a long-range cue to attract flies from a distance, while visual cues such as motion, size, shape and dark colour function to attract horse flies from shorter distances. Horse fly development sites are freshwater and salt water marshes and streams, moist forest soils and even moist decomposing wood. Females usually deposit egg masses on wet soil or vegetation that overhangs water. Larvae are active in moist or wet organic matter and look similar to house fly maggots.

Diet and biting behaviour: Adult horse-flies feed on nectar and plant exudates, and some are important pollinators of certain specialised flowers. Both males and females engage in nectar feeding, but in addition to this, females of most species are anautogenous, meaning they require a blood meal before they are able to reproduce effectively. To obtain the blood, the females bite animals, including humans, while the males are harmless. It takes the female about six days to fully digest its blood meal and after that it needs to find another host. It seems that the flies are attracted to a potential victim by its movement, warmth, and surface texture, and by the carbon dioxide it breathes out. The flies mainly choose large mammals such as cattle, horses, camels, and deer, but few are species specific. They have also been observed feeding on smaller mammals, birds, lizards and turtles, and even on animals that have recently died. Because their bite is irritating to the victim, they are often brushed off, and may have to visit multiple hosts to obtain sufficient blood. This behaviour means that they may carry disease-causing organisms from one host to another.

The mouthparts of females are of the usual Dipteran form and consist of a bundle of six chitinous stylets that, together with a fold of the flesh labium, form the proboscis. On either side of theseare two maxillary palps. When the insect lands on an animal it grips the surface with its clawed feet, the labium is retracted, the head is thrust downwards and the stylets slice into the flesh. Some of these have sawing edges and muscles can move them from side-to-side to enlarge the wound. Saliva containing anticoagulant is injected into the wound to prevent clotting. The blood that flows from the wound is lapped up by another mouthpart which functions as a sponge. Horsefly bites can be painful for a day or more; fly saliva may provoke allergic reactions such as hives and difficulty with breathing. Tabanid bites can make life outdoors unpleasant for humans, and can reduce milk output in cattle. They are attracted by reflections from water which are polarized, making them a particular

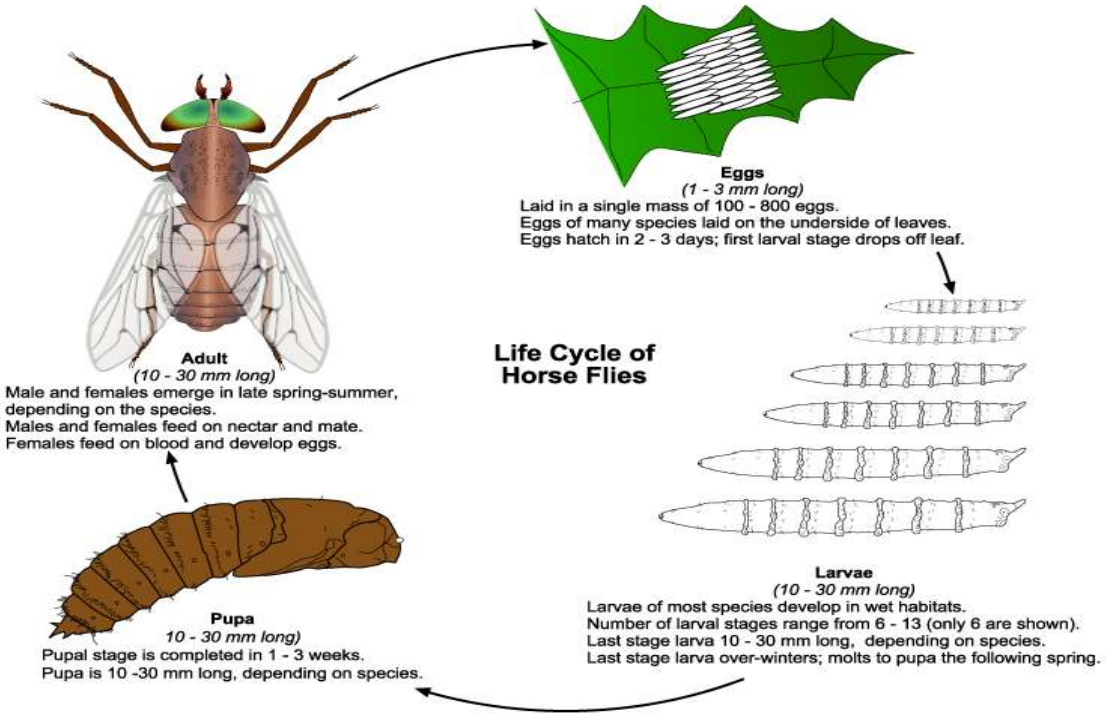
nuisance near swimming pools. Since tabanids prefer to be in sunshine, they normally avoid shaded places such as barns, and are inactive at night. Attack patterns vary with species: Large species of ankles, legs or backs of knees; have a high buzzing note. It has been suggested that the striped hides of reduce their attractiveness to horsehides. The closer together the stripes, the fewer flies are visually attracted; the zebra's legs have particularly fine striping, and this is the shaded part of the body that is most likely to be bitten by the other, unstriped equids. This does not preclude the possible use of stripes for other purposes such as signalling or camouflage.

Life Cycle:

Mating often occurs in swarms, generally at landmarks such as day, and type of landmark, used for mating swarms is specific to particular species. Eggs are laid on stones or vegetation near water, in clusters of up to one thousand, especially on emergent water plants. The eggs are white at first but darken with ages. They hatch after about six days, the emerging larvae using a special hatching spike to open the egg case. The larvae fall into the water or onto the moist ground below.

Chrysops species develop in particularly wet locations while *Tabanus* species prefer drier places. The larvae are legless grubs, tapering at both ends. They have small heads and eleven or twelve segments and moult six to thirteen times over the course of up to a year or more. In temperate species, the larvae have a quiescent period during winter (diapause) while tropical species breed several times a year. In the majority of species they are white, but in some, they are greenish or brownish, and they often have dark bands on each segment. A respiratory siphon at the hind end allows the larvae to obtain air when submerged in water. Larvae of nearly all species are carnivorous, often cannibalistic in captivity, and consume worms, insect larvae, and arthropods. The larvae may be parasitized by nematodes, flies of the families Bombyliidae and Tachinidae; and Hymenoptera in the family Pteromalidae. When fully developed, the larvae move into drier soil near the surface of the ground to pupate.

The pupae are brown and glossy, rounded at the head end and tapering at the other end. Wing and limb buds can be seen and each abdominal segment is fringed with short spines. After about two weeks, metamorphosis is complete, the pupal case splits along the thorax and the adult fly emerges. Males usually appear first, but when both sexes have emerged, mating takes place, courtship starting in the air and finishing on the ground. The female needs to feed on blood before depositing her egg mass.



Pathogenesis:

The Tabanidae flies in themselves are responsible for painful, irritating bites which can cause distress to the animal in question. They are most active on hot, sunny days and locate their prey by sight using their large bulbous eyes. As well as causing irritation when biting the flies act as mechanical vectors for a number of other pathogens;

a. Bacteria such as anthrax and pasteurellosis

b. Viruses such as Equine infectious anaemia and African horse sickness

c. Rickettsiales, such as anaplasmosis

The Tabanidae flies may also act as intermediate host for some trypanosome species.

Disease transmission:

Tabanids are known vectors for some blood-borne bacterial, viral, protozoan and worm diseasesof mammals, such as the equine infectious anaemia virus and various species of *Trypanosoma* which cause diseases in animals and humans. Tabanids are known to transmit anthrax among cattle and sheep, and tularemia between rabbits and humans. Blood loss is a common problem in some animals when large flies are abundant. There are anecdotal reports of horse-fly bites leading to fatal anaphylaxis in humans, an extremely rare occurrence.

Control:

Controlling horse flies is nearly impossible. The use of insecticides to kill larvae is not an option because the vast majority of species develop in natural habitats in which insecticides cannot be applied due to environmental concerns. Even if they could be used, insecticides would be ineffective in controlling larvae because they are widely dispersed in a developmental site. The use of insecticides against adult horse flies is not a realistic option because they are relatively large to very large and unaffected by the rate of insecticide that can be applied according to product label. At best, an insecticide application aimed at adults might produce a minor and temporary reduction in biting. A number of trapping devices have been used to capture adults, but their value is limited to sampling. At best, trapping devices produce temporary, minor relief from female horse flies.

Malaise traps are most often used to capture them and these can be modified with the use of baits and attractants that include carbon dioxide or octenol. A dark shiny ball suspended below them that moves in the breeze can also attract them and forms a key part of a modified "Manitoba trap" that is used most often for trapping and sampling Chemical: Cattle can be treated with pour-on pyrethroids which may repel the flies, and fitting them with insecticide impregnated eartags or collars has had some success in killing the insects. Again, repellents, including those containing DEET, have very little or no effect in deterring adult horse flies. Wearing a thick long sleeve shirt, thick pants, and a heavy hat may provide some protection against bites when entering habitats that support large numbers of adult horse flies, but they can be very annoying as they attempt to take blood meals.

Pediculus humanus capitis

Common Name : Head louse

Introduction:

The head louse(*Pediculus humanus capitis*) is an obligate ectoparasite of humans that causeshead lice infestation (*pediculosis capitis*). Head lice are wingless insects spending their entire lifeon the human scalp and feeding exclusively on human blood. Humans are the only known hostsof this specific parasite, while chimpanzees host a closely related species, *Pediculus schaeffi*.Other species of lice infest most orders of mammals and all orders of birds, as well as other partsof the human body.

Lice differ from other hematophagic ectoparasites such as fleas in spending their entire life cycleon a host. Head lice cannot fly, and their short stumpy legs render them incapable of jumping, oreven walking efficiently on flat surfaces.The non-disease-carrying head louse differs from the related disease-carrying body louse(*Pediculus humanushumanus*) in preferring to attach eggs to scalp hair rather than to clothing.

The two subspecies are morphologically almost identical but do not normally interbreed,although they will do so in laboratory conditions. From genetic studies, they are thought to havediverged as subspecies about 30,000–110,000 years ago, when many humans began to wear asignificant amount of clothing. A much more distantly related species of hair-clinging louse, thepubic or crab louse (*Pthirus pubis*), also infests humans. It is visually different from the othertwo species and is much closer in appearance to the lice which infest other primates.

Head lice (especially in children) have been, and still are, subject to various eradicationcampaigns. Unlike body lice, head lice are not the vectors of any known diseases. Except for raresecondary infections that result from scratching at bites, head lice are harmless, and they havebeen regarded by some as essentially a cosmetic rather than a medical problem. It has even beensuggested that head lice infestations might be beneficial in helping to foster a natural immuneresponse against lice which helps humans in defence against the far more dangerous body louse,which is capable of transmission of dangerous diseases.

Systematic position:

Kingdom: Animalia
Phylum: Arthropoda
Class: Insecta
Order: Phthiraptera
Family: Pediculidae
Genus: *Pediculus*
Species: *humanus*
Subspecies: ***P. h. capitis***

Morphology:

Like other insects of the suborder Anoplura, adult head lice are small (2.5–3 mm long), dorsoventrally flattened (see anatomical terms of location), and entirely wingless. The thoracic segments are fused, but otherwise distinct from the head and abdomen, the latter being composed of seven visible segments. Head lice are grey in general, but their precise colour varies according to the environment in which they were raised. After feeding, consumed blood causes the louse body to take on a reddish colour.

Head

One pair of antennae, each with five segments, protrude from the insect's head. Head lice also

have one pair of eyes. Eyes are present in all species within *Pediculidae* (the family of which the head louse is a member) but are reduced or absent in most other members of the Anoplura suborder. Like other members of Anoplura, head lice mouth parts are highly adapted for piercing skin and sucking blood. These mouth parts are retracted into the insect's head except during feeding.

Thorax

Six legs project from the fused segments of the thorax. As is typical in Anoplura, these legs are short and terminate with a single claw and opposing "thumb". Between its claw and thumb, the louse grasps the hair of its host. With their short legs and large claws, lice are well adapted to clinging to the hair of their host. These adaptations leave them incapable of jumping, or even walking efficiently on flat surfaces. Lice can climb up strands of hair very quickly, allowing them to move quickly and reach another host.

Abdomen

There are seven visible segments of the louse abdomen. The first six segments each have a pair of spiracles through which the insect breathes. The last segment contains the anus and (separately) the genitalia.

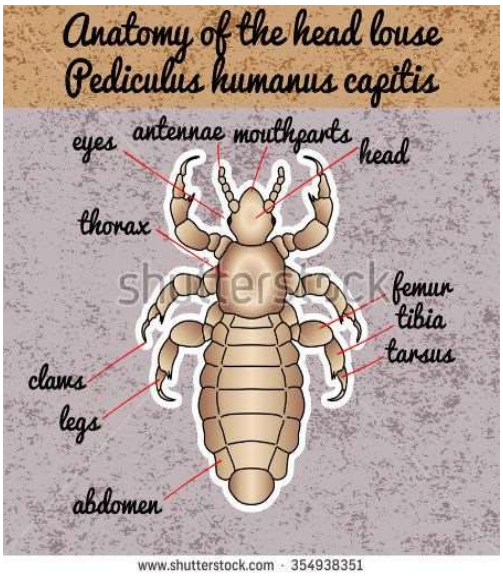


Figure: External morphology of Head louse

Sex differences:

In male lice, the front two legs are slightly larger than the other four. This specialized pair of legs is used for holding the female during copulation. Males are slightly smaller than females and are characterized by a pointed end of the abdomen and a well-developed genital apparatus visible inside the abdomen. Females are characterized by two gonopods in the shape of a W at the end of their abdomen.

Louse eggs (nit):

Like most insects, head lice are oviparous. Females lay about 3–4 eggs per day. Louse eggs are attached near the base of a host hair shaft. Egg-laying behaviour is temperature dependent and likely seeks to place the egg in a location that will be conducive to proper embryo development (which is, in turn, temperature dependent). In cool climates, eggs are generally laid within 3–5 mm of the scalp surface. In warm climates, and especially the tropics, eggs may be laid 6inches (15 cm) or more down the hair shaft. To attach an egg, the adult female secretes a glue from her reproductive organ. This

glue quickly hardens into a "nit sheath" that covers the hair shaft and large parts of the egg except for the operculum, a cap through which the embryo breathes. The glue was previously thought to be chitin-based, but more recent studies have shown it to be made of proteins similar to hair keratin. Each egg is oval-shaped and about 0.8 mm in length. They are bright, transparent, tan to coffee coloured so long as they contain an embryo but appear white after hatching. Typically, a hatching time of six to nine days after oviposition is cited by authors. After hatching, the louse nymph leaves behind its egg shell (usually known as nit), still attached to the hair shaft. The empty egg shell remains in place until physically removed by abrasion or the host, or until it slowly disintegrates, which may take 6 or more months.

Nits

The term nit refers to an egg without embryo or a dead egg. Accordingly, on the head of an infested individual the following eggs could be found:

Life Cycle:

Head lice, like other insects of the order Phthiraptera, are hemimetabolous. Newly hatched nymphs will moult three times before reaching the sexually-mature adult stage. Thus, mobile head lice populations contain members of up to four developmental stages: three nymphal instars, and the adult (imago). Metamorphosis during head lice development is subtle. The only visible differences between different instars and the adult, other than size, is the relative length of the abdomen, which increases with each moult. Aside from reproduction, nymph behaviour is similar to the adult. Nymphs feed only on human blood (hematophagia), and cannot survive long away from a host.

The time required for head lice to complete their nymph development to the imago depends on feeding conditions. At minimum, eight to nine days is required for lice having continuous access to a human host. This experimental condition is most representative of head lice conditions in the wild. Experimental conditions where the nymph has more limited access to blood produces more prolonged development, ranging from 12 to 24 days.

Nymph mortality in captivity is high—about 38%—especially within the first two days of life. Inthe wild, mortality may instead be highest in the third instar. Nymph hazards are numerous. Failure to completely hatch from the egg is invariably fatal and may be dependent on the humidity of the egg's environment. Death during moulting can also occur, although it is reportedly uncommon. During feeding, the nymph gut can rupture, dispersing the host's blood through out the insect. This results in death within a day or two. It is unclear if the high mortality recorded under experimental conditions is representative of conditions in the wild.

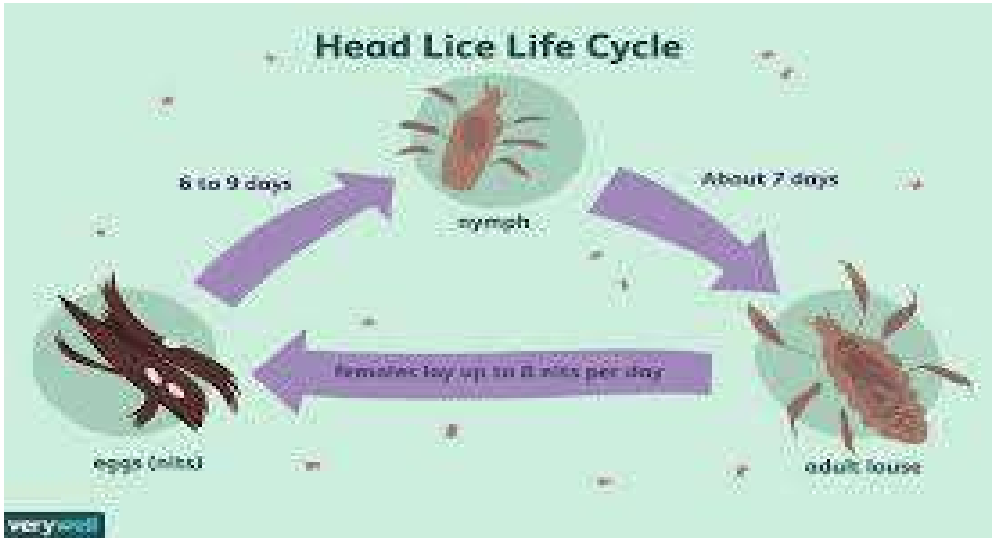


Figure: Life cycle of *Pediculus humanu*

Reproduction and lifespan:

Copulation in *Pediculus humanushumanus* (*Pediculus humanus capitis* is similar). Female is on top, with the male below. Dilation of the female's vagina has already occurred, and the male's dilator rests against his back (dorsal surface), out of the way. The male vesica, which contains the penis proper (not seen), is fully inserted into the vagina. Note the male's attachment with his specialized claws on the first leg pair to the specialized notch on the female's third leg pair. Adult head lice reproduce sexually, and copulation is necessary for the female to produce fertile eggs. Parthenogenesis, the production of viable offspring by virgin females, does not occur in *Pediculushumanus*. Pairing can begin within the first 10 hours of adult life. After 24 hours, adult lice copulate frequently, with mating occurring during any period of the night or day. Mating attachment frequently lasts more than an hour. Young males can successfully pair with older females, and vice versa.
Experiments with *Pediculus humanus humanus* (body lice) emphasize the attendant hazards of lice copulation. A single young female confined with six or more males will die in a few days, having laid very few eggs. Similarly, death of a virgin female was reported after admitting a male to her confinement. The female laid only one egg after mating, and her entire body was tinged with red—a condition attributed to rupture of the alimentary canal during the sexual act. Old females frequently die following, if not during, intercourse. A single louse has a thirty-day life cycle beginning from the moment the nit is laid until the adult louse dies.

Habit:

All stages are blood-feeders and bite the skin four to five times daily to feed. They inject saliva which contains an anti-coagulant and suck blood. The digested blood is excreted as dark red frass. Although any part of the scalp may be colonized, lice favour the nape of the neck and the area behind the ears, where the eggs are usually laid. Head lice are repelled by light and will move towards shadows or dark-coloured objects in their vicinity.

Disease caused by head lice:

Head lice are not known to transmit any disease and therefore are not considered a health hazard. Head lice infestations can be asymptomatic, particularly with a first infestation or when an infestation is light. Itching ("pruritus") is the most common symptom of head lice infestation and is caused by an allergic reaction to louse bites. It may take 4–6 weeks for itching to appear the first time a person has head lice.

Other symptoms may include:

- a tickling feeling or a sensation of something moving in the hair;
- irritability and sleeplessness; and
- sores on the head caused by scratching. These sores caused by scratching can sometimes become infected with bacteria normally found on a person's skin.

Transmission:

Lice have no wings or powerful legs for jumping, so they move by using their claw-like legs to transfer from hair to hair. Normally head lice infest a new host only by close contact between individuals, making social contacts among children and parent-child interactions more likely routes of infestation than shared combs, hats, brushes, towels, clothing, beds or closets. Head-to head contact is by far the most common route of lice transmission.

Diagnosis of head lice infestation:

Head lice are most frequently located on the scalp behind the ears and near the neckline at the back of the neck. Head lice hold on to hair with hook-like claws that are found at the end of each of their six legs. Head lice are rarely found on the body, eyelashes, or eyebrows. Head lice can be detected by looking closely through the hair and scalp for nits, nymphs, or adults. Locating a nymph or adult may

be difficult; there are usually only a few of them, and they can move quickly from searching fingers. However, the presence of nits close to the scalp confirms that a person is infested. If the nits are located more than ¼ inch from the scalp, the infestation is probably an old one. If you are not sure whether or not a person has head lice, the diagnosis should be made by a health care professional, school nurse, or a professional from the local health department or agricultural extension service. The nits of head lice are easily visible with a microscope.

Probable Questions:

1. Describe the morphology of *Phlebotomus s*p.
2. Describe habit and habitat of *Phlebotomus s*p.
3. Describe host preference of *Phlebotomus s*p.
4. Describe life cycle of *Phlebotomus s*p with suitable diagram.
5. Describe pathogenicity and role in disease transmission of *Phlebotomus s*p.
6. Describe morphology of *Glossinia* sp.
7. Describe life cycle of *Glossinia* sp.
8. Describe role of *Glossinia* sp in disease transmission.
9. How *Glossinia* sp population can be controlled ?
10. Describe life cycle of *Tabanus* sp.
11. Describe morphology of *Tabanus* sp.
12. Describe habit and habitat of *Tabanus* sp.
13. Describe pathogenicity of *Tabanus* sp.
14. describe role of *Tabanus* sp in disease transmission.
15. How *Tabanus* sp can be controlled?
16. Describe morphology of head louse.
17. Describe life cycle of head louse.
18. Describe pathogenicity of head louse.
19. How head louse infection can be diagnosed?

Suggested Readings:

1. The Insects by Chapman
2. Modern Entomology by D.B. Thembare
3. Economic Zoology by Shukla and Upadhyay
4. The Insects by Gullan and Carnston
5. Introduction to Economic Zoology by Sarkar, Kundu and Chaki.
6. A textbook of Economic Zoology by Aminul Islam.

Unit-VI

Parasitic Acarines: General remarks on ticks in relation to morphology, habitat, life-cycle and diseases caused by them

Objective: In this unit you will know about Parasitic acarines such as Ticks. Yu will learn about their habit, habitat, taxonomy, morphology

Introduction: Ticks are small arachnids, part of the order Parasitiformes. Along with mites, they constitute the subclass Acari. Ticks are ectoparasites, living by hematophagy on the blood of mammals, birds, and sometimes reptiles and amphibians. Ticks are vectors of a number of diseases that affect both humans and other animals.

Systematic position:

Kingdom: Animalia
Phylum: Arthropoda
Class: Arachnida
Subclass: Acari
Superorder:Parasitiformes
Order: Metastigmata=Ixodida
Superfamily: Ixodoidea
Families: a. Ixodidae – hard ticks
b. Argasidae – soft ticks
c. Nuttalliellidae

Taxonomy:

There are three families of ticks. The two big ones are the sister families of Ixodidae (hard ticks)and Argasidae (soft ticks). The third one is Nuttalliellidae, which comprises a single species, *Nuttalliellanamaqua*, and is the most basal lineage. Ticks are closely related to the numerous families of mites, within the subclass Acarina. The Ixodidae, which include over 700 species, are known as 'hard ticks' because they have a *scutum* or hard shield, which the Argasidae lack. This shield generally can resist the force of ahuman's soft-soled footwear, especially on soft ground; it requires a hard sole on a hard surface to crush the tick. However, stepping on an engorged tick, filled with blood, kills it easily, though messily. In nymphs and adults of the Ixodidae, a prominent *capitulum* (head) projects forwards from the body; in this they differ from the Argasidae. They differ too, in their life cycle; Ixodidae that attach to a host will bite painlessly and generally unnoticed, and they remain in place until they engorge and are ready to change their skin; this process may take days or weeks. Some species drop off the host to moult in a safe place, whereas others remain on the same host and only drop off once they are ready to lay their eggs. The Argasidae are known regionally as 'soft ticks' or 'tampans'. The family includes about 200 species, but the proper composition of the genus is under review. The genera accepted as of 2010are *Antricola*, *Argas*, *Nothoaspis*, *Ornithodoros* and *Otobius*. The features that most obviously distinguish the Argasidae from the Ixodidae are that they have no *scutum* and the *capitulum* is concealed beneath the body. The Argasidae also differ from the Ixodidae in their habits and ecology. Many of them feed primarily on birds, though some Ornithodoros, for example, feed on mammals and are extremely harmful. Both groups feed rapidly, typically biting painfully and gorging within minutes, and none of the species will stick to the host in the way that hard ticks do. Unlike the Ixodidae that have no fixed dwelling place except on the host, they live in sand orin crevices or similar shelters near animal dens or nests, or in human dwellings where they might come out nightly to attack roosting birds, or emerge only when they smell carbon dioxide in the breath of their hosts and emerge from the sand to attack them. Species common in NorthAmerica

primarily parasitise birds, and very rarely attack humans or other mammals. The family *Nuttalliellidae* contains only a single species, *Nuttalliellanamaqua*, a tick found in southern Africa from Tanzania to Namibia and South Africa. It can be distinguished from Ixodidae and Argasidae ticks by a combination of characteristics, including the position of the stigmata, lack of setae, strongly corrugated integument, and the form of the fenestrated plates.

Habit and habitat:

Tick species are widely distributed around the world, but they tend to flourish more in countries with warm, humid climates, because they require a certain amount of moisture in the air to undergo metamorphosis, and because low temperatures inhibit their development from egg to larva. Ticks of domestic animals are especially common and varied in tropical countries, where they cause considerable harm to livestock by transmission of many species of pathogens and also causing direct parasitic damage.

For an ecosystem to support ticks, it must satisfy two requirements: the population density of host species in the area must be high enough, and humidity must be high enough for ticks to remain hydrated. Due to their role in transmitting Lyme disease, ixodid ticks, particularly *I. scapularis*, have been studied using geographic information systems (GIS), to develop predictive models for ideal tick habitats. According to these studies, certain features of a given microclimate – such as sandy soil, hardwood trees, rivers, and the presence of deer – were determined to be good predictors of dense tick populations.

Morphology:

Ticks, like mites, have bodies which are divided into two primary sections: the anterior *capitulum* (or *gnathosoma*), which contains the head and mouthparts; and the posterior *idiosoma* which contains the legs, digestive tract, and reproductive organs.

Like all arachnids, adult ticks have eight legs. The legs of Ixodidae and Argasidae are similar in structure. Each leg is composed of six segments: the coxa, trochanter, femur, patella, tibia, and tarsus. Each of these segments is connected by muscles which allow for flexion and extension, but the coxae have limited lateral movement. When not being used for walking, the legs remain tightly folded against the body. Larval ticks hatch with six legs, acquiring the other two after a blood meal and moulting into the nymph stage. In addition to being used for locomotion, the tarsus of leg I contains a unique sensory organ, the Haller's organ, which can detect odours and chemicals emanating from the host, as well as sensing changes in temperature and air currents.

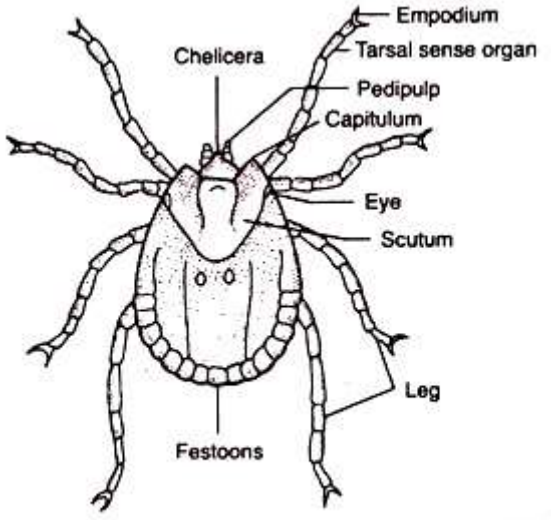


Fig. 15.11: Tick (Dorsal view)

The basic characters of soft and hard ticks are more or less same but there are many distinguishing features which are tabulated below:

Characteristics	Hard tick (Ixodidae)	Soft tick (Argasidae)
1. Body covering	1. Body is covered with hard cuticle but devoid of tubercles and granulations.	1. Body is covered with leathery cuticle which consists of tubercles, granulations or circular disc.
2. Capitulum	2. Terminal end can be seen in dorsal view.	2. Subterminal end cannot be seen in dorsal views.
3. Palpi	3. Rigid but not leg like.	3. Leg like with subequal segments.
4. Scutum	4. Well developed scutum covers the back of the male but only the anterior portion of the female.	4. Scutum is absent.
5. Silvery markings on scutum	5. Scutum is always with silvery markings, **ornates**.	5. Absence of ornates or silvery markings.
6. Festoon	6. Scutum is prolonged beyond the body in male and this portion is called **festoon**.	6. Festoon is absent in male.
7. Mouth parts	7. Mouth parts anterior in position and are directed forward.	7. Mouth parts ventral in position.
8. Camerostome	8. Camerostome is a groove in which mouth parts are lodged.	8. Absent.
9. Pedipalpi	9. Segments of pedipalpi are fixed.	9. Movable.

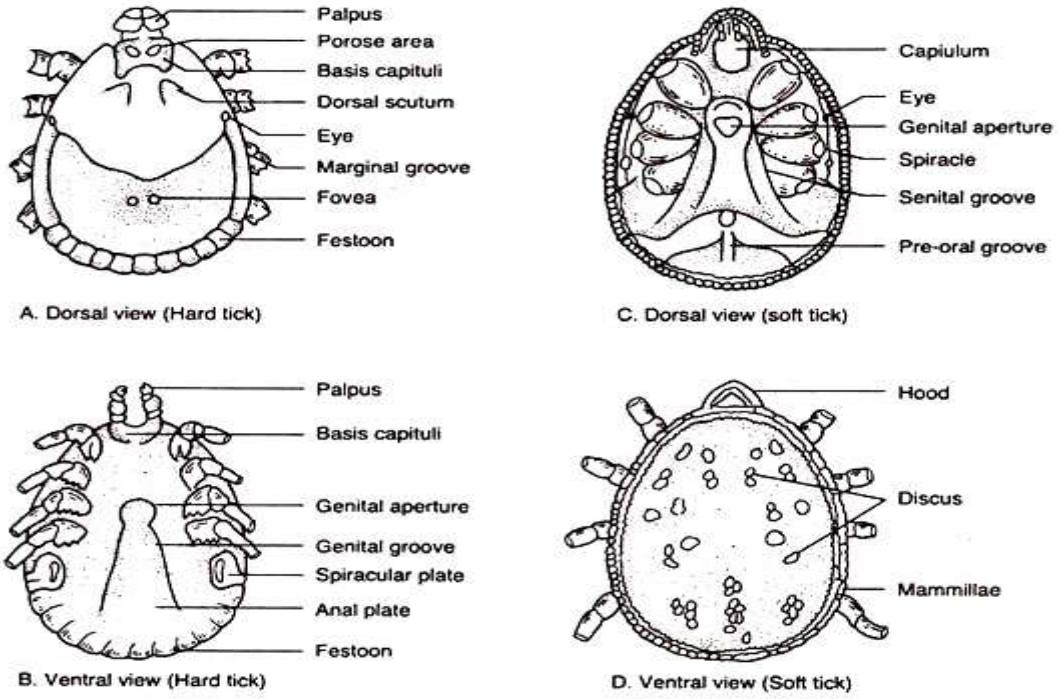


Fig. 15.12: Dorsal and ventral views of hard and soft ticks. A and B of ♂ ixodid (*Dermacentor*); C and D of argasid (*Ornithodoros*)

Life Cycle:

Both ixodid and argasid ticks undergo three primary stages of development: larval, nymphal, andadult.

a. Ixodidae

Ixodid ticks require three hosts, and their life cycle takes at least one year to complete. Up to 3,000 eggs are laid on the ground by an adult female tick. When larvae emerge, they feed primarily on small mammals and birds. After feeding, they detach from their host and moult to nymphs on the ground, which then feed on larger hosts and molt to adults. Female adults attach to larger hosts, feed, and lay eggs, while males feed very little and occupy larger hosts primarily for mating.

b. Argasidae :

Argasid ticks, unlike ixodid ticks, may go through several nymphal stages, requiring a meal of blood each time. Their life cycles range from months to years. The adult female argasid tick can lay a few hundred to over a thousand eggs over the course of her lifetime. Larvae feed very quickly and detach to molt to nymphs. Nymphs may go through as many as seven instars, each requiring a blood meal. Both male and female adults feed on blood, and they mate off the host. During feeding, any excess fluid is excreted by the coxal glands, a process which is unique to argasid ticks.

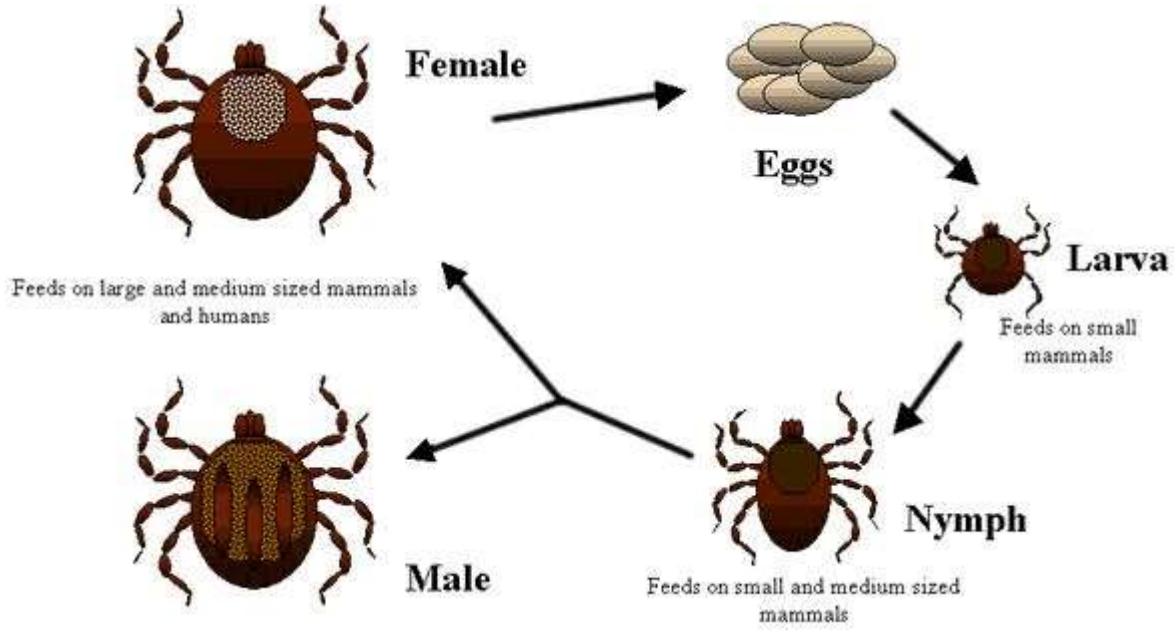


Figure: Life cycle of Tick

Role of Ticks as Transmitters of Disease:

Ticks play an important role as vectors of disease to domestic animals and to somewhat less extent to man.

They transmit six principal types of organisms of diseases, as:

(i) Spirochaetes of relapsing fever,

(ii) Rickettsias of spotted fever;

(iii) Babesiidae,

(iv) Pasteurella tularensis, the bacterium of tularemia,

(v) Anaplasma and

(vi) Filtrable virus and encephalomyelitis virus.

Many soft and hard ticks harbour and transmit a bacilli of the genus *Salmonella* which cause para-typhoid like disease in rodents. Species of *Ornithodoros* can also harbour and transmit Leptospiras. One of the most dreaded tick of this genus is *O. moubata* which is the vector for *Spirochaetaduttoni*, the pathogen that causes African relapsing fever. The infection is transmitted by the bite of both male and female ticks during all their active stages.

The attack of relapsing fever in human takes place from 5 to 10 days after the tick has bitten. *O. moubata* appears to be essentially a parasite of human being. It is a man- to tick-to man vector of relapsing fever. Once the *Spirochaetaduttoni*, the pathogens are ingested by the ticks, they penetrate the stomach wall to reach the body cavity where they multiply. Then the pathogens invade the salivary and coxal glands of the ticks. The pathogens are transmitted to the man by both the sexes of ticks. Infection starts by the injection into the host's skin of the Spirochaetes (pathogens) along with the saliva and coxal fluid of the ticks. Once infected, the ticks remain so, and the infection may be transmitted from generation to generation.

In addition to Rocky mountain fever, the tick *Dermacentorandersoni* also acts as the vector for several human diseases like Indian tick typhus, Colorado tick fever, Tick paralysis, Powassan encephalites etc. In Rocky mountain fever the infection is acquired from the reservoir animals by a feeding tick, *Dermacentorandersoni* in any stage of its life history and is passed on from stage to stage. For example the pathogen, *Rickettsia rickettsii* infected blood is ingested by the larva, the infection is transferred into nymph and then to the adult which in turn may infect. It has been reported that some infected adult females will pass the pathogen through their eggs to the larvae of the next generation. The pathogen of this Rocky mountain fever is transferred to the host through saliva of the tick while biting.

Control of Ticks:
In general ticks can be controlled by the following methods:

A. General control: It is of three kinds

1. Insecticidal control:
The ticks and mites can be destroyed by dusting or spraying of insecticides like malathion, lindane, chlorodane at rates of 0.5-1 kg per acre.

2. Environmental protection:
Before planning a control programme a thorough knowledge of the habit and habitats of ticks and mites must be gained. Animal hosts should be destroyed. The cracks and crevices in the field particularly near buildings should be reduced by filling.

3. Protection of workers:
Workers should be encouraged to wear protective clothing impregnated with an insect repellent, like benzyl benzoate, indalone or diethyltoluamide etc.

B. Specific control:

1. Chigger mite infestations in gardens, lawns and general premises can be utterly destroyed by applying suitable chemicals (chlorodane, toxaphen or lindane) either by a spraying method or by a dusting process to the infested areas.

2. Reproduction of rodents should be avoided.

3. Old tree snags in vicinity which may harbour nesting squirrels and chipmunks should not be encouraged.

C. Personal control:

1. To avoid the infected person and making arrangement for proper treatment.

2. Avoid sleeping in the same bed with the infected person.

3. Use Dettol soap during bathing.

Suggested Readings:

1. The Insects by Chapman
2. Modern Entomology by D.B. Thembare
3. Economic Zoology by Shukla and Upadhyay
4. The Insects by Gullan and Carnston
5. Introduction to Economic Zoology by Sarkar, Kundu and Chaki.
6. A textbook of Economic Zoology by Aminul Islam

Probable Questions:

1. Describe the life cycle of ticks.
2. How the tick population is controlled?
3. How the diseases are transmitted by ticks?
4. What are the differences between Hard tick and Soft tick?
5. Describe the morphology of ticks.
6. Write down about habit and habitat of Ticks.

HARD CORE THEORY PAPER (ZHT- 309)

Arthropod of Economic Importance

& Biodiversity and Resource management

Group B: Biodiversity and Resource management

Module	Unit	Content	Credit	Class	Time (h)	Page No.
ZHT - 309 (Arthropod of Economic Importance & Biodiversity and Resource management)	VII	Meanings of Biodiversity: Levels of species diversity and relationship; geographic distribution of biological diversity; biological hotspots.	2.0	1	1	
	VIII	Measuring biodiversity; interrelationship between diversity measures; pattern of local and regional biodiversity.		1	1	
	IX	Threats to species diversity: natural and human induced threats and vulnerability of species extinction; Red data book; Rarity, endemism, effective and minimum viable population, fragmentation of population and metapopulation		1	1	
	X	Problems of genetic diversity; bottleneck; genetic drifts; inbreeding depression.		1	1	
	XI	Biodiversity Resource Management: values and uses of biological diversity, invertebrate diversity as bioindicator; putting a price on biological diversity; pollinating insect diversity and their management and utilization in sustainable agriculture.		1	1	
	XII	Vermiculture: Types of earthworms and their utilization; use in sustainable agriculture.		1	1	

Unit-VII

Meanings of Biodiversity: Levels of species diversity and relationship; geographic distribution of biological diversity; biological hotspots

Objective: In this unit you will learn about different levels of species diversity and relationship. You will also learn about geographic distribution of biological diversity and also about biological hotspots.

Concept of Biodiversity:
It has been estimated that more than 50 million species of plants, animals and micro-organisms are existing in the world. Out of these, about 1.4 million species have been identified so far. Each species is adapted to live in specific environment, from mountain peaks to the depth of seas, from polar ice caps to tropical rain forests and deserts. All this diversity of life is confined to only about one kilometer thick layer of lithosphere hydrosphere and atmosphere which form biosphere.

Though the study of environment and ecology is quite old, the term biodiversity has been introduced by Walter Rosen in 1986. Biological diversity or Biodiversity is defined as the variety and variability among the living organisms and the ecological complexes in which they occur.

It refers to the variability's among species of plants, animals and microorganisms; ecosystems; ecosystem including terrestrial, aerial, marine and other aquatic system and ecological complexes of which they are part. In simpler terms, biodiversity is the assemblage of different life forms (Fig. 19.1).

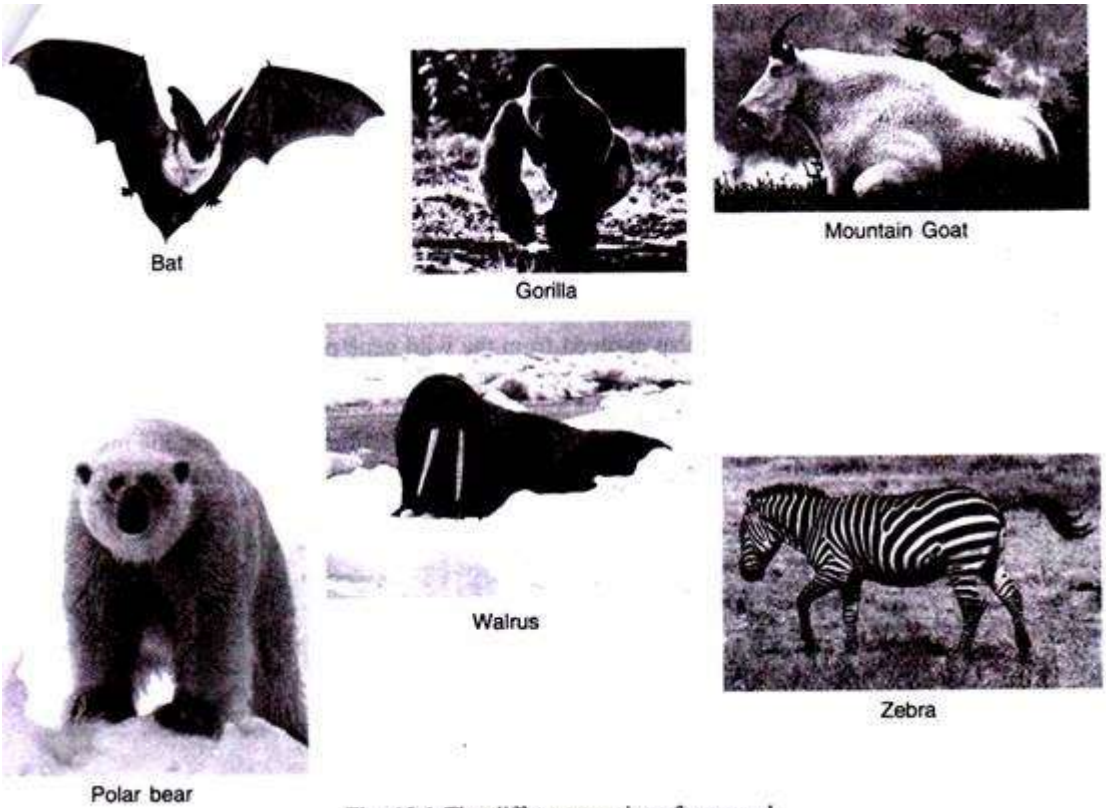


Fig. 19.1 The different species of mammals

If reflects the number of different organisms and their relative frequencies in an ecological system. It includes the organisation of organisms at many levels ranging from complete ecosystems to the

chemical components that form the molecular basis of heredity. Thus, biodiversity is sum of all the genes, varieties, species, populations in different ecosystems and their relative abundance.

Scientists are aware of the immense potentials of various life-forms existing on the earth. Our planet's requirements and services depend mainly on the biological resources. Biological resources not only provide us nourishment, clothing, housing, fuel and medicine but also meet our several other requirements. Therefore the knowledge of biodiversity is of immense utility in planning sustainable livelihood and conserving the natural resources.

Types of Biodiversity:

Biodiversity is of three types:
1. Species diversity

2. Genetic diversity

3. Ecological diversity

1. Species Diversity:
According to Biological Species Concepts (BSC), species is a basic unit of classification and is defined as a group of similar organisms that interbreed with one another and produce offspring's and share a common lineage. Species diversity refers to biodiversity at the most basic level and is the 'variety and abundance of different types of individuals of a species in a given area'. It includes all the species on Earth, ranging from plants such as bacteria, viruses, fungi, algae, bryophytes, pteridophytes, gymnosperms, angiosperms and all the species of animals including unicellular protozoans to mammals.

Certain regions support a more diverse populations than others. Regions that are rich in nutrients and have well balanced climatic factors, such as moderate temperature, proper light and adequate rainfall, show high degree of diversity in their life forms. The tropical areas support more diverse plant and animal communities than the desert and polar areas, as for examples, tropical forest has a higher species diversity as compared to a timber plantation. The regions that are rich in species diversity are called hotspots of biodiversity.

(i) Species Diversity:
Evolution of species diversity has probably been possible because of habitat diversity on earth. It refers to the variety of species within a region. This diversity could be measured on the basis of number of species in a region. The term biodiversity is commonly used as a synonym of species diversity.

It actually refers to species richness, in terms of number of species in a site or habitat. Global diversity is typically represented in terms of total number of species of different taxonomic groups. As mentioned before, an estimated 1.4 million species have been identified to date. Species diversity, again, is studied at three levels: alpha diversity (number of species coexisting at a site), beta diversity (difference in species complement between patches) and gamma diversity (number of species in a large area, e.g. a country).
This series can further be extended to delta diversity for biomes (biomes are climatically and geographically defined areas of ecologically similar climatic conditions such as communities of plants, animals and soil organisms and are often referred to as ecosystems) and omega diversity for the entire biosphere.

Some authors call it taxon diversity (variety of taxa within a community of an area). It is generally studied at the species level and hence called species diversity. When the taxonomic levels such as genus and family are considered, the term taxon diversity is more appropriate. This term is similar to taxic diversity.

2. Genetic Diversity:

'Genetic diversity pertains to the range of diversity in the genetic resources of the organisms'. Every individual member of a plant or animal species differs from other individuals in its genetic constitution. Each individual has specific characters, which is due to the genetic makeup or code. The genes present in the organisms can form infinite number of combinations that causes genetic variability.

Thus, we find that each human, who is representative of the same species, i.e. Homo sapiens, is distinct from another. Similarly, there are many varieties within the same species such as rice, wheat, apples, mangoes, etc. that differ from one another in shape, size, colour of flowers and taste of fruits and seeds due to the variations at the genetic level.

The term 'gene pool' has been used to indicate the genetic diversity in the different species (Fig. 19.2). This also includes the diversity in the wild species, which through intermixing in nature over millions of years have given rise to newer varieties. The domesticated varieties of agricultural crops and animals have also evolved from the wild gene pool.

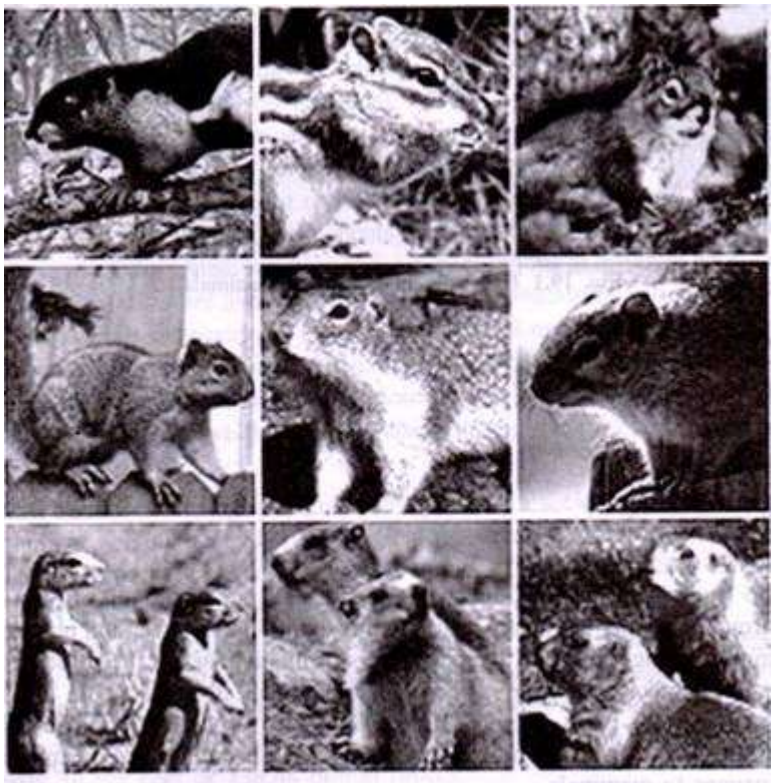


Fig. 19.2 Genetic diversity in squirrels

The genetic variability is essential for healthy breeding population, the reduction in genetic variability among breeding individuals leads to inbreeding which in turns can lead to extinction of species. In the recent decades, a new science named 'biotechnology' has emerged. It manipulates the genetic materials of different species through various genetic re-combinations to evolve better varieties of crops and domestic animals.

Within a species there are a number of subspecies, varieties (subspecies and varieties are recognizable morphological variations within a species), forms (form is generally used to recognize and describe sporadic variations in a single morphological feature) or strains which slightly differ from each other.

These differences are due to slight variations in their genetic organization. This diversity in the genetic make-up of a species is referred to as genetic diversity. A species with a large number of varieties or strains is considered to be rich and diverse in its genetic organization. Genetic variations arise in individuals of a species by genie or chromosomal mutations. Genetic variation within populations is considered a "prerequisite for adaptation and evolutionary change", and as such an important aspect of biodiversity.

Genetic variation is often expressed in terms of alleles (genes occupying the same locus in a chromosome) and is mainly studied at the population level. Genetic variations can be measured by different recent techniques such as allozyme analysis, DNA fingerprinting, polymerase chain reaction, restriction site mapping and DNA sequencing.

Diversities go on increasing at the micro level. Differences in the level of varieties are followed by differences among the subspecies, varieties and species. Accumulation of these differences at infra-specific level will automatically lead to distinctive character at the species level.

3. Ecological/Ecosystem Diversity:

Each ecosystem consists of organisms from many different species, living together in a region connected by the flow of energy and nutrients. The Sun is the ultimate source of energy for all the ecosystems. The Sun's radiant energy is converted to chemical energy by plants. This energy flows through the different systems when animals eat the plants and then are eaten, in turn, by other animals. Fungi and bacteria derive energy from the decomposing dead organisms, releasing nutrients back into the soil as they do so.

An ecosystem, therefore, is a collection of living components, like microbes, plants, animals, fungi, etc. and non-living components, like climate, matter and energy that are connected by energy flow. Ecological diversity refers to the 'variability among the species of plants and animals living together and connected by flow of energy and cycling of nutrients in different ecosystems or ecological complexes'. It also includes variability within the same species and variability among the different species of plants, animals and microorganisms of an ecosystem. Thus, it pertains to the richness of flora, fauna and microorganisms with in an ecosystem or biotic community.

The richness of the biosphere in terms of varied life forms is due to the variations in the ecosystems. The earth has a number of ecosystems like grasslands, forests, semi arid deserts, marine, freshwater, wetland, swamp, marshlands (Fig. 19.3) etc. each one having its distinct floral, faunal and microbial assemblages. Ecological diversity represents an intricate network of different species present in local ecosystems and the dynamic interaction among them. The ecological diversity is of great significance that has developed and evolved over millions of years through interactions among the various species within an ecosystem.

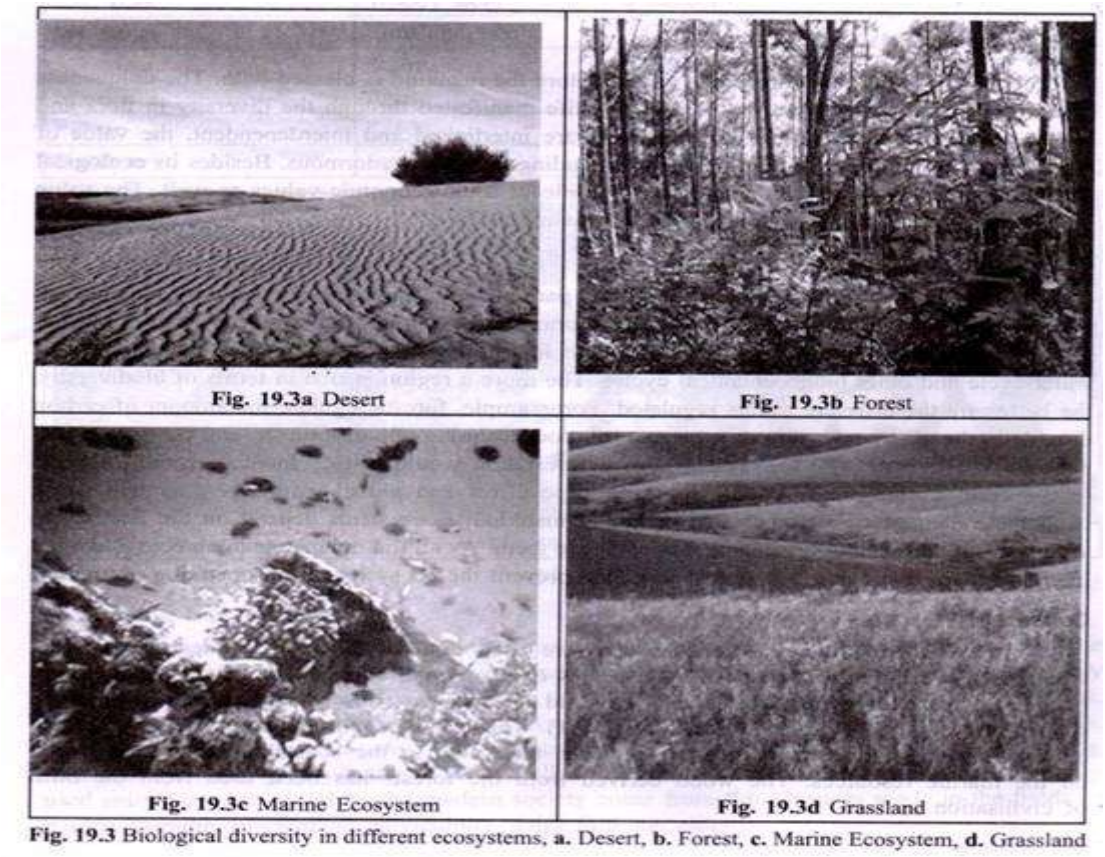


Fig. 19.3a Desert

Fig. 19.3b Forest

Fig. 19.3c Marine Ecosystem

Fig. 19.3d Grassland

Fig. 19.3 Biological diversity in different ecosystems, **a.** Desert, **b.** Forest, **c.** Marine Ecosystem, **d.** Grassland

In ecosystem, there may exist different land-forms, each of which supports different and specific vegetation. Ecosystem diversity in contrast to genetic and species diversity is difficult to measure since the boundaries of the communities which constitute the various sub-ecosystems are not distinct. Ecosystem diversity could best be understood if one studies the communities in various ecological niches within the given ecosystem, each community is associated with definite complexes.

These complexes are related to composition and structure of biodiversity. Loss of ecosystem diversity may be considered as ultimate cause of loss of species and genetic diversity. Community diversity is a synonym of ecosystem diversity and is defined as the diversity of community types within larger areas (ecological, units). It should not be confused with habitat variety which is an expression mainly used for different species of animals which have different habitats.

It has been observed and reported that there is no direct effect of the number of species on ecosystem processes. The effect on the ecosystem arises from functional differences between species. It is suggested that ecosystem with lots of functional traits will operate more efficiency in terms of productivity, resilience and resistance to invaders.

Consequently, it was suggested (Hooper, 1998) that functional diversity should be measured in a species pool that summarizes the extent of functional differences. In simple form, functional diversity is the range of functions that are performed by organisms in a system.

The species within a habitat or community can be divided into different functional types such as feeding guilds or plant growth forms or into functionally similar taxa such as suspension feeders or deposit feeders. Functionally similar species may be from quite different taxonomic entities. A common measure of functional diversity is the number of functional groups represented by species in a community.

To cluster species into functional groups, first a set of characters significant for ecosystem functioning is measured for each species obtaining a trait matrix. The trait matrix is then converted into a distance matrix. Finally, the distance matrix is clustered with standard multivariate methods to divide species among functional groups.

Biomes of the World :

There are manly 8 biomes in the world which are described below:

Biome # 1. Tundra:

The literal meaning of word Tundra is north of the timberline. The tundra extends above 60°N latitude. It is almost treeless plain in the far northern parts of Asia, Europe and North America. A tundra consists of plains characterised by snow, ice and frozen soil most of the year. The permanent frozen soil of tundra is called permafrost.

Winters are very long on the tundra with little daylight. In contrast summers are short but there are many daylight hours. Precipitation is low, amounting to only 25 cm or less per year, because cold air can hold relatively little moisture.

The ground is soggy in the summer because moisture cannot soak into the permanently frozen ground. Ponds, small lakes and marshes are abundant due to the nearly flat terrain.

There are no upright trees on the tundra. Only trees such as dwarf willows and birches, which grow low to the ground, can escape the drying effect of the wind which upright trees would experience. This biome consists mainly of mosses, grasses, sedges, lichens and some shrubs. Seasonal thawing of the frozen soil occurs only up-to a few centimetres depth, which permits the growth of shallow rooted plants.

Fig. 12.20. Tundra biome.

Carbon, arctic hare and musk ox are important herbivores of tundra biome. Some important carnivores that prey on the herbivores are the arctic fox, arctic wolf, bobcat and snowy owl. Polar bears live along coastal areas, and prey on seals.

Because of the severe winters, many of the animals are migratory and move from one region to another with the change in seasons. Many shorebirds and water fowls, such as ducks and geese, nest on the tundra during the summer but migrate south for the winter. The tundra make a very delicate ecosystem, and may be recovered from any disturbance very slowly.

Biome # 2. Northern Conifer Forest:

The northern coniferous forest or taiga is a 1300-1450 km wide band south of the tundra. This extends as an east-west band across North America, Europe and Asia. This area also has long, cold winters, but summer temperatures may reach 10-12°C, and the summer and the growing season are longer than in the tundra. Precipitation is higher than in the tundra, ranging from 10 to 35 cm annually.

The moisture is the combined result of summer rains and winter snows. Lakes, ponds and bogs are abundant. The duration of growing period of plants is only about 150 days. Since five physical conditions are variable, the organisms are resistant to fluctuations of temperature.

The taiga makes really a northern forest of coniferous trees such as spruce, fir, pine, cedar and hemlock. In disturbed areas, deciduous trees such as birch, willow and poplar are abundant. In certain areas the trees are so dense that little light may reach the floor of the forest. Vines, maple and spring wild flowers are common. Mosses and ferns also grow in moist areas.

The common smaller mammals are herbivores, such as squirrels, snowshoe hare, and predatory martens. Important migratory herbivores include moose, elk, deer and carbon. Moose and carbon migrate to the taiga for winters and to the tundra for summers.

Important predators are the timber wolf, grizzly bear, black bear, bobcat and wolverine. Many insects are found during the warmer months. Migratory shore birds and waterfowls are abundant during summer months.

Fig. 18.22. Temperate deciduous forest biome.

Biome # 3. Temperate Deciduous Forests:

The deciduous forests are found in the temperate regions of north central Europe, east Asia and the eastern United States, that is, south of the taiga in the Northern Hemisphere. Such forests occur in regions having hot summers, cold winter, rich soil and abundant rain. Annual rainfall is typically around 100 cm per year.

Common deciduous trees are the hardwoods such as beech, maple, oak, hickory and walnut. They are broad-leaved trees. The trees shed their leaves in the late fall so the biome has an entirely different appearance in the winter than in the summer.

The fallen leaves provide food for a large variety of consumer and decomposer populations, such as millipedes, snails and fungi living in or on the soil. The temperate deciduous forest produces flowers, fruits and seeds of many types which provide a variety of food for animals.

The common herbivores of this biome are deer, chipmunks, squirrels, rabbits and beavers. Tree-dwelling birds are abundant in number and diversity. Important predators are—black bears, bobcats, and foxes. Predatory birds are also found, such as hawks, owls and eagles. The coldblooded or ectothermic animals, such as snakes, lizards, frogs, and salamanders are also common.

The temperate deciduous forest makes a very complex biome. Many changes take place during the year, and a large variety of species inhabit the soil, trees and air.

Fig. 12.23. Stratification in a tropical rain forest strata : 1. Tallest trees; II. dense canopy of trees ; III. short trees ; IV. shrubs ; V. herbs and decaying.

Biome # 4. Tropical Rain Forest:

This biome is situated in the equatorial regions having the annual rainfall more than 140 cm. However, the tropical rain forest makes an important biome across the earth as a whole. This biome is found in Central America, the Amazon Basin, Orinocon Basin of South America, Central Africa, India and Southeast Asia.

Tropical rain forests have high rainfall, high temperature all year, and a great variety of vegetation. Plant life is highly diverse reaching up-to a framework of 200 species of trees per hectare. The warm, humid climate supports broad- leaved evergreen plants showing peculiar stratification into an upper storey and two or three understoreys.

The tallest trees make an open canopy, but the understoreyed plants block most of the light from the jungle floor. The climbers and lianas reach the highest level of the trees in search of light.

An enormous variety of animals lives in the rain forest, such as insects, lizards, snakes, monkeys and colorful birds. The ant eaters, bats, large carnivorous animals, and a variety of fish in the rivers are quite common. About 70-80 per cent of the known insects are found in tropical rain forests. Such rich animal diversity is linked to plant-animal interaction for pollination and dispersal of fruits and seeds.

Biome # 5. Chapparal:

This biome is also known as mediterranian scrub forest. This is marked by limited winter rain followed by drought in the rest of the year. The temperature is moderate under the influence of cool, moist air of the oceans. The biome extends along the mediterranian.

Pacific coast of North America, Chile, South Africa and South Australia. This biome has broad-leaved evergreen vegetation. The vegetation is generally made up of fire resistant resinous plants and drought-adapted animals. Bush fires are very common in this biome.

Biome # 6. Tropical Savannah:

The savannahs are warm climate plants characterized by coarse grass and scattered trees on the margins of tropics having seasonal rainfall. Primarily they are situated in South America, Africa and Australia. However, there is no savannah vegetation in India. The average total rainfall in such regions is 100 to 150 cm. There is alternation of wet and dry seasons.

Plants and animals are drought tolerant and do not have much diversity. The animal life of tropical savannah biome consists of hoofed herbivorous species, such as giraffe, zebra, elephant, rhinoceros and several kinds of antelope. Kangaroos are found in the savannahs of Australia.

Biome # 7. Grassland:

Some grasslands occur in temperate areas of the earth and some occur in tropical regions. Temperate grasslands usually possess deep, rich soil. They have hot summers cold winters and irregular rainfall. Often they are characterized by high winds. The main grasslands include the prairies of Canada and U.S.A., the pampas of South America, the steppes of Europe and Asia, and the veldts of Africa.

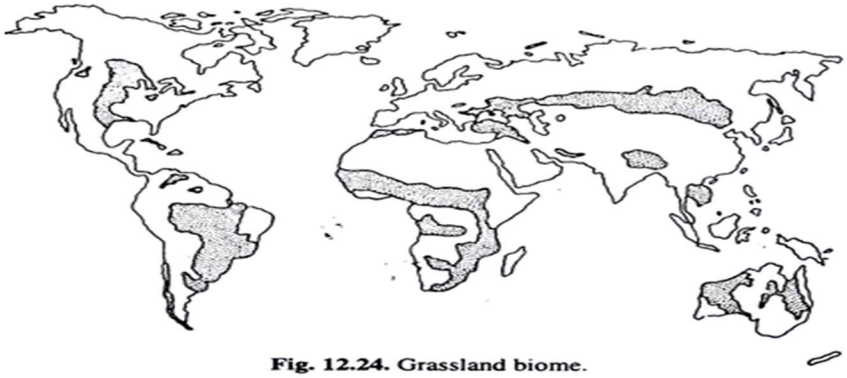


Fig. 12.24. Grassland biome.

The dominant plant species comprise short and tall grasses. In tall-grasses prairies in the United States, important grasses are tall bluestem, Indian grass and slough grass. Short-grass prairies generally have blue grama grass, mesquite grass and bluegrass. Many grasses have long, well-developed root systems which enable them to survive limited rainfall and the effects of fire.

The main animals of this biome are-the prong-horned antelopes, bison, wild horse, jack rabbit, ground squirrel and prairie dogs. Larks, the burrowing owl and badgers are also found. Important grassland predators include coyotes, foxes, hawks and snakes.

Biome # 8. Desert:

The desert biome is characterised by its very low rainfall, which is usually 25 cm per year or less. Most of this limited moisture comes as short, hard showers. Primarily the deserts of the world are located in the south-west U.S.A., Mexico, Chile, Peru, North Africa (Sahara desert), Asia (Tibet Gobi Thar) and central Western Australia. Deserts generally have hot days and cold nights, and they often have high winds.

The reason for the difference of temperature between day and night is due to the lack of water vapour in the air. Deserts are characterised by scanty flora and fauna. Desert organisms must meet some initial requirements if they are to survive. The plants must be able to obtain and conserve water.

In order to meet these requirements, many adaptations have been made by desert plants. Such adaptations are—reduced leaf surface area, which reduces evaporation from the plants, loss of leaves during long dry spell; small hairs on the leaf surfaces, and the ability to store large amount of water. The examples of important desert plants are—yuccas, acacias, euphorbias, cacti, many other succulents and hardy grasses. Many of the small plants are annuals.

Fig. 12.25. Desert biome.

Animals also must meet the requirements of heat, cold and limited water. Many desert animals are nocturnal in habit, and are active mainly at night. Many reptiles and small mammals burrow to get away from the intense heat of midday. The other common desert animals are the herbivorous kangaroo, rat, ground squirrel, and jack rabbit.

The important predators are—coyotes, badgers, kit fox, eagles, hawks, falcons and owls. Ants, locusts, wasps, scorpions, spiders, insect-eating birds, such as swifts and swallows, seed-eating quails, doves and various cats are other common desert animals.

Biological Hotspots:

Hotspots are the areas that are biologically rich and exhibit high species diversity most of which are endemic in nature. Endemic species are those that are restricted to a specific area. Hotspots are characterised by richness in floral and faunal wealth.Though the area covered by the hotspots represent only two per cent of the world's land area, it preserves about 50% of the total terrestrial biodiversity.

This term was introduced by Myers in 1988, who has presently recognized twenty-five such hotspots of biodiversity on a global level. These hotspots include 49955 endemic species which account for about 20% of world's total plant species, Mittermeir and Werner (1990) on the basis of species richness put forth the concept of mega diversity centres. The recognized 12 countries/regions are mega diversity centres. These are Mexico, Columbia, Peru, Ecuador, Brazil, Zaire, Madagascar China, India, Malaysia, Indonesia and Australia.

List of Global Hotspots of Biodiversity:

1. Tropical Andes

2. Madagascar

3. Brazil's Atlantic forest region

4. The Philippines

5. Meso-American forests

6. South Africa's Cape Floristic region

7. Wallacea (Western Indonesia)

8. Western Sunda (Indonesia, Malaysia and Brunei)

9. Brazil's Cerrado

10. Polynesia and Micronesian island complex

11. The Darien and Choco of Panama, Colombia, Western Ecuador

12. The Eastern Mediterranean region

13. The Western Ghats of India and the Island of Sri Lanka

14. Indo-Burma Eastern Himalayas

15. The Guinean forest of West Africa

16. New Caledonia

17. South-eastern Australia and Tasmania

18. Caribbean

19. Central Chile

20. California Floristic Province

21 . Eastern Arc and Coastal forest of Tanzania / Kenya

22. Succulent Karoo

23. Caucasus

24. South Central China

25. New Zealand.

Hotspots in India:

Of the twenty-five hotspots of biodiversity, recognized in the world, two are found in India, which extend into the neighbouring countries:
(i) The Indo-Burma region covering the Eastern Himalayas and (ii) the Western Ghats – Sri Lankan region.

The hotspots are rich in floral wealth, reptiles, amphibians, mammals and also in their endemism.

The botanical hotspots of India include:

(1) Western Ghats,

(2) North-East India,

(3) Himalayas and

(4) Andaman and Nicobar Islands

Eastern Himalayas:

As compared to the Western Himalayas that are colder and drier, the Eastern Himalayan ranges are much wetter with suitable climatic conditions. This supports endemism and biodiversity. The Eastern Himalayas comprise of .parts of Nepal, Bhutan, Sikkim, Arunanchal Pradesh and extends up to Burma. The forest vegetation ranges from tropical rain forests to temperate alpine forests. Rhododendron is the dominant tree of this region.

The topography in the Eastern Himalayan region is quite varied that helps biodiversity and endemism. The rugged mountains and valleys support the virgin forests which are rich in many endemic plant species. In the Indian part of the Eastern Himalayan hotspot, about 5800 plant species are found of which around 2000 are endemic. Sikkim is one of the most blessed Indian states so far as endemism is concede; as of the 4250 plant species found there, 60% are endemic to the region.

Palaeobotanists consider the Indo-Burma region to be one of the centres of origin for the flowering plants (angiosperms). Many primitive angiosperm families occurring there, such as Magnoliaceae, Winteraceae, etc. support this contention. The Eastern Himalayas are home to around 8000 species of angiosperms of which nearly 40% are endemic. The characteristic floras are Rhododendron, Alnus, Betula, Magnolia, etc.

The animals include members of the goat family, antelopes, musk deer, snow leopard, brown bear, black bear and the red panda. Due to continuous habitat destruction and hunting, many of these species are highly endangered. Andaman and Nicobar Islands are rich in littoral and Island type floras. About 2500 species of flowering plants have been recorded from there, of which 250 species are endemic. The important hotspots of Andaman & Nicobar Islands are North Andaman, spike Island, Table Island. South reef Island, Little and Great Nicobars.

Western Himalayas are well-known for Alpine flora which abounds in Gymnosperms. Some 5000 species of flowering plants are known to occur in this region of which 800 species are endemic. The valley of flowers, Pithoragarh, Gori Valley, Mandal Chopta Valley, Karakoram and Laddakh are some of the major hotspots of this region.

Western Ghats:

The Western Ghats run parallel to the west coast of India and constitute more than 16,000 km strip of forests in the states of Maharashtra, Goa, Karnataka, Tamil Nadu and Kerala. Locally they are also known as the Sahyadris. The Western Ghats are characterised by low hills; however, it achieves the highest elevation of 2675 m at Annamalai up to an elevation of 500 m the forests are evergreen in nature, while the forests occurring between 500-1500 m altitude are of semi-evergreen type.

The Western Ghats by virtue of having a humid tropical climate and geological stability supports one of the most biodiversity rich areas of the country. According to an estimate, of the 17,000 flowering plants species reported from India, more than 4,500 occur in the Western Ghats region. The Botanical Survey of India has listed 518 endangered species endemic to Peninsular India, the majority of which occur in the Western Ghats.

The dominating plant families are Acanthaceae, Graminae, Orchidaceae, Rubiaceae, Labiatae, Compositeae and Leguminoseae. More than 200 species of rare orchids, many of them are endemic, are found in Western Ghats. Many economically important plants such as banana, rice, black pepper, ginger, etc. have spread to other parts of the country from here.

The Western Ghats are home to a rich variety of fauna which are unique and many are endemic to this region. Nilgiri Langur, Lion-tailed Macaque, tiger, leopard, elephant, rare species of tortoise and other amphibians represent the faunal wealth. Many varieties of birds and fishes are also found here.

Sacred Forests:

These real hotspots of biodiversity are protected and worshiped by tribals due to religious sanctity. These are known as Devaskadu in Karnataka Devarahati in Maharashtra and Lakyntok in Meghalaya. These forests contain many rare endangered and endemic species.

Mangroves:

Mangroves are salt tolerant forest ecosystems found in Saline habitat, tropical and subtropical intertidal zones near estuaries. The total area under mangrove vegetation in India is estimated to be 6740 km^ and of this 4200 km^2 is covered by mangrove forest of sundarban alone and the second being in the Andaman & Nicobar islands.

Table 19.3 The major hotspots of endemic and genetic diversity of India

S.No.	Hotspots	Bio-geographic Zone
1.	Karakoram and Ladakh	Trans Himalaya
2.	Kumaon and Garhwal Himalaya	Western Himalayas
3.	Siwaliks	Himalaya
4.	Sikkim Himalaya	Eastern Himalaya
5.	Arunanchal Pradesh	Eastern Himalaya
6.	Lushai Hills	North-eastern India
7.	Tura-Khasi Hills	Meghalaya
8.	Aravallis	Semi-arid zone
9.	Bundelkhand	Central India Plateau
10.	Chota-Nagpur Plateau	Deccan Plateau
11.	Panchmarhi - Satpura ranges	Deccan Plateau
12.	Simlipal and Jeypore Hills of Orissa	Deccan (Eastern Ghats)
13.	Bastar and Koraput Hills	Deccan Plateau
14.	Vishakhapatnam Hills and Araku Valley	Eastern Ghats
15.	Tirupati - Cuddappa Hills	Eastern Ghats
16.	Marathwada Hills	Deccan Plateau
17.	Saurashtra Kutch	Deccan Plateau
18.	Mahabaleshwar - Khandala ranges	Western Ghats
19.	Agumbe - Phonda ranges	Western Ghats
20.	Ratnagiri and Kolaba ranges	Western Ghats
21.	Nilgiris	Western Ghats
22.	Silent valley and Wynaad	Western Ghats
23.	Annamalai	Western Ghats
24.	Idduki - Sabarigiri	Western Ghats
25.	Kalakad and Agastyamalai Hills	Western Ghats
26.	Andaman and Nicobar	Islands

There are two main centres of diversity, the Agastyamalai hills and Silent valley:

1. About 62 per cent of known amphibian species are endemic with the majority occurring in the Western Ghats. Nearly 50 per cent of lizards of India are endemic with high degree of endemism in the Western Ghats.

2. There are currently seven national parks in Western Ghats with a total area of 2,073 sq km and 39 wildlife sanctuaries covering an area of about 13,862 sq km.

3. The status of management of wildlife sanctuaries in this part of India varies enormously. Nilgiri wildlife sanctuary, for example, has no human inhabitant, while the Paramikulam wildlife sanctuary in Kerala includes considerable area of commercial plantation with heavy resources exploitations (Joshi and Joshi, 2004).

India has a large network of protected areas that gives priority to conserve and protect the remaining biodiversity centres (natural forests). Altogether, there are 14 biosphere reserves, out of them three are in the world network of biosphere reserve—Sunderban, Gulf of Mannar and Nilgiris. Also, there are about 100 national parks and about 500 wildlife sanctuaries.

The forests in India range from tropical evergreen forests in Andaman and Nicobar Islands, Western Ghats and north-eastern states to dry alpine scrub in high Himalaya. Between the two extremes, the country has semi-evergreen rain forests, deciduous monsoon forests, thorn forests, subtropical pine forests in lower mountain zone and temperate mountain forests. Western and Central India is characterized by dry and thorn forests (Table 1.4).

Table 1.4
General Distribution of Forest Types in India

Forests Type	*Area of Occurrences*
Tropical Wet Evergreen Forests	North-east and South India, Andaman and Nicobar Islands
Tropical Semi-Evergreen Forests	South and East India
Tropical Moist Deciduous Forests	Central and East India
Tropical Dry Deciduous Forests	West and Central India
Tropical Thorn Forests	West and Central India
Tropical Dry Evergreen Forests	Central and South India
Subtropical Broad-Leaved Forests	South India
Subtropical Pine Forests	Sub-Himalayan Regions
Subtropical Dry Evergreen Forests	North-east and South India
Montane Wet Temperate Forests	Himalaya and Nilgiris
Himalayan Moist Temperate Forests	Temperate Areas of Himalaya
Sub-Alpine Forests	Himalaya
Himalayan Dry Forest and Alpine Scrubs	Himalaya
Littoral and Swap Forests	Along the Coasts

North-eastern states, viz., Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland, Sikkim and Tripura, parts of states to the western side of Western Ghats, coastal plains and islands, etc., have very rich forests and biodiversity. Each of these states has more than 70 per cent area under forest cover.

These regions are mainly having tropical evergreen forests. Some tribal and hill states, viz., Orissa, Chhattisgarh, Uttarakhand and Himachal Pradesh have also a large area under forest cover. On the other end, Punjab, Haryana, Uttar Pradesh, Bihar and Rajasthan, each having less than 10 per cent of their geographical area under forest cover, are very poor in terms of forest cover.

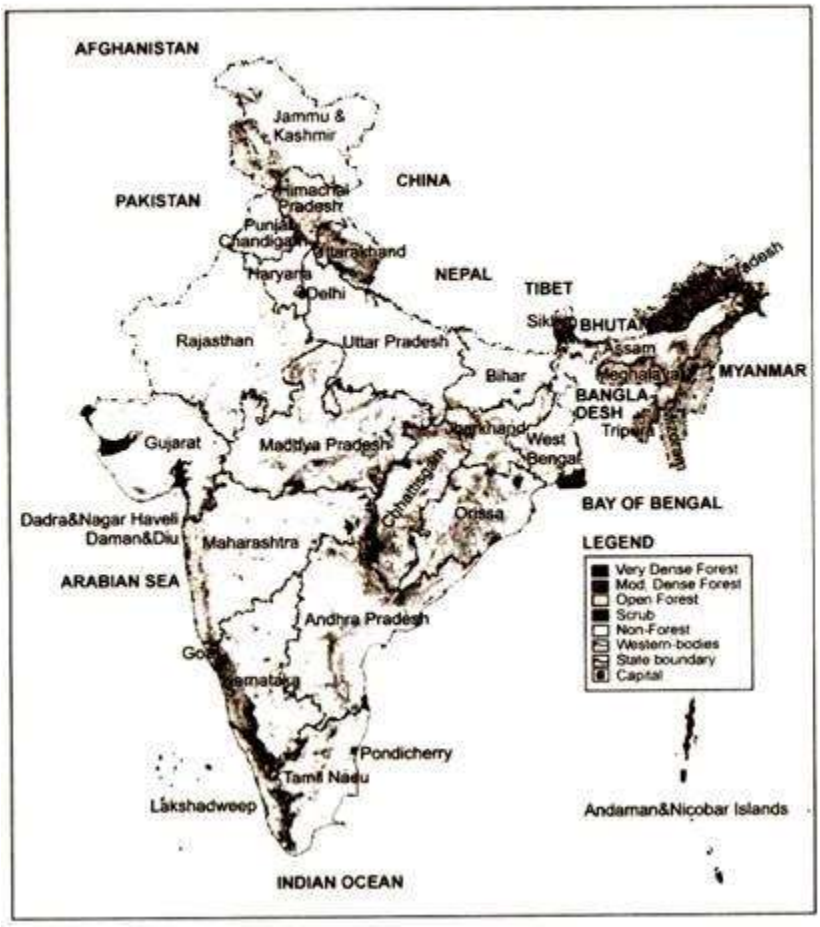
Table 1.5
Spatial Distribution of Forests in India, 2005

States/UTs	Forest Cover (sq km)	Percentage	Tree Cover (sq km)	Percentage	Forest and Tree Cover (%)
Andhra Pradesh	44,372	16.13	7,640	2.78	18.91
Arunachal Pradesh	67,777	80.93	446	0.53	81.46
Assam	27,645	35.24	1,484	1.89	37.13
Bihar	5,579	5.92	2,522	2.68	8.60
Chattisgarh	55,863	41.32	4,492	3.32	44.64
Delhi	176	11.87	107	7.20	19.07
Goa	2,164	58.45	268	7.24	65.69
Gujarat	14,715	7.51	7,621	3.89	11.40
Haryana	1,587	3.59	1,565	3.54	7.13
Himachal Pradesh	14,369	25.81	709	1.27	27.08
Jammu & Kashmir	21,273	9.57	5,633	2.53	12.10
Jharkhand	22,591	28.34	3,080	3.86	32.20
Karnataka	35,251	18.38	5,467	2.85	21.23
Kerala	15,595	40.13	2,632	6.77	46.90
Madhya Pradesh	76,013	24.66	6,267	2.03	26.69
Maharashtra	47,476	15.43	8,978	2.92	18.35
Manipur	17,086	76.53	142	0.63	77.16
Meghalaya	16,988	75.74	405	1.80	77.54
Mizoram	18,684	88.63	122	0.58	89.21
Nagaland	13,719	82.75	238	1.43	84.18
Orissa	48,374	31.07	4,589	2.95	34.02
Punjab	1,558	3.09	1,823	3.62	6.71
Rajasthan	15,850	4.63	8,379	2.45	7.08
Sikkim	3,262	45.97	27	0.38	46.35
Tamil Nadu	23.044	17.72	5,621	4.32	22.04
Tripura	8,155	77.77	134	1.28	79.05
Uttar Pradesh	14,127	5.86	8,203	3.40	9.26
Uttarakhand	24,442	45.70	658	1.23	46.93
West Bengal	12,413	13.99	2,269	2.56	16.55
Andaman and Nicobar	6,629	80.36	53	0.65	81.04
Chandigarh	15	13.16	9	7.61	20.77
Dadra and Nagar Haveli	221	45.01	28	5.66	50.67
Daman and Diu	8	7.14	9	7.76	14.90
Lakshadweep	25	78.13	4	13.33	91.46
Pondicherry	42	8.75	42	8.66	17.41
Total	6,77,088	20.60	91,663	2.79	23.39

).

Geographical distribution of forest cover is depicted in Figure 1.7. It can be concluded from the figure that Eastern Himalaya, Western Himalaya, Western Ghats, islands have substantial area under forest cover. Eastern parts of Central Indian upland, i.e., Orissa, Chattisgarh, Jharkhand, Eastern Madhya Pradesh, etc., are also characterized by dense forests. North-western parts of the country, Indo-Gangetic plain and rain shadow parts of South India are very poor in terms of forest cover (Figure 1.7).

Figure 1.7
Spatial Distribution of Forests in India



The number of plant (flowering and non-flowering) species in India is estimated to be over 47,000 representing about 12 per cent of world's flora. These are categorized in different taxonomic divisions including over 1,500 flowering plants.

Estimates for lower plants are: 64 gymnosperms, 2,843 bryophytes, 1,012 pteridophytes, 1,940 lichens, 12,480 algae and 23,000 fungi. Some 5,150 species of flowering plants are endemic to the country. Among the endemic species, 2,532 species are found in Himalaya and adjoining areas followed by 1,782 species in peninsular India.

About 1,500 endemic species are facing varying degrees of threat. In India, tropical forests harbour around 89,451 animal and 49,219 plant species. Forests are rich in floral and faunal diversity and are regarded as home of the biodiversity because most of the biodiversity occurs in the forests particularly tropical forests among terrestrial systems have a great number of species.

Suggested readings:

1. Biodiversity - Use and Conservation R.P. Singh and J.P. Singh

2. Textbook of Biodiversity by K.V. Krishna

3. Biodiversity – an Introduction by Kevin. J. Gaston and John. I. Spice

Probable questions:

1. What do you mean by Biodiversity.
2. What are different levels of biodiversity.
3. What are the characteristics of Tundra biome.
4. What are the characteristics of Desert biome.
5. What are the characteristics of grassland biome.
6. What are the characteristics of Temperate deciduous forest biome.
7. What are the characteristics of Tropical rain forest biome.
8. What do you mean by Biological Hotspots. State its two characteristics.
9. Write down the importance of Western Ghats as Biological Hotspots in India.
10. Write down the importance of Eastern Himalayas as Biological Hotspots in India.

Unit-VIII

Measuring Biodiversity; interrelationship between diversity measures; pattern of local and regional biodiversity

Objective: In this unit you will learn about how to measure biodiversity and species diversity of a particular area. You will also learn about interrelationship between them and pattern of local and regional biodiversity.

Introduction:

Any measure of species diversity, by itself, does not convey much information; we appreciate its significance only when we compare with any other measure.

Measures of species diversity can be divided into three categories (Magurran, 1988).

These are:

(i) Species richness indices,

(ii) Species abundance models, and

(iii) Species proportional abundance based indices

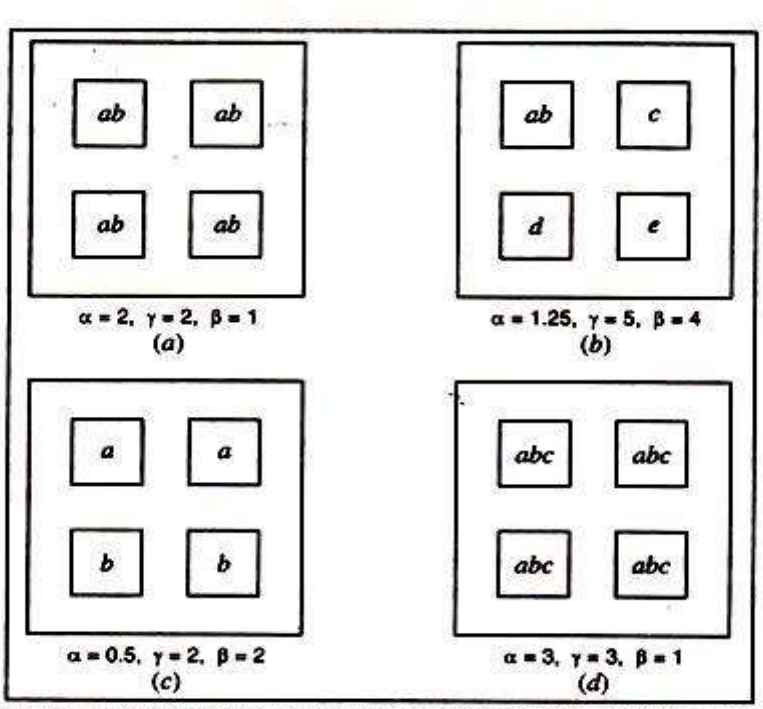


Fig. 7.1. Relationship between alpha, gamma and beta diversity (after Ricklefs and Miller, 2002). Each large box (region) has four small boxes (habitats). In (*a*), the diversity of each habitat (alpha diversity) is the smae for all 4 habitats, each contains species a and b, species richness of 2. The regional diversity (gamma) is 2. The beta diversity is gamma/alpha, 2/2 = 1. In (*b*), alpha diversity is 2 for one habitat (species a and b) and 1 for the other three (species c, d and e occur alone in a habitat), yielding an average a diversity of 1.25; gamma diversity is 5 (No. of habitats × average alpha diversity), so beta diversity is gamma/alpha, 5/1.25 = 4. In (*c*) average alpha diversity is 2/4 = 0.5, gamma diversity 2, beta diversity 2/1 = 2. In (*d*) alpha diversity is 12/4 = 3, gamma diversity 3, beta diversity is gamma/alpha, 3/3 = 1. Regions (*a*) and (*d*) have different gamma diversities but the same beta diversity, indicating little species turnover in those areas.

Species Richness Indices:

Species richness, as measure of diversity, has been used by ecologists. Species density or the number of species per m^2 is most commonly used to measure species richness. However, species richness increases with sample size. The smallest sample size may be 1 km^ and the largest may be the entire region or country.

Rarefaction:

As the sample sizes are always unequal, Sanders technique called Rarefaction is used to cope with this difficulty.

Sanders's formula, as modified by Hurlbert (1971) is as follows:

$$E(S) = \sum\left\{1-\left[\binom{N-N_i}{n}\Big/\binom{N}{n}\right]\right\}$$

where $E(S)$ = expected number of species in the rarefied sample

n = standardized sample size

N = the total number of individuals recorded in the sample to be rarefied

N_i = the number of individuals in the *ith* species in the sample to be rarefied

The simplest approach is to take the number of individuals in the smallest sample as the standardized sample size.

This may be explained with the help of the following example:

If in one catch of fish we obtain 9 species with 23 individuals, and in another catch from the same area made for the same duration we obtained only 13 individuals belonging to 6 species, Hurlberts' formula may be used to find out the number of species we would have expected in the first catch if it too had only 13 individuals. Thus, expected number of species for the first catch x is 6.6 species (Table 7.4).

Table 7.4. Rarefaction with the help of Hurlbert's formula (see text for details)

Species	*Catch X*	*Catch Y*
A	9	1
B	3	0
C	0	1
D	4	0
E	2	0
F	1	0
G	1	1
H	0	2
I	1	0
J	0	5
K	1	3
L	1	0
Total No. of species (S)	9	6
Total No. individuals (N)	23	13

1. The term $\binom{x}{y}$ is a 'combination' which is calculated as follows.

$$\binom{x}{y} = \frac{x!}{y!(x-y)!}$$

x ! is a factorial. For example $5! = 5 \times 4 \times 3 \times 2 \times 1 = 120$

With these points in mind the computations can proceed

2.The first step is to take each species abundance from catch X and insert it in the formula

$$\left\{1 - \left[\binom{N - N_i}{n} \Big/ \binom{N}{n}\right]\right\}$$

Thus, for the species A which was represented by 9 individuals, the calculations are

$$\left\{1 - \left[\left(\frac{14!}{13! \times 1!}\right) \Big/ \left(\frac{23!}{13! \times 10!}\right)\right]\right\} = \{1 - [14/1144066]\}$$

$$= 1 - 0.00 = 1.00$$

The result for each species is listed and summed to give the expected species number for catch X

Ni	
9	1.00
3	0.93
4	0.98
2	0.82
1	0.57
1	0.57
1	0.57
1	0.57
1	0.57
Expected No. of Species for catch X	E(S) = 6.58 or 6.6

Menhinick's Index (I M_n):

This index is based on the ratio of number of species (S) and the square root of the total number of individuals (N).

$I_{Mn}=S/\sqrt{N}$ or $D_{Mn} = S/\sqrt{N}$
It is claimed that this index may be used to compare samples of different sizes and that the effect of the number of individuals is reduced. However, some authors have shown that this index is not independent of sample size.

Using the data given in Table 7.4, the value of IM_n for catch x and catch y will be 1.88 and 1.66 respectively.

Margalefs index (I_{Mg}):
This index also relates the number of species to the number of individuals.

$I_{Mg} = S - 1/\log_e N$ or $D_{Mg}= (S - 1)/\ln N$

The index is influenced by sample size. However, some authors have demonstrated that both this and Manhinick's index are insensitive to changes in community structure.

Using the data given in Table 7.4, the value of for sample x and sample y will be 2.55 and 1.95 respectively.

Species Abundance Models:

No community has species of equal abundance. Some species are very abundant, others may have medium abundance and still others may be rare or represented by only a few individuals. This observation led to the development of species abundance models.

Species diversity data is frequently described by one or more patterns of distribution (Piclou, 1975), diversity is usually examined in relation to the following four models:

(a) The geometric series

(b) The log normal distribution

(c) The logarithmic series

(d) The broken stick model (the random niche boundary hypothesis)

When plotted on a rank abundance graph, the four models represent a progression ranging from the geometric series where a few species are dominant with the remaining fairly uncommon, through the log series and log normal distributions where species of intermediate abundance become more common and ending in the conditions represented by the broken stick model in which species are equally abundant as may be hardly observed.

Species Proportional Abundance Based Indices:

These indices provide an alternative approach to the measurement of diversity. These indices are called heterogeneity indices (Peet 1974) as they take both species richness and evenness into consideration. South wood (1978) called them nonparametric indices in view of the fact that no

assumptions are made about the shape of the underlying species abundance distribution. The following indices are used.

Simpson's Index:

This index relates the contribution made by each species to the total number of individuals present.

$I = \Sigma p_i^2$

Where pi is the proportion of individuals in the ith species. The equation given by Wilhm (1967) is the following:

$I = \Sigma (ni(ni-1)/N(N-1)$

Where pi = the number of individuals in the ith species and N= the total number of individuals. The values of Simpson's index range from zero to 1 (unity) and are inversely proportional to the wealth of species (As I increases, diversity decreases). Pielou (1969) has given the following form of equation.

$I = 1- \Sigma (ni(ni-1)/N(N-1)$

Therefore, index is usually expressed as 1 – I or l/I. The reciprocal form of Simpson's index ensures that the value of the index increases with diversity.

Shannon Index:

The index independently derived by Shannon and Wiener from the application of information theory is known as the Sharmon index of diversity. It is sometimes incorrectly referred to as the Shannon – weaver index (Krebs, 1985).

The index assumes that:

(a) All species are represented in the sample, and

(b) Individuals are randomly sampled from an 'indefinitely large' population (Pielou, 1975).

It is calculated from the equation: $H' = -\Sigma pi \ In \ pi$

Where pi is the proportion of individuals found in the ith species. It is estimated as (ni/N). N is total number of individuals in S species. The value of Shannon index usually varies between 1.5 and 3.5 and rarely exceeds 4.5. The value of H' is related to species richness but is also influenced by the underlying species abundance distribution. May (1975) has shown that if the underlying distribution is log normal, 10 species will be required to give a value of H' < 5.0. Log_2 is often used to calculate Shannon index. Usually the index is obtained from the series.

$H' = -\Sigma pi \ In \ pi – S-1/N+1-\Sigma pi^{-1} /12N^{2} + \Sigma(pi^{-1} – pi^{-2})/12N^{3}$

Brief outlines of the two types of diversity indices of biodiversity are discussed in this article.

The two types are: (1) Dominance Indices, and (2) Information-Statistic Indices.

1. Dominance Indices:

Dominance indices are weighted toward the abundance of the commonest species. A widely used dominance index is Simpson's diversity index. It takes into account both richness and evenness.

Simpson's Diversity Indices:

The term "Simpson's diversity index" can actually refer to any one of 3 closely related indices.

Simpson's Index (D):

Simpson's index measures the probability that any two individuals drawn at random from an infinitely large community will belong to same species. There are two versions of the formula for calculating D.

Either is Acceptable but is to be Consistent:

$$D = \sum (n/N)^2 \qquad D = \frac{\sum n(n-1)}{N(N-1)}$$

where, n = the total number of individuals of each species, N = the total number of organisms of all species.

The value of D ranges between 0 and 1.

With this index, 0 represents infinite diversity and 1, no diversity. That is, the bigger the value of D, the lower the diversity. This does not sound logical, so to get over this problem, D is often subtracted from 1 or the reciprocal of the index is taken.

Simpson's Index of Diversity 1-D:

This index represents the probability that two individuals randomly selected from a community will belong to different species. The value of this index also ranges between 0 and 1, but here, the greater the value, the greater the diversity.

Simpson's Reciprocal Index 1/D:

The value of this index starts with 1 as the lowest possible figure. This figure would represent a community containing only one species. The higher the value, the greater would be the diversity. The maximum value is the number of species in the sample. For example, if there are five species in the sample, then maximum value is 5.

The name Simpson's diversity index is often very loosely applied and all three related indices described above (Simpson's index, Simpson's index of diversity and Simpson's reciprocal index) have been quoted under term, depending on authors.

As an example, let us consider the following table:

Species	Number(n)	n(n – 1)
A	2	2
B	8	56
C	1	0
D	1	0
E	3	6
Total (N)	15	64

Putting the values into the formula for Simpson's index:

$$D = \frac{\sum n(n-1)}{N(N-1)} = \frac{64}{15 \times 14} = 0.3 \text{ (Simpson's index)}$$

Then, Simpson's index of diversity 1 – D = 0.7 and Simpson's reciprocal index 1/D = 3.3.

All these three values represent the same biodiversity. It is, therefore, important to ascertain which index has actually been used in any comparative studies of biodiversity. The disadvantage of Simpson's index is that it is heavily weighed toward the most abundant species, as are in all dominance indices.

The addition of rare species with one individual will fail to change the index. As a result, Simpson's index is of limited value in conservation biology if an area has many rare species with just one individual.

2. Information-Statistic Indices:

Information-statistic indices can take into account rare species in a community. Information- statistic indices are based on the rationale that diversity in a natural system can be measured in a way that is similar to the way information contained in a code or message is measured.

By analogy, if we know how to calculate the uncertainty of the next letter in a coded message, then we can use the same technique to calculate the uncertainty of the next species to be found in a community.

Shannon Index:

A widely used diversity index is Shannon index.

The Index is given by:

$$H_s = \sum_{i=1}^{s} p_i \ln p_i$$

where, p_i is the proportion of individuals found in the i^{th} species and In denotes natural logarithm.

The following table gives an example:

	Species	Abundance	p_i	$p_i \ln p_i$
	A	50	0.5	– 0.347
	B	30	0.3	– 0.361
	C	10	0.1	– 0.230
	D	9	0.09	– 0.217
	E	1	0.01	– 0.046
Total	5	100	1.00	– 1.201

Putting the values into the formula for Shannon index, $H_s = 1.201$

Even the rare species with one individual (species E) contributes some value to the Shannon index, so if an area has many rare species, their contributions would accommodate. Shannon index has a minus sign in the calculation, so the index actually becomes 1.201, not-1.201. Values of Shannon index for real communities are often found to fall between 1.5 and 3.5. The value obtained from a sample is in itself of no significance. The index becomes useful only while comparing two or more sites.

Brillouin Index:

A second information-statistic index, designed to reflect species abundance.

The Brillouin index and is given by:

$$H_B = \frac{\ln(N!) - \sum \ln(ni!)}{N}$$

where, N is the total number of individuals in the community, ni is the number of individuals in the i^{th} species.

The following table gives an example:

Species	No. of individuals	$\ln(n_i!)$
A	5	4.79
B	5	4.79
C	5	4.79
D	5	4.79
E	5	4.79
	N = 25	Σ ln(n!) = 23.95

Putting the values into the formula for Brillouin index, we get

$$H_B = \frac{\ln(25!) - 23.95}{25} = \frac{58 - 23.95}{25} = 1.362$$

This index describes a known population. There is no room for uncertainty while using this index. It places more emphasis on species richness and is moderately sensitive to sample size.

Zoological Realms:

Sclater (1857) was the first one to give concepts of zoogeography and divided the continental masses into six Realms based on his studies on the bird fauna under two Creatio or centres of Creation, namely, Palaeogeana (Old world) and Neogeana (Newworld).

A.R. Wallace (1876), who is considered father of modern zoogeography agreed with Sclater's classification but proposed the name ORIENTAL instead of INDIAN and AFRICAN instead of ETHIOPIAN regions because the earlier names represented countries and not the zoogeographical regions.

The widely accepted modern classification of land masses into regions is given below which is based on Wallace (1876) and Darlington (1957).

1. Realm MEGAGEA

a. PALEARCTIC (Europe, Russia, Mediterranean).

b. NEARCTIC (North America up to the middle of Mexico).

c. AFRICAN (=ETHIOPIAN) (Africa south of Sahara).

d. ORIENTAL (Tropical Asia south of 30° latitude).

2. Realm NEOGEA

e. NEOTROPICAL (South America, tropical Mexico and Caribbean Islands).

3. Realm NOTOGEA

f. AUSTRALIAN (Australia, Tasmania, New Guinea and New Zealand).

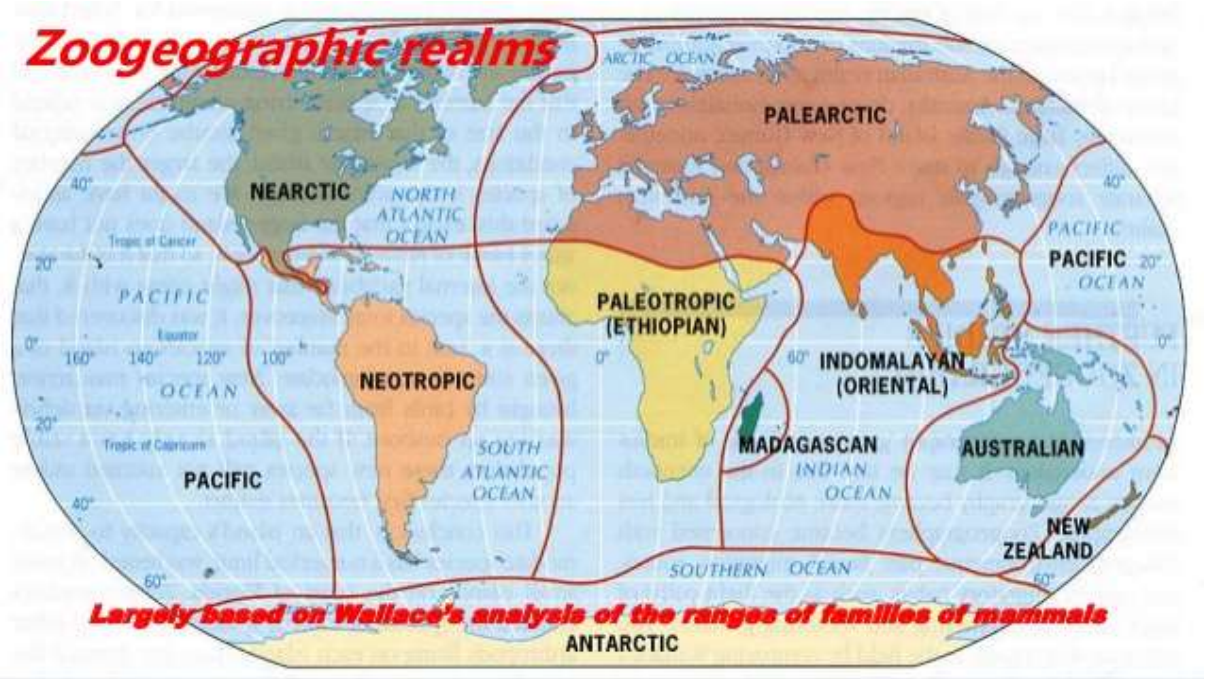


Figure: Zoogeographical Realms of the Earth

A. Palaearctic Realms

This region includes Europe, Russia up to pacific coast and Mediterranean up to Sahara. Climate is temperate and polar in the north. Eastern Asia is temperate with deciduous forests. In northern zone there are grasslands (steppe) and interior portion is arid.

Mammals: There are 33 families of land mammals. Animals of word-wide distribution which amounts to one-third of families are rabbits, mice dog family, shrews, squirrels and cat family. Animals that are restricted to the Old World include hedge hog, porcupine, civets, giant panda (*Ailuropoda*), hyena and pigs.

Four families are shared with Nearctic: beavers, jumping mice, flying squirrels, mole (*Talpa*) and four shared with African region. Endemic mammals: mole rat (*Spalacidae*) and Camel (*Seleviniidae*), dormice. African elements are wild horses, the prezevalski's horse is the only truly wild horse in the world.

Aves: There are 53 families of birds most of which are migratory. All birds have wide distribution and are shared with Nearctic, Oriental and African regions, e.g. pheasants, wrens, finches, warblers, sea birds, geese, birds of prey, cranes, terns, gulls etc. Hedge sparrow is restricted to this region.

Reptiles: There is no endemic reptilian family. Lizard, *Sinisaurus*, and *Alligartor sinensis* are endemic in China. There are lizards, snakes, *Typhlops* and sand boa, *Trionyx* and emydine turtles.

Amphibia: There are common newts, crested newt (*Triton*), Spanish newt and alpine newt. The colourless Proteus is blind and lives in European caves. There are European salamanders, *Salamandra salamandra* and *S. atra* and a species of giant salamander (*Megalobatrachus*) in Japan and China that attains a length of over 5 feet. Anurans are represented by frogs, toads, tree frogs. Male of the midwife toad (*Alytes obstetricans*), which is found in France and Italy carried eggs wrapped around his hind legs. Amphibians show affinities with Nearctic Region.

Fishes: Fish fauna also shows affinities with Nearctic. There is no endemic fish and carp is the dominant family. There are carps, salmon, pikes, perches, eels and Petromyzon that migrates from sea to the rivers to breed and the ammocoete larva, commonly known as sand sleeper lives in mud for several years in European rivers. Few species of toothless sturgeons immigrate from sea to the rivers of Japan and Russia for laying eggs which are harvested to prepare a delicacy called caviar.

Subregions of Palaearctic Realm

a. **European.** Northern and central Europe. Black sea. The fauna includes hedge hog, shrew, mole and myogale (a mammal).

b. **Mediterranean.** Southern Europe. Arabian, Asia Minor, Afghanistan, Baluchistan and parts of Russia. Fauna includes civets, hyena, hyrax.

c. **Siberian.** Northern Asia north of Himalaya having extreme climatic conditions. Fauna includes yak, musk deer, mole, freshwater seal (*Phocasibirica*) found in Baikal lake.

d. **Manchurian.** Mongolia, Japan, Korea, Manchuria, Tibet and northern China. The fauna includes Tibetan langur (*Rhinopithecus*), giant panda (*Ailuropus*), Chinese water deer (*Hydropotes*), tufted deer (*Elaphodus*)

B. Nearctic Realms

This region includes the north American continent up to the middle of Mexico. Climate is temperate with an arctic edge. There are grasslands in the middle of the continent.Western part is arid with mountains and coniferous forests.

Mammals: There are 24 families of land mammals. Endemic mammals include rodents, pocket mice (*Heteromyidae*), pocket gophers (*Geomyidae*) and mountain beavers (*Aplodontidae*). Pronghorns and Sewellel (*Antilocapridae)* are endemic artiodactyls. Palaearctic elements include beavers, moles (*Talpidae*), pikas and jumping mice. Neotropical species that have crossed over to North America are marsupials such as opossum (*Didelphis virginiana*), shrew opossum (*Lestorosinca*); 9-banded armadillo (*Dasypus*) and tree porcupine. There are some mammals of wide ranging distribution, namely, shrews, rabbits, squirrels, mice, cats, bats, bears, deers and bovids.

Birds: There are 49 families of birds in this region out of which 39 are widelydistributed.Exclusive birds are: red cardinals, humming birds, tanagers and wild turkeys(*Meleagrididae*). Golden plover migrates from Europe.

Reptiles: There are turtles, non-poisonous garter snakes, rattle snakes, geckos, horned lizards, limbless lizard (*Ophisaurus*), horned toad (*Phrynosoma*) and *Alligator mississipiensis*. Gila monster (*Heloderma*) is exclusive to this region.

Amphibia: Urodeles include salamanders, hellbender (*Cryptobranchus*), neotenic larva called axolotl, eel-like siren, the Congo eel *(Amphiuma*) and tiger salamanders, *Ambystoma tigrinum*. Newts include Smooth newt, great crested newt, banded newt, alpine newt, Bosca's newt. There are dusky salamanders, red-backed salamander and Jordan's salamander. Anurans include American Bell toad, *Liopelma*, North American bull frog and Leopard frog found in grassy meadows. *Rana cascadae* and *Bufo boreas* inhabits Cascade Mountains of Oregon, USA. The desert spade-foot toad is adapted to the arid climate of north western America. Other toad species are: Oak toad, southwestern toad and the giant toad which is 20 cm long.

Fishes: There are many carps (*Cyprinidae*) and perches. Holostei are endemic that include only two surviving ganoid fishes, one species of bowfin (*Amia calva*) found in American lakes and 7 species of garpike *(Lepidosteus*) found in American rivers. These fishes possess sharp teeth to seize and gulp prey with extraordinary swiftness. Paddlefish (*Polyodon*) occurs in Mississippi river of America and represents Chondrostei. Another species of paddlefish occurs in China. These fishes have paddlelike snout that carries sensory organs for locating prey by detecting its electrical fields. Ameiurid catfishes, moon-eyes *(Hiodontidae*) and bass family with genera *Morone* and *Upiblema* are also endemic. The fauna of Nearctic Region is rich in reptiles and is a complex of tropical and temperate animals.

Subregions of Nearctic Realm

a. Californian: Narrow strip between Sierra Nevada and Cascade Range. From Vancouver to British Columbia. Fauna includes 86 families of vertebrates that include vampire and free-tailed bats.

b. Rocky Mountains: This includes dry mountainous region east of California. Fauna contains 107 families of terrestrial vertebrates. There are pronghorns (*Antilocapra*), mountain goat (*Haplocerus*), American bison, prairie dogs (*Cynomus*) and Heloderma.

c. Alleghany: This includes eastern region of USA. The fauna includes opossum, star nosed mole, vampire bats, turkeys and mud eel(*Siren*).

d. Canadian: This sub region includes Canada, Greenland and Alaska. The fauna is poor and

resembles palaearctic region. There are reindeer, sheep, bisons, lemmings, polar beer, elk and arctic fox.

C. African (Ethiopian) Realms

This region includes continental Africa south of Sahara desert. This is mainly a tropical region having evergreen forests and grasslands in the central and eastern parts. There is desert in the north.

Mammals: There are 38 families of mammals, out of which 12 are exclusive and the rest are shared with Neotropical and Oriental Regions. Animals having worldwide distribution include shrews, rabbits, squirrels, cricetid mice, dogs, mired mice, cats and bovids, antilopes. The exclusive animals include giraffes, hippopotamus, Aardvark or Cape anteater belonging to Tubulidentata, rock hyrax (*Hyracoidea*), golden mole (*Chrysochloridae*), elephant shrew, small deer-like water chevrotain, aye-aye, bush babies and lemurs in Madagascar. There are 6 endemic families of rodents and 3 of insectivores. There are no camels, bears and tigers in this region. Animals shared with Oriental are, lorises, monkeys, apes, pangolins, Cheetah, elephants and rhinoceroses. Shared with Palaearctic are dormice, jerboa (*Dipodida*e), wild horses.

Aves: Bird fauna has affinities with Oriental Region. There are cuckoos, woodpeckers, hornbill, sunbirds, herons, orioles, birds of prey, storks, parrots, pigeons, fowls, pitta, guinea fowl, hornbill, swallows and bee-eaters. There are 6 exclusive families that include ostrich, secretary bird (*Secretariidae*), hammer head that feed on frogs and fish, crested touracos (*Turacidae*), ground hornbill, mouse birds and helmet shrike. Honey guide feeds on honey bee larvae and guides honey collecting tribals to bee hives. Two species of oxpeckers, namely, yellow billed and red billed feed on ticks and other ectoparasites of rhinoceroses. The crocodile bird dares to enter the mouth of crocodiles to feed on leeches.

Reptiles: Crocodiles and turtles abound and few lizards belong to families Lacertidae and Agamidae. Iguanid horned lizards are absent. Spiny lizard of family Cordylidae is restricted to this region. Chameleon also occurs in the Oriental Region. Snakes include pythons, *Typhlops* and biting vipers. Crocodiles include, *Crocodylus niloticus*, *Osteolaemus* in West Africa and *Osteoblepharon* in Congo.

Amphibia: There are no urodeles but frogs and toads abound such as the African ridged frog and African shovel-nosed frog. Family Hylidae of tree frogs is absent, replaced by *Polypedatidae*. The flying frog is the African rhacophorid. Genera Rana and Bufo are absent. Phrynomerid tree frogs are endemic. Xenopus and aquatic clawed toads present. Limbless amphibians are present.

Fishes: Lung fishes have two species of *Protopterus* that live in the rivers and lakes of tropics. Chondrostei is represented by 10 species of Bichir (*Polypterus*). Electric eel of family Mormyridae has electric organs in tail. There are cat fishes, carps, characins and generally the fish fauna is diverse. Fauna shows overwhelming similarity with the Oriental Region.

Subregions of African Region

1. East African: This includes tropical Africa and tropical Arabia. Fauna contains 145 families of vertebrates. There are rhinoceros, zebra, giraffe, cheetah, spotted hyena and lions.

2. West African: Western Africa up to Congo includes forests. Fauna has 134 families of vertebrates that include, gorilla, chaimpanzee, monkeys, baboons, flying squirrel.

3. South African: Southern portion of Africa. Fauna contains 133 families of vertebrates. There are ostriches and secretary birds. Mammals include golden mole, elephant shrew, jumping mice, aardvark and naked mole rat that leads subterranean existence.

4. Malagasy: Madagascar, Mauritius, Seychelles and neighbouring islands. Fauna has 86 families out of which 8 are endemic. There are aye-aye, lemurs and common tenrec *(Tenrec ecaudatus).* Helmet birds and cuckoo rollers and rough-tailes snakes (Uropeltidae) are endemic. The flightless bird Dod*o* which was related to flightless pigeon became extinct in 1681 due to hunting by man and egg predation by dogs, pigs and monkeys.

D. Oriental Realms

This region includes Indian subcontinent, southern China south of 30o latitude, Malaya, Philippines and Indonesian islands up to Wallace's Line. There are rain forestsin the east, high mountains in the north and the western part is arid. Along the south western coast there is a forested low mountainous belt.

Mammals: There are 30 families of mammals out of which 5 are endemic that include *Cynocephalus (=Galeopithecus),* which is called Calugo, a gliding mammal with furred membrane stretched between fore limb and hind limb. Tree shrews (Tupaiidae) and arboreal tarsiers (Tarsiidae) of Philippines are restricted primates. Spiny dormouse is arboreal rodent. Twenty five percent of the fauna is shared with Africa that includes, old world monkeys, lorises, apes (Gibbon and Orang-Utan), pangolin or scaly anteater *(Manis),*bamboo rat (Rhizomyidae), Elephant *(Elephas maximus), Rhinoceros unicornis* andalion population in Gir forest. Fauna shared with Palaearctic includes hedge hog, porcupine, civets, Lynx, hyenas,pigs, bear *(Ursusarctos),* red panda.Tapir*(Tapirus)* found in Sumatra and Borneo is Neotropical element. There are moles, tapirs, bears and deers which are absent in Africa.

Aves: Out of 66 families of birds 53 are eurytopic or widespread. Woodpeckers and barbets are widespread. There are also peacocks, argus pheasants, cattle egrets *(Bubulcus ibis)* and jungle fowl. Sunbirds, hornbills, parrots and cuckoos and shared with Africa. Shared with Palaearctic are pheasants. Exclusive birds are fairy blue bird *(Irena puella)* which is found in Philippines where it follows troupes of monkeys to feed on insects disturbed by their movement. There are 4 genera and 14 species of leaf birds. Whitehead's trogon *(Harpacteswhiteheadi)* is found in Indonesia and monkey-eating eagle is endangered species found in the dense forests of Philippines.

Reptiles: There are plenty of lizards, turtles, poisonous snakes, pythons and crocodiles. Lizards belong to Agamidae and Varanidae and include geckos, skinks, calotes, draco and chameleon. There are king cobras, common cobras, typhlops, xenopeltid snakes, uropeltid snakes and sea snakes (Hydophidae). Crocodiles include *Crocodylus porosus, C. palustris, Gavialis gangeticus. Tomistoma*is found in Sumatra and Borneo. *Alligator sinensis*is found in southern China.

Amphibia: Frogs and toads bound. Caecilians are represented by *Ichthyophis* and *Gegenophis* found in Indo-Malayan region. Tree frogs belong to family Polypedatidae and Hylidae family is absent. Flying frog, *Rhacophorus malabaricus* found in Western Ghats in India. Tailed Amphibia are few and found in northern Indo-china. Fire-bellied toads are exclusive and extend to Palaearctic range.

Fishes: Fishes are dominated by carps and catfishes (Cypriniformes). Loaches, mullets and mud-eels are exclusive to this region. One species of the Chondrostean paddlefish *(Polyodon)* occurs in Yangtze River of China. Another species of this group occurs in American rivers. Fauna shows similarity with the Ethiopian Region because of their proximity and similar environmental conditions.

Subregions of Oriental Realms

a. Indian: Indian subcontinent up to the foot of Himalaya and south up to Mysore. East and Western Ghats are tropical rain forests. There are rocky hills in the central part and desert in the north-west.

Fauna is varied. Tibetan Wild Ass found in Ladakh and Himachal Pradesh. Wild Ass found in Runn of Kutch. Golden Langur *(Presbytesgeei)* found in Assam. Indian Pangolin *(Manis crassicauda)* and great Indian Bustard *(Ardeotisnigriceps).*

b. Indo-Ceylonese: Sri Lanka and southern parts of India containing Tamil Nadu and Kerala. Fauna includes lorises and elephants. Slender Loris (Loris tardigradus), slow loris (*Nycticebuscaucang*), lion-tailed monkey (*Macacasilenus*) and giant squirrel (*Ratufamacroura*).

c. Indo-Chinese: China south of 30o latitude, Burma and Thailand. Fauna includes panda, gibbons (Hylobates), flying lemur, lynx, bear (*Ursus*), Chinese pangolin, red panda, snow leopard (*Panthera uncia*) and clouded leopard. Red-shanked douc langur is completely arboreal and endemic to Southeast Asia and Cat Ba Langur is endemic in Vietnam. There are Indo-Chinese warty pig and Javan warty pig, Sumatran and Annamite striped rabbits. Crested Argus pheasant (*Rheinardiaocellata*) is endemic to Laos and Vietnam and possesses 70 inches long tail feathers, longest for any bird in the world. Rhinoceros snake (*Rhynchophis boulengeri*), the green, arboreal snake and green pricklenape lizard (*Acanthosauracapra*) are endemic to Indo-china. Whipping frog (*Polypedatesdugritei*) also present.

d. Indo-Malayan: Malayan peninsula and islands of Malay Archipelago and Indonesia. Fauna includes Orang-Utan, proboscis monkey, Malayan badger, Tupaia, gibbons, flying lemurs, tapirs and broad bills. *Rhinoceros sondaicus* is the lesser one-horned rhinoceros which is found in Java and *Rhinoceros unicornis* is found in India and Tarai regions of Nepal. Swamp deer (*Cervus duvauceli*).

E. Neotropical Realms

South America, most of Mexico, West Indies, Caribbean islands. Mostly tropical but the southern part extends into temperate zone. Rain forests on the western side. Grasslands in the middle in Argentina. Andes mountain on the western coast.

Mammals: There are 32 families of mammals of which 16 are unique. Widely distributed animals are shrews, rabbits, squirrels, mice, dogs, bears, cats and deers. Camels are represented by two species of *Llama: L. vicuna* and *L. guanaco.* Llama and Alpaca are domesticated breeds of these species. There are three species of tapirs of which one species also occurs in the Oriental Region. Monkeys include: spider monkeys, squirrel monkeys, howlers, capuchin, marmosets which belong to families Cebidae and Hapalidae of suborder Platyrrhina. Endemic mammals include, six-banded armadillo *(Euphractus),* armadillo *(Dasypus),*two-toed sloth *(Choloepus),* 3-toed sloth *(Bradypus),* 3 species of anteaters,*(Myrmecophaga),* 11 endemic families of rodent Caviomorpha and five endemic families of bats that include disc-winged bats, furipterid bats and vampirebats (Desmodontidae), the last one is also a carrier of rabies. Marsupials belong to the family Didelphidae (12 genera) that includes common opossum *(Didelphis)* and water opossum *(Chironectes)* and family Coenolestidae (3genera) includes opossum rat *(Ceonolestes).* The common opossum has also spread to the Nearctic Region where it has adapted to varied climatic conditions. There are no hedgehogs, moles, beavers, hyenas, bovids and horses in this region.

Aves: Almost 50% of the avian fauna is endemic and unique due to which South America is known as *The Bird Continent.* Out of 67 families of birds, 25 are endemic to the region. There are partridge-like tinamous, toucans that carry enormous beaks, trumpeters, hoatzin, cock of the rock *(Rupicola),* oil birds and several species of macaws, such as yellow macaw, Hahn's macaw, red bellied macaw and red and blue macaw. Quail is the only member of Galliformes here. Bee hummingbird found in Cuba measures only 6 cm and is the smallest bird. Ostriches are represented by *Rhea americana.* Common birds include herons, ibis, storks, ducks, hawks, owls, plover, cuckoos. There is scarcity of song birds.

Reptiles: There are plenty of snakes, iguanid lizards, *Crocodilus*, *Caiman* (alligator)and turtles. Xantusiid lizards are endemic. There are tree boas, anacondas, pit vipers and coral snakes. Mud turtles (Pelomedusidae) are shared with Africa and snake-neckturtles, *Chelodina* (Chelidae) with Australia.

Amphibia: There are hylid tree frogs such as Brazilian tree frog *Hyla, Hylodes,* Cubantree frog, Venezuelan tree frog that deters predators by foul odour and the poisonous *Phyllomedusa* found in Amazon. *Leptodactylus* deposits its eggs in frothy mass in holes on muddy banks of rivers and ponds and *Hylafaber* makes crater-like nests. The Tungara frog is noisy and makes foam nests. Tiny frogs belong to the genera *Phyllobates, Dendrobates* and *Agalychnis.* The yellow frog is the largest and most toxic and its poison is used by Colombia tribes to poison their blowgun darts. The dart poison frog is also highly poisonous. The tongue and toothless Surinam toads show parental care. The small Chilean frog carries eggs in the gular pouch while the Brazilian tree frog, *Hylagoeldii* carries eggs on the back. Caecilians are represented by *Typhlonectes. Oedipus* is the only tailed amphibian (Urodela) found in South America.

Fishes: There are no carps (Cyprinidae) and other fish fauna is endemic. There is electric eel (Gymnotidae), cat fishes *(Diplomystes, Nematogenys)* and characin fish(Piranhas). Lung fishes are represented by *Lepidosiren paradoxa* found in Amazon River. The fauna of Neotropical Region is rich in endemic families; almost 40 families are endemic out of 155 families of vertebrates. Other fauna is shared with Nearctic and other tropics.

Subregions of Neotropical Reals

a. Chilian: Western coast of South America, embracing summits of Andes, Peru andBolivia. Fauna includes chinchilla, Llama, oil birds and Rhea.

b. Brazilian: Tropical forests up to Isthmus of Panama. Also open plains and pasturelands. Fauna includes New World Monkeys, vampire bats, tree porcupine, sloths, armadillo, opossum, tapirs, cavia, spiny mice.

c. Mexican: Mexico and northern lands of isthmus of Panama and rocky mountains. Fauna includes mud terrapins, tapirs and Plethodontids.

d. Antilean or West Indies: Caribbean Islands except Tobago and Trinidad. Contains mountainous and rocky areas covered with forests. Fauna is poor and native mammals are absent.

F. Australian Realm

This region includes Australia, Tasmania, New Guinea, New Zealand and islands east of Wallace's Line. New Guinea is tropical with rain forests. Eastern Australia is covered with lush green forests, Western Australia is desert and there are grasslands in the middle.

Mammals: Eight of the 9 families of marsupials are unique. There are 52 genera of 6families of marsupials that are unique and are not found in the Neotropical Region. Marsupials show parallelism with their counterpart placentals in other parts of the world in their evolutionary modifications. Placental mammals that were introduced by man or some of them immigrated themselves include rodents and bats, rabbits, foxes, rats, mice, dingo dogs, cats, pigs and murid mice. Monotremes are represented by *Ornythorhynchus* and *Echidna.*

Birds: There are 58 families of birds out of which 44 are widely distributed. Species having wide range are trogons *(Harpactes),* hawks, kingfisher, cuckoos, parrots and pigeons. Shared with Oriental are frogmouths, wood-swallows, flowerpeckers and megapods. There are 10 families of endemic birds

which include cassowary, emu, kiwi, lyrebird, bower birds, honey suckers, birds of paradise, magapods and cookabura or laughing jackass that feeds on lizards and snakes. Birds not present are pheasants, finches, barbets and woodpeckers.

Reptiles: There are pythons, biting snakes, elepid coral snakes, geckos, skinks, agamid lizards, scale-footed lizard *(Pygopus)* and Komodo Dragon (Varanidae),crocodiles and turtles. Chelyd turtles and snake-necked turtles *(Chelodina)* possess strikingly long necks. *Sphenodon pounctatus*is found exclusively in New Zealand.

Amphibia: There are no tailed amphibians. Common toads are absent and frogs are few. Tree frogs belong to family Hylidae which is also found in the New World andPalaearctic but absent in African and Oriental Regions. The Australian Green Tree frog secretes mosquito repellent compound from the skin glands. The Australian frogs, *Rheobatrachussilus* and *R. vitellinus*carry tadpoles in their stomachs. Somespecies of frogs are adapted to live in deserts, e.g. *Notaden, Neobatrachus* and *Helioporus*.

Fishes: Fresh water fishes are rare and belong to family Osteoglossidae. Lung fish (*Neoceratodus*) is restricted to Burnett and Mary rivers in Queensland. Fauna of this region is poor in freshwater fishes, Amphibia and reptiles. There is uniqueness of mammals and affinities exist with the Oriental and South American faunas.

Subregions of Australian Realm

a. Australo-Malayan: Malayan Archipelago, Moluccas, Solomon Islands, New Guinea. Fauna includes 130 families of vertebrates. Birds are crowned pigeons, birds of paradise, honey eaters, cuckoos, bover birds, cassowaries. There are fly river turtles, flying phalangers and tree frogs.

b. Australian: Australia and Tasmania. There are 98 families of vertebrates and the region is home of monotremes and marsupials. Monotremes include short-nosed echidna, long-nosed echidna and duck-billed platypus. Marsupials include marsupial rat kangaroos, Tasmanian devil, koala, the horny possum, marsupial mole and rabbit. Tasmanian wolf (*Thylacinus cynocephalus*) became extinct in 1936.There are scrub birds, lyre birds, emus and plenty of cobras. Tailed amphibians are absent.

c. Polynesian: Polynesia and the adjoining islands. Fauna is poor containing only 53families. Tooth-billed pigeon is unique.

d. New Zealand: New Zealand, Norfolk Island, Auckland and Campbell Island. Fauna includes murid bats, owl parrots, nester parrots, kiwi, Sphenodon and frog (*Liopelma*).Flightless owl parrot or Kakapo feeds on leaves, shoots, berries, fruits and moss. The only carnivorous parrot called KEA was a vegetarian earlier but after introduction of sheep in New Zealand has learnt to cut sheep skin and feed on flesh. Giant Moa (*Dinornis maximus*) that reached a height of 3 metres contained 22 species in New Zealand which became extinct all by 1600 AD.

Suggested readings:

1. Biodiversity - Use and Conservation R.P. Singh and J.P. Singh

2. Textbook of Biodiversity by K.V. Krishna

3. Biodiversity – an Introduction by Kevin. J. Gaston and John. I. Spice

Probable questions:

1. What is species richness index. How it is measured?
2. What is Menhinick's Index?
3. What is Margalef's index ?
4. What is Simpson's Index? How it is calculated ?
5. What is Shanon's Index? How it is calculated ?
6. State the faunal characteristics of Ethiopian realms.
7. State the faunal characteristics of Oriental realms.
8. State the faunal characteristics of Neotropical realms.
9. State the faunal characteristics of Palaearctic realms.
10. State the faunal characteristics of Nearctic realms.
11. State the faunal characteristics of Australian realms.
12. What is the significance of zoogeographical realms?

Unit-IX

Threats to species diversity: natural and human induced threats and vulnerability of species extinction; Red data book; rarity, endemism, effective and minimum viable population, fragmentation of population and metapopulation

Objective: In this section you will learn about natural and human induced threats and vulnerability of species extinction; Red data book; rarity, endemism, effective and minimum viable population, fragmentation of population and metapopulation.

Introduction:

Some of the main threats to biodiversity are:

1. Human Activities and Loss of Habitat, 2. Deforestation, 3. Desertification, 4. Marine Environment, 5. Increasing Wildlife Trade and 6. Climate Change. 7. Invasive Species. 8. Pollution. 9. Population Growth and Over-consumption:

1. Human Activities and Loss of Habitat:

Human activities are causing a loss of biological diversity among animals and plants globally estimated at 50 to 100 times the average rate of species loss in the absence of human activities. Two most popular species in rich biomes are tropical forests and coral reefs.

Tropical forests are under threat largely from conversion to other land-uses, while coral reefs are experiencing increasing levels of over exploitation and pollution. If current rate of loss of tropical forests continues for the next 30 years (about 1 percent per year), the projected number of species that the remaining forests could support would be reduced by 5 to 10 percent relative to the forest in the absence of human disturbance.The rate of decline would represent 1000 to 10,000 times the expected rate of extinction without deforestation by humans. Some studies suggest that, globally, as many as one half of all mammal and bird species may become extinct within 200 to 300 years.

Biodiversity loss can result from a number of activities, including:

(a) Habitat conversion and destruction;

(b) Over-exploitation of species;

(c) Disconnected patches of original vegetation; and

(d) Air and water pollution.

Over the coming decades, human-inducted climate change increasingly become another major factor in reducing biological/biodiversity. These pressures on biodiversity are, to a large extent, driven by economic development and related demands including the increasing demand for biological resources.

Activities that reduce biodiversity, jeopardize economic development and human health through losses of useful materials, genetic stocks, and the services of intact ecosystems. Material losses include food, wood, and medicines, as well as resources important for recreation and tourism. Losing genetic diversity, like losing species diversity, makes it even more likely that further environmental disturbance will result in serious reductions in goods and services that ecosystems can provide.

Decreased biodiversity also interferes with essential ecological services such as pollination, maintenance of soil fertility, flood controls, water purification, assimilation of wastes and the cycling of carbon and other nutrients.

2. Deforestation:

Forest ecosystems contain as much as 80 percent of the world's terrestrial biodiversity and provide wood fiber and biomass energy as well as critical components of the global cycles of water, energy and nutrient. Forest ecosystems are being cleared and degraded in many parts of the world.

Current projections suggest that demand for wood will roughly double over the next 50 years, which will make increasing use of sustainable forest practices more difficult. In addition to threats to biodiversity and potential shortages in the supply of forest products, the degradation of forests represents an enormous potential source of green house gas emissions. Forest ecosystems contain about three times the amount of carbon currently present in the atmosphere and about one-third of this carbon is stored above ground in trees and other vegetation and two-third is stored in the soil. When forests are cleared or burned, much of this carbon is released into the atmosphere. According to current estimates, tropical deforestation and burning account for about one quarter of carbon emissions into the atmosphere from human activities.

3. Desertification:

Desertification and deforestation are the main causes of biodiversity loss. Both processes are decisively influenced by the extension of agriculture. The direct cost of deforestation is reflected in the loss of valuable plants and animal species. Desertification process is the result of poor land management which can be aggravated by climatic variations. Converting wild lands to agriculture often involves ploughing the soils which leads in temperate regions to an average decline in soil organic matter between 25 and 40 per cent over twenty five years. Decreasing soil organic matter is always a clear indication of soil degradation, and often is accompanied by reductions in water infiltration, fertility, and ability to retain fertilizers. Ploughing also exposes soils to wind and water erosion, resulting in large-scale pollution of freshwater resources.

4. Marine Environment:

Oceans play a vital role in the global environment. Covering 70 per cent of the earth's surface, they influence global climate, food production and economic activities. Despite these roles, coastal and marine environment are being rapidly degraded in many parts of the globe. In coastal areas, where human activities are concentrated, pollution, over-exploitation of resources, development of critical habitats such as wetlands, and mangroves, and water-flow from poor land-use practices have led to drastic reductions in near shore fisheries production and aquatic biodiversity.

5. Increasing Wildlife Trade:

According to Nick Barnes, "Trade is another cause of biodiversity depletion that gives rise to conflict between North and South." Global trade in wildlife is estimated to be over US $ 20 billion annually. Global trade includes at least 40,000 primates, ivory from at least 90,000 African elephants, 1 million orchids, 4 million live birds, 10 million reptile skins, 15 million furs and over 350 million tropical fish.

6. Climate Change:

As climate warms, species will migrate towards higher latitudes and altitudes in both hemisphere. The increase in the amount of CO_2 in the air affects the physiological functioning of plant and species composition. Moreover, aquatic ecosystems, particularly coral reefs, mangrove swamps, and coastal wetlands, are vulnerable to changes in climate.
In principle, coral reefs, the most biologically diverse marine systems, are potentially vulnerable to changes in both sea level and ocean temperature. While most coral systems should be able to grow at a sufficient pace to survive a 15 to 95 cm sea-level rise over the next century, a sustained increase of several degrees centigrade would threaten the long-term viability of many of these systems.

7. Invasive Species:

Invasive species are 'alien' or 'exotic' species which are introduced accidentally or intentionally by human. These species become established in their new environment and spread unchecked, threatening the local biodiversity. These invasive alien species have been identified as the second greatest threat to biodiversity after habitat loss.

8. Pollution:

Pollution is a major threat to biodiversity, and one of the most difficult problems to overcome; Pollutants do not recognize international boundaries. For example, agricultural run-off, which contains a variety of fertilizers and pesticides, may seep into ground water and rivers before ending up in the ocean. Atmospheric pollutants drift with prevailing air currents and are deposited far from their original source.

9. Population Growth and Over-consumption:

From a population of one billion at the beginning of the 19th century, our species now numbers more than six billion people. Such rapid population growth has meant a rapid growth in the exploitation of natural resources— water, foods and minerals. Although there is evidence that our population growth rate is beginning to slow down, it is clear that the exploitation of natural resources is currently not sustainable. Added to this is the fact that 25 per cent of the population consumes about 75 per cent of the world's natural resources. This problem of over-consumption is one part of the broader issue of unsustainable use.

Reasons for species extinction

Main reasons for extinction are either natural or manmade. Through evolution, new species arise through the process of speciation and species become extinct when they are no longer able to survive in changing conditions or against superior competition. A typical species becomes extinct within 10 million years (1 crore year) of its first appearance although some species, called living fossils, survive virtually unchanged for hundreds of millions of years. Extinction, though, is usually a natural phenomenon; it is estimated that 99.9% of all species that have ever lived are now extinct.

Various anthropogenic activities causing extinction are manmade reasons. Only recently scientists have become alarmed at the high rates of recent extinctions due to various anthropogenic activities. Some of these anthropogenic activities include intentional or accidental introduction of invasive alien species, over exploitation and unscientific collection of Non-Timber Forest Produce (NTFPs) including medicinal plant, climate change, unsustainable tourism, habitat destruction, encroachment etc.

The IUCN Red List :

The IUCN is perhaps best known for its Red List of Threatened Species (also known as the Red List). The Red List, established in 1964, provides the conservation status of plant and animal species around the world. IUCN members determine the risk of a species' extinction by utilizing criteria such as population size, subpopulations, the number of mature individuals, generation, the decline in population size, extreme fluctuations in population size, fragmented populations and habitats, habitat area size, and distribution of the population.

The Red List provides scientifically based information about species' survival, promotes public education about biodiversity, influences governmental policies, and offers advice about conservation efforts. The category assigned to each species is reassessed every 5 to 10 years by the IUCN Species Survival Commission Specialist Groups. This list is generally accepted as the most comprehensive information on the health and conservation of the world's species.

IUCN Categories: The IUCN Red List assigns a specific category to each of the evaluated species. The categories and their meanings are as follows:

a. Extinct (EX): A taxon is extinct when there is no reasonable doubt that the last individual has died.

b. Extinct in the wild (EW) : When a species only survive in cultivation, in captivity or as naturalized population (or populations) well outside from their past range.

c. Critically Endangered (CR): A taxon is called critically endangered when it is facing a high risk of extinction in the wild in immediate future. These species have 50% or higher probability of extinction within 5 years or 2 generations whichever is longer.

d. Endangered : The taxon is endangered when it is in danger of extinction and whose survival is not possible if the causal factors continue operating. These species have 20% or greater probability of extinction within 20 years or 10 generation whichever is longer.

e. Vulnerable (VU) : The taxon is vulnerable when it is not critically endangered or endangered but is facing the high risk of extinction in the wild. These species have 10% or greater chance of extinction within 100 years.

f. Near Threatened (NT) :A taxon is near threatened when it has been evaluated but does not qualify for critically endangered, endangered, vulnerable category but is close to qualifying for or is likely to qualify for a threatened category in the near future.

g. Least Concern (LC) :A taxon is least concern when it has been evaluated but does not qualify for critically endangered, endangered, vulnerable or near threatened category. Currently these taxa are widespread and abundant.

h. Data Deficient (DD):A taxon is data deficient when there is inadequate information to make a direct or indirect assessment of its risk of extinction based on its distribution and population status.

i. Not Evaluated (NE): A taxon is not evaluated when it has not yet been assessed against the criteria.

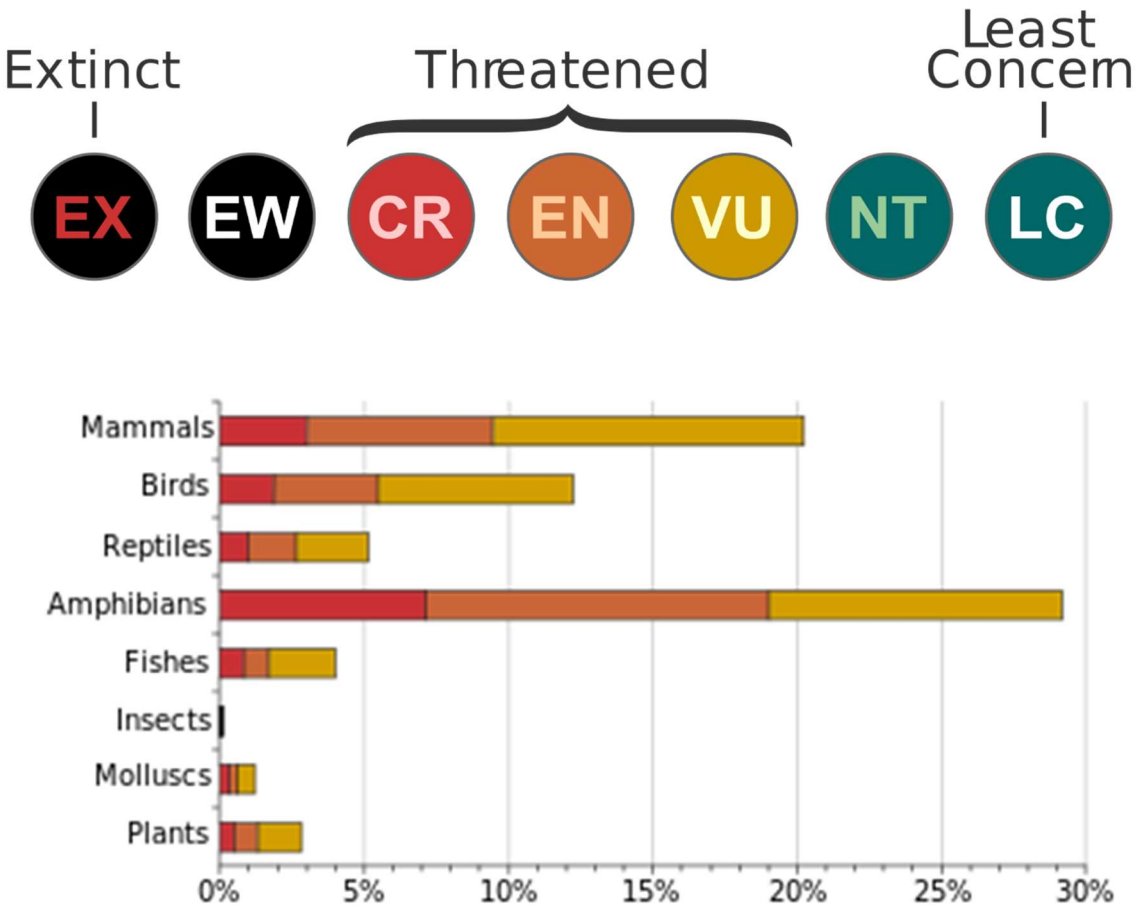


Fig: The percentage of species in several groups which are listed as critically endangered, endangered, or vulnerable on the 2007 IUCN Red List

Other categories :

a. Indeterminate: The taxon that is suspected of belonging to any of the threatened category but for which sufficient information is not available currently is called indeterminate taxon.

b. Lower risk : A taxon is in lower risk when it has been evaluated but does not qualify for any of the threatened category (critically endangered, endangered, vulnerable or near threatened) or data insufficient.

c. Rare: The taxon with small populations in the world that are not at present endangered or vulnerable are called rare taxon. These taxa are usually localized within restricted geographical areas or habitats or are thinly scattered over a more extensive range.

d. Out of danger : The taxon formerly included in any one of the extinction prone categories (critically endangered, endangered, vulnerable or near threatened) but which are now considered as relatively secured because of the effective conservation measures or the previous threat to their survival has been removed.

e. Endemic: Taxon with restricted geographical distribution are called endemic taxon. Due to such restricted distribution with small population size, they are vulnerable to both natural and anthropological threats of extinction.

What is Red Data Book?

The Red Data Book is a public document which is created for recording endangered and rare species of plants, animals, fungi as well as some local subspecies which are present in a particular region. The Red Data Book helps us in providing complete information for research, studies and also for monitoring the programs on rare and endangered species and their habits.

This book is mainly created to identify and protect those species which are on the verge of extinction. Brief History of the Red Data Book The name of this book has its origins from Russia, it was originally known as the Red Data Book of the Russian Federation or the RDBRF. The book was based on research conducted between 1961 and 1964 by biologists in Russia. Hence, it is also called as the Russian Red Data Book.

Currently, the International Union for Conservation of Nature maintains the Red Data Book. IUCN is the world's most detailed inventory centre of the global conservation status of biological species. The **International Union for Conservation of Nature** (**IUCN**) was founded in 1964 with an aim to maintain a complete record of every species that ever lived.

The Red Data Book contains the complete list of threatened species. The main aim behind this documentation is to provide complete information for research and analysis of different species.

The Red Data Book contains colour-coded information sheets, which are arranged according to the extinction risk of many species and subspecies.

- Black represents species which are confirmed to be extinct.
- Red represents species that are endangered
- Amber for those species whose status is considered to be vulnerable
- White is assigned for species that are rare
- Green for species that were formerly endangered, but their numbers have started to recover
- Grey coloured for the species that are classified as vulnerable, endangered, or rare but sufficient information is not available to be properly classified.

In a nutshell, the Red Data Book indexes species as:

- Threatened
- Not threatened
- Unknown

Furthermore, The Red Data Book also has information as to why a species has become extinct along with the population trends and the extent of its range (distribution).

Advantages of the Red Data Book:

- It helps in identifying all animals, birds and other species about their conservation status.
- It is used to evaluate the population of a particular species.
- The data available in this book can be used to evaluate the taxa at the global level.
- With the help of this book, we can estimate the risk of taxa becoming globally extinct.
- Provides a framework or guidelines for implementing protective measures for endangered species.

Disadvantages of the Red Data Book :

- The information available in the Red Data Book is incomplete. Many species, both extinct and extant are not updated in this book.
- The source of the book's data has been speculated and has been mired in controversy.
- This book maintains the complete record of all animals, plants, other species but it has no information about the microbes.

Red Data Book of India

Red Data Book of India includes the conservation status of animals and plants which are endemic to the Indian subcontinent. The data for this book is provided through surveys which are conducted by the Zoological Survey of India and the Botanical Survey of India under the guidance of the Ministry of Environment, Forest and Climate Change.

Critically endangered mammals as per the Red Data List of India include:

- Kondana Rat
- Malabar Civet
- Kashmir Stag
- River Dolphins

Critically endangered arthropods include:

- Rameshwaram Parachute spider
- Peacock Tarantula

Critically endangered fish include:

- Pookode Lake barb
- Ganges River shark
- Pondicherry shark

Critically endangered amphibians and reptiles include:

- Gharial
- White-spotted bush frog
- Toad-skinned frog

Endemism

Definition :

Endemism is the condition of being *endemic*, or restricted in geographical distribution to an area or region. The area or region can vary in size, and is defined or identified in different ways. Endemism is an ecological classification in that it describes the range or distribution of a species, or group of species. For instance, entire families of different species of birds are endemic to the island of Madagascar. The term endemism can applied to many things, including diseases and natural phenomenon. Endemism in these cases refers to the "normal" or standard level of some measured observation within a specific geographic region or area.

Endemism is not to be confused with *indigenous*, a term which refers to the origins of a species. Indigenous refers to where a group originated. A species can be both endemic and indigenous to an area. However, some species thrive and exceed the bounds of their original indigenous location. This means that the species is no longer endemic, but is still indigenous to the original area. Once a species has reached a wide-spread, global distribution it is said to be *cosmopolitan*. Animals like whales, once indigenous to a specific mainland in the form of their 4-legged ancestors, are now cosmopolitan in distribution.

Endemic Species:

An endemic species is a species which is restricted geographically to a particular area. Endemism in a species can arise through a species going extinct in other regions. This is called *paleoendemism*. Alternatively, new species are always endemic to the region in which they first appear. This is called *neoendemism*. Both forms of endemism are discussed in more detail under the heading "Types of Endemism", below.

Endemic species, regardless of how they came to be restricted to a particular area, experience the same threats to their existence. The smaller the region, the more dire the threat toward the survival of the species. Any action that reduces the size of the land, or divides it in any way can significantly affect the normal patterns of the endemic species. While endemism and being *endangered* or *threatened* are different things, being endemic to a small area is often a warning sign that a species may become threatened or endangered.

This is not always the case, as many globally distributed species are also considered threatened or endangered. In recent years, many sharks have joined the list. While they are distributed throughout many of the ocean's waters, the harvesting of shark fins for soup has decimated their populations globally. Endemism sometimes protects species from being exploited globally, simply because of the fact that the species only exists in a small area. This can even make the species easier to protect, because the land can be placed under a *conservation easement* to restrict the construction and human impact on the land.

Endemic Disease:

Scientists studying *epidemiology*, or disease outbreaks, have a similar definition of endemism. An *endemic disease* is a disease seen at consistent levels in specific location. For instance, *endemic relapsing fever* is a disease seen in Europe and in North America. The disease is not seen in any sort of observable amounts in other parts of the world. Other diseases, which are new to an area or are spiking in their prevalence, are known as *epidemic diseases*.

There are many endemic diseases, and their endemism has roots in the species and vectors which promote these diseases. In the case of relapsing fever, a *vector* carries the bacterium of the *Borrelia* species. There are several vectors which can carry these bacteria, mostly including ticks and lice. The species of ticks and lice which carry these bacteria are endemic to the Northern Hemisphere. *Borrelia* bacteria are also responsible for *Lyme disease*, a disease endemic to the Northern Hemisphere. A map of Lyme disease is shown below, and corresponds to the endemism seen in tick and lice species.

While Lyme disease and relapsing fever are endemic to these areas, they are not endemic to say, Australia. If there were even a few cases of Lyme disease in Australia, the disease would be considered epidemic, because the normal level of Lyme disease in Australia is zero.

Types of Endemism:

a. Paleoendemism

There are two basic ways for a species to show endemism to a certain region. Basically, the difference between the two is whether the species is newly emerging, or historic and declining. Paleoendemism describes the later. In this form of endemism, a species which was once widespread has been reduced to a much smaller range. This is the case for many large predators today.

Before humans, large predators were widely distributed across the globe. As human society became more organized, large predators were driven away from society, and out of their historic ranges. Those which have not gone *extinct* are now restricted to limited ranges. Conservation efforts for these animal focus on protecting the current range and expanding it to encompass the historic range. This is hard however, as humans often oppose the re-introduction of large predators. Without protections from hunters, the species will easily be pushed back to their endemic range.

b. Neoendemism

On the opposite hand, new species are branching off the evolutionary tree every day. These species are both endemic and indigenous to the location in which they first appeared. They are restricted to a geographical location simply because that is where they started. This is known as neoendemism. There are many species, found on islands, which show this form of endemism.

Islands provide an interesting and isolated grounds for the development of new species. While the species on the island are now endemic, their ancestors were likely not. Take the Galapagos finches, as an example. The Galapagos archipelago contains many islands. Many thousands of years ago, a single finch species arrived on the islands. At first, it spread across the island as one species. However, evolution has now separated the birds so much that they represent different species. The differences in the vegetation on the islands divided the ancestor into many smaller species, which show endemism to the island they are found on.

Minimum Viable Population Size :

The idea that small populations are more vulnerable to extinction can be found in the work of MacArthur and Wilson's (1967) Theory of Island Biogeography. They proposed a model, which indicates that the probability of extinction varies with population size. An isolated island represents equilibrium between the number of immigrating species and number of species becoming extinct. Smaller islands have less number of species and, more important, smaller populations will have shorter time to extinction. This model implies that below a certain threshold population size of individuals for island species the expected time to extinction will be very short. And above that threshold the population will have relatively longer time of persistence. Shaffer (1981) defined MVP for any given species in any given habitat as the smallest isolated population having a 99% chance of remaining extant for 1000 years despite the foreseeable effects of demographic, environmental, genetic stochasticity, and natural catastrophes. Thus to avoid extinctions, the population must be sufficient to withstand such random events. Demographic stochasticity includes random factors that affect the birth rate and the death rate of population. If more animals die and few animals born, extinction can occur before the population can recruit themselves to a safe number again. Random variation in sex ratio and reproductive successes in females also lead population to decline and extinction. The effect of demographic stochasticity is greatest in small populations. Environmental stochasticity includes variations that are external to the population like rainfall, temperature, availability of food, and population of competitors, predators and diseases. Environmental stochasticity affects the population by influencing the demographic parameters. Natural catastrophes

include fires, floods, earthquakes, and volcanic eruptions. Genetic stochasticity refers to the random processes involved in passing genes from one generation to the next. Genes may be lost from a small population and the gene frequencies may be changed due to drift or inbreeding.

Effective Population Size:

The concept of effective population size is fundamental to MVP size. The effective population size is the actual number of individuals that can breed to produce viable offspring. In other words the effective population size is the ideal population, which is able to maintain the same genetic diversity as the real population. Therefore, it is always necessary to find out the effective population number before the MVP size for that population can be estimated. The effective population size takes into several basic assumptions (Mace 1986):- a) random mating, b) no migration, c) no mutation, d) no selection, and e) non-overlapping generation. If any of these assumptions are violated in a population, the effective population size will differ from the census population size. In the real world, a population will almost never follow all the above assumptions at the same time and therefore the census population is usually greater than the effective population size. A census population consisting of the effective population size to avoid extinction over a given time is taken as the MVP for that population. Simberloff (1988) has pointed out two types of effective population size- the inbreeding effective population size, and the variance effective population size. The inbreeding effective population size is the size of an ideal population with the same rate of decrease in homozygosity as in the particular population, while the variance effective population size is the size of an ideal population with the same rate of variance due to drift as in the particular population. These two population sizes can be similar when population size is constant, and sometimes can be different. In a growing population the variance effective population will tend to be greater than inbreeding effective population size. In a declining population, the opposite occurs. In addition to random mating, no migration, no mutation, no selection and nonoverlapping generation, the effective population size will also vary because of different mating behaviours in monogamous and polygamous system.

Metapopulation:

In ecology, a regional group of connected populations of a species. For a given species, each metapopulation is continually being modified by increases (births and immigrations) and decreases (deaths and emigrations) of individuals, as well as by the emergence and dissolution of local populations contained within it. As local populations of a given species fluctuate in size, they become vulnerable to extinction during periods when their numbers are low. Extinction of local populations is common in some species, and the regional persistence of such species is dependent on the existence of a metapopulation. Hence, elimination of much of the metapopulation structure of some species can increase the chance of regional extinction of species.

The structure of metapopulations varies among species. In some species one population may be particularly stable over time and act as the source of recruits into other, less stable populations. For example, populations of the checkerspot butterfly (*Euphydryaseditha*) in California have a metapopulation structure consisting of a number of small satellite populations that surround a large source population on which they rely for new recruits. The satellite populations are too small and fluctuate too much to maintain themselves indefinitely. Elimination of the source population from this metapopulation would probably result in the eventual extinction of the smaller satellite populations.

In other species, metapopulations may have a shifting source. Any one local population may temporarily be the stable source population that provides recruits to the more unstable surrounding populations. As conditions change, the source population may become unstable, as when disease increases locally or the physical environment deteriorates. Meanwhile,

conditions in another population that had previously been unstable might improve, allowing this population to provide recruits.

Suggested Readings:

1. Akçakaya, H.R. and Ferson, S. 2001. RAMAS® Red List: Threatened Species Classifications under Uncertainty. Version 2.0. Applied Biomathematics, New York.

2. Akçakaya, H.R., Ferson, S., Burgman, M.A., Keith, D.A., Mace, G.M. and Todd, C.A. 2000. Making consistent IUCN classifications under uncertainty. Conservation Biology 14: 1001-1013.

3. Baillie, J. and Groombridge, B. (eds). 1996. 1996 IUCN Red List of Threatened Animals. IUCN, Gland, Switzerland.

4. Burgman, M.A., Keith, D.A. and Walshe, T.V. 1999. Uncertainty in comparative risk analysis of threatened Australian plant species. Risk Analysis 19: 585-598.

5. Fitter, R. and Fitter, M. (eds). 1987. The Road to Extinction. IUCN, Gland, Switzerland.

6. Gärdenfors, U., Hilton-Taylor, C., Mace, G. and Rodríguez, J.P. 2001. The application of IUCN Red List Criteria at regional levels. Conservation Biology 15: 1206-1212.

Probable Questions:

1. Discuss the causes of loss of biodiversity.
2. Write a short note on Red Data Book of IUCN.
3. Define nine IUCN categories.
4. State advantages and disadvantages of Red data book.
5. Define and explain metapopulation.
6. Define and explain Effective Population Size.
7. Define and explain Minimum Viable Population Size.
8. Define Endemism and explain it.
9. Write short notes on Endemic species with examples.
10. Write short note on Endemic disease.
11. Discuss about types of endemism.

Unit-X

Problems of genetic diversity ; bottleneck; genetic drifts; inbreeding depression

Objective: In this unit you will learn about Genetic Drift, Founder Effect and Bottle Neck Effect

Introduction:

Natural selection is an important mechanism of evolution. But is it the *only* mechanism? Nope! In fact, sometimes evolution just happens by chance.

In population genetics, evolution is defined as a change in the frequency of alleles (versions of a gene) in a population over time. So, evolution is any shift in allele frequencies in a population over generations – whether that shift is due to natural selection or some other evolutionary mechanism, and whether that shift makes the population better-suited for its environment or not.

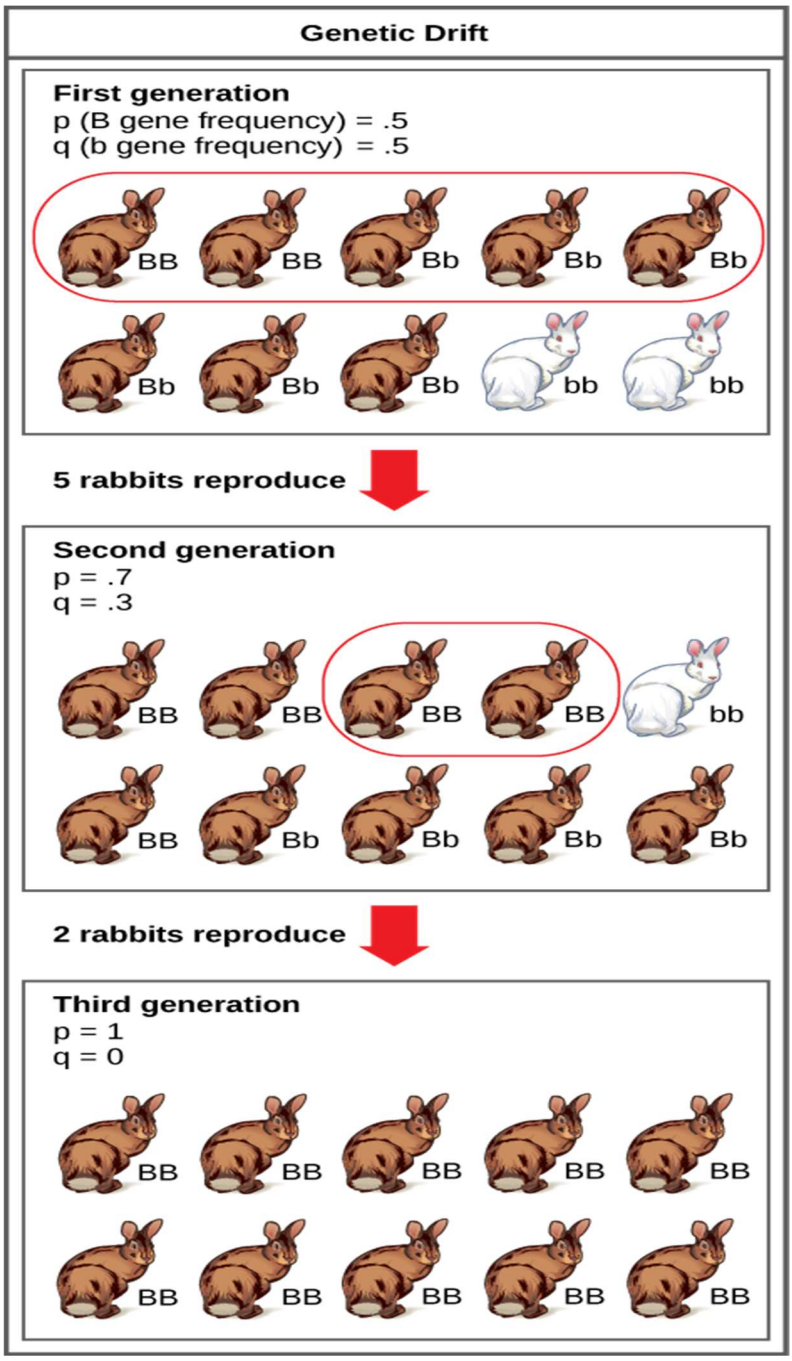
What is genetic drift?

Genetic drift is change in allele frequencies in a population from generation to generation that occurs due to chance events. To be more exact, genetic drift is change due to "sampling error" in selecting the alleles for the next generation from the gene pool of the current generation. Although genetic drift happens in populations of all sizes, its effects tend to be stronger in small populations.

In a small population (having less than hundred members) of a species change rather than natural selection may play an important role in determining in the composition of the next generation In a small interbreeding population, there is a relatively high probability of heterozygous gene pair to become homozygous by chance. this may lead to the accumulation of certain disadvantageous traits and the group with such traits may be eliminated. The tendency in small populations to become homozygous for one allele or other by chance rather than selection has been called genetic drift.

Genetic drift example

Let's make the idea of drift more concrete by looking at an example. As shown in the diagram below, we have a very small rabbit population that's made up of 888 brown individuals (genotype *BB* or *Bb*) and 222 white individuals (genotype *bb*). Initially, the frequencies of the *B* and *b* alleles are equal.



What if, purely by chance, only the 555 circled individuals in the rabbit population reproduce? (Maybe the other rabbits died for reasons unrelated to their coat color, e.g., they happened to get caught in a hunter's snares.) In the surviving group, the frequency of the *B* allele is 0.70.70, point, 7, and the frequency of the *b* allele is 0.30.30, point, 3.

In our example, the allele frequencies of the five lucky rabbits are perfectly represented in the second generation, as shown at right. Because the 555-rabbit "sample" in the previous generation had different allele frequencies than the population as a whole, frequencies of *B* and *b* in the population have shifted to 0.70.70, point, 7 and 0.30.30, point, 3, respectively.

From this second generation, what if only two of the *BB* offspring survive and reproduce to yield the third generation? In this series of events, by the third generation, the *b* allele is completely lost from the population.

Population size matters

Larger populations are unlikely to change this quickly as a result of genetic drift. For instance, if we followed a population of 100010001000 rabbits (instead of 101010), it's much less likely that the *b* allele would be lost (and that the *B* allele would reach 100\%100%100, percent frequency, or **fixation**) after such a short period of time. If only half of the 100010001000-rabbit population survived to reproduce, as in the first generation of the example above, the surviving rabbits (500500500 of them) would tend to be a much more accurate representation of the allele frequencies of the original population – simply because the sample would be so much larger.

This is a lot like flipping a coin a small vs. a large number of times. If you flip a coin just a few times, you might easily get a heads-tails ratio that's different from 505050 - 505050. If you flip a coin a few hundred times, on the other hand, you had better get something quite close to 505050 - 505050 (or else you might suspect you have a doctored coin)!

Allele benefit or harm doesn't matter

Genetic drift, unlike natural selection, does not take into account an allele's benefit (or harm) to the individual that carries it. That is, a beneficial allele may be lost, or a slightly harmful allele may become fixed, purely by chance.

A beneficial or harmful allele would be subject to selection as well as drift, but strong drift (for example, in a very small population) might still cause fixation of a harmful allele or loss of a beneficial one.

Genetic Drift vs. Gene Flow :

The concept of genetic drift is often confused with the concept of gene flow in biology. Gene flow **is** the movement of genes between populations, species, or between organisms**.** For instance, bacterial cells are able to transfer genes between different cells as a method of gaining antibiotic resistance. Populations of organisms exhibit gene flow when individuals from one population migrate and breed with a new population. Gene flow does not analyse the allele frequency of genes. Rather, it is a concept which describes the movement of genes between populations. By contrast, genetic drift describes the random selection of genes within a population, not attributable to natural selection forces.

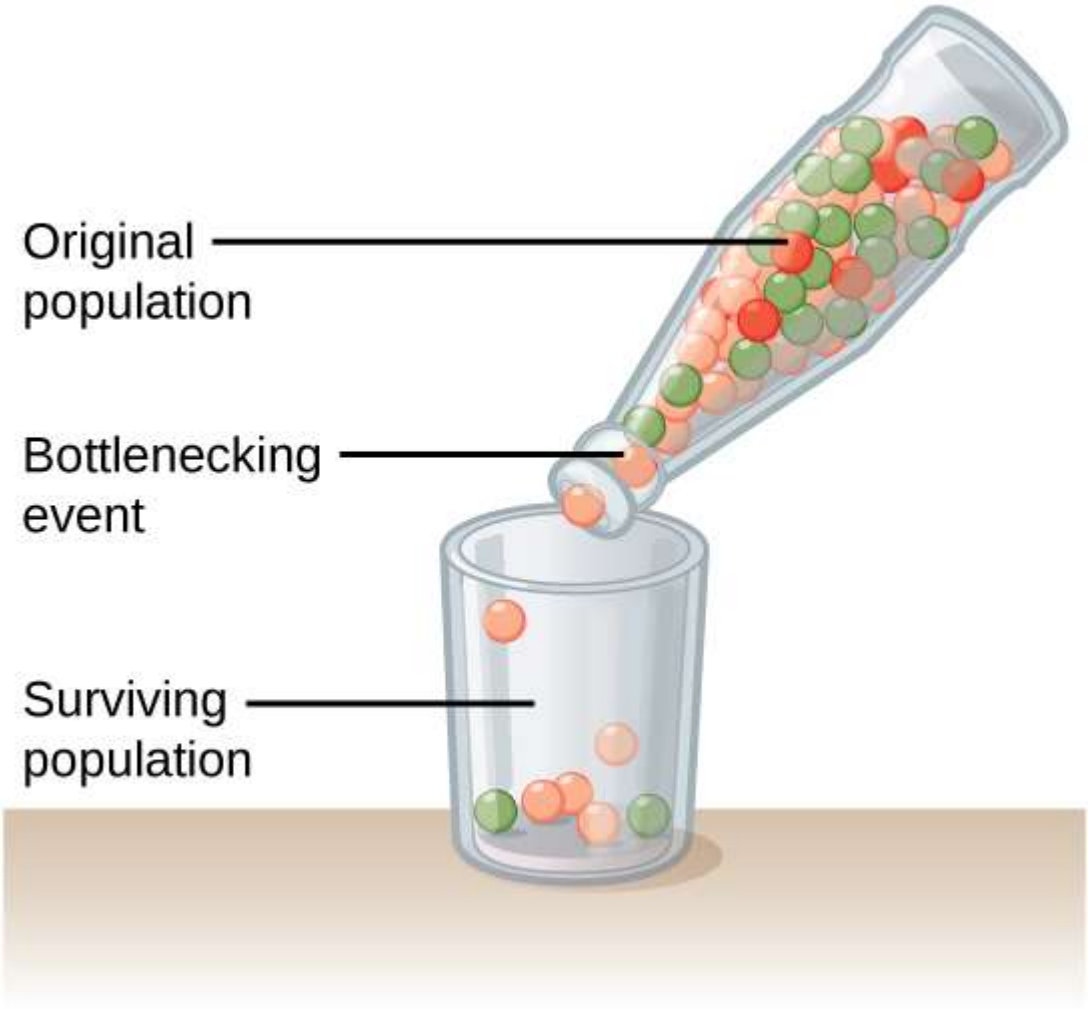
The bottleneck effect

The bottleneck effect is an extreme example of genetic drift that happens when the size of a population is severely reduced. Events like natural disasters (earthquakes, floods, fires) can decimate a population, killing most individuals and leaving behind a small, random assortment of survivors.

The allele frequencies in this group may be very different from those of the population prior to the event, and some alleles may be missing entirely. The smaller population will also be more susceptible to the effects of genetic drift for generations (until its numbers return to normal), potentially causing even more alleles to be lost.

How can a bottleneck event reduce genetic diversity? Imagine a bottle filled with marbles, where the marbles represent the individuals in a population. If a bottleneck event occurs, a small, random assortment of individuals survive the event and pass through the bottleneck (and into the cup), while

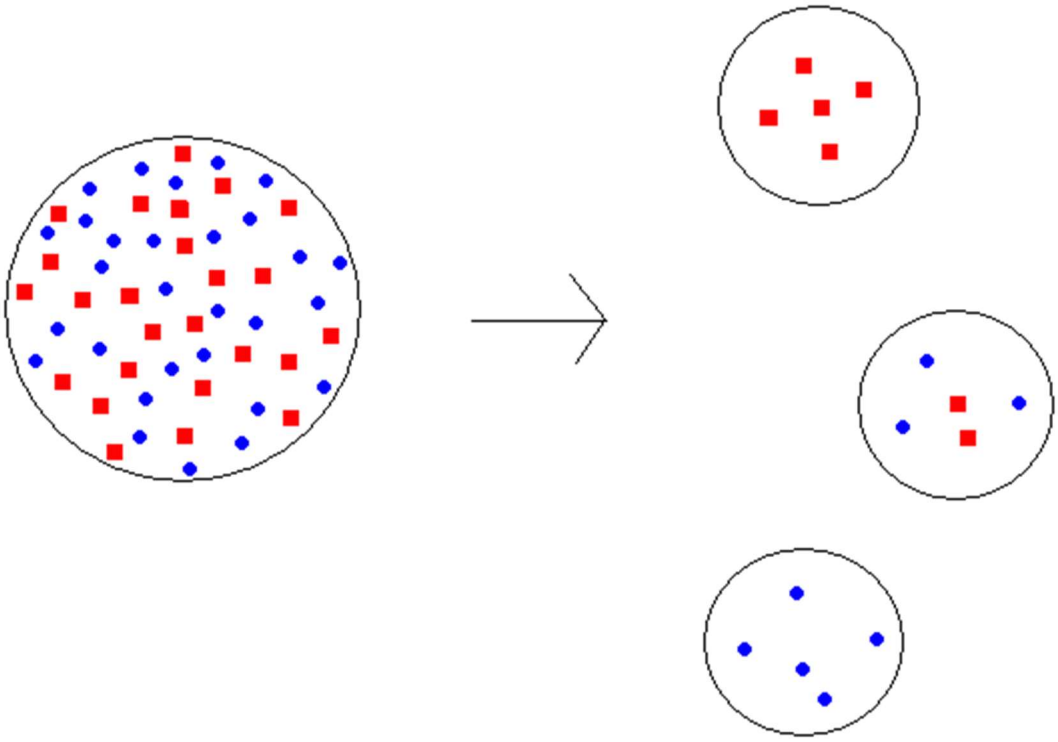
the vast majority of the population is killed off (remains in the bottle). The genetic composition of the random survivors is now the genetic composition of the entire population.



A population bottleneck yields a limited and random assortment of individuals. This small population will now be under the influence of genetic drift for several generations.

The founder effect:

The founder effect is another extreme example of drift, one that occurs when a small group of individuals breaks off from a larger population to establish a colony. The new colony is isolated from the original population, and the founding individuals may not represent the full genetic diversity of the original population. That is, alleles in the founding population may be present at different frequencies than in the original population, and some alleles may be missing altogether. The founder effect is similar in concept to the bottleneck effect, but it occurs via a different mechanism (colonization rather than catastrophe).



Simplified illustration of the founder effect. The original population consisting of equal amounts of square and circle individuals fractions off into several colonies. Each colony contains a small, random assortment of individuals that does not reflect the genetic diversity of the larger, original population. These small colonies will be susceptible to the effects of genetic drift for several generations.

In the figure above, you can see a population made up of equal numbers of squares and circles. (Let's assume an individual's shape is determined by its alleles for a particular gene).

Random groups that depart to establish new colonies are likely to contain different frequencies of squares and circles than the original population. So, the allele frequencies in the colonies (small circles) may be different relative to the original population. Also, the small size of the new colonies means they will experience strong genetic drift for generations.

Difference between Natural Selection and Genetic Drift:

Natural selection and genetic drift both result in a change in the frequency of alleles in a population, so both are mechanisms of evolution. However, the two processes differ in how they cause allele frequencies to change. Genetic drift causes evolution by random chance due to sampling error, whereas natural selection causes evolution on the basis of fitness.

In natural selection, individuals whose heritable traits make them more fit (better able to survive and reproduce) leave more offspring relative to other members of the population. That is, an individual with higher fitness is more likely to pass on its genetic material (alleles) to the next generation. The alleles that helped make this individual more fit will likely benefit the offspring in a similar way and should increase in frequency in the population over time. Thus, evolution by natural selection is not dependent on chance; it depends on an allele's effect on reproductive success. Alleles that improve fitness are likely to increase in frequency, while alleles that reduce fitness will decrease in frequency.

Genetic drift does not take into account an allele's effect on fitness because it is a random process. Think back to the rabbit population discussed above. What if the white rabbits were more fit than the brown rabbits (better able, on average, to survive and reproduce in the environment in which they lived)? In the example, the only two white rabbits in the population failed to reproduce, resulting in a loss of the beneficial alleles they carried. This result was purely due to chance and illustrates how genetic drift can result in the loss of beneficial alleles from a small population.

Environmental Variance

Genes are not the only players involved in determining population variation. Phenotypes are also influenced by other factors, such as the environment (Figure 6). A beachgoer is likely to have darker skin than a city dweller, for example, due to regular exposure to the sun, an environmental factor. Some major characteristics, such as sex, are determined by the environment for some species. For example, some turtles and other reptiles have temperature-dependent sex determination (TSD). TSD means that individuals develop into males if their eggs are incubated within a certain temperature range, or females at a different temperature range.

Geographic separation between populations can lead to differences in the phenotypic variation between those populations. Such geographical variation is seen between most populations and can be significant. One type of geographic variation, called a cline, can be seen as populations of a given species vary gradually across an ecological gradient. Species of warm-blooded animals, for example, tend to have larger bodies in the cooler climates closer to the earth's poles, allowing them to better conserve heat. This is considered a latitudinal cline. Alternatively, flowering plants tend to bloom at different times depending on where they are along the slope of a mountain, known as an altitudinal cline.If there is gene flow between the populations, the individuals will likely show gradual differences in phenotype along the cline. Restricted gene flow, on the other hand, can lead to abrupt differences, even speciation.

Adaptive Evolution:

Natural selection only acts on the population's heritable traits: selecting for beneficial alleles and thus increasing their frequency in the population, while selecting against deleterious alleles and thereby decreasing their frequency—a process known as adaptive evolution. Natural selection does not act on individual alleles, however, but on entire organisms. An individual may carry a very beneficial genotype with a resulting phenotype that, for example, increases the ability to reproduce (fecundity), but if that same individual also carries an allele that results in a fatal childhood disease, that fecundity phenotype will not be passed on to the next generation because the individual will not live to reach reproductive age. Natural selection acts at the level of the individual; it selects for individuals with greater contributions to the gene pool of the next generation, known as an organism's evolutionary (Darwinian) fitness.

Fitness is often quantifiable and is measured by scientists in the field. However, it is not the absolute fitness of an individual that counts, but rather how it compares to the other organisms in the population. This concept, called relative fitness, allows researchers to determine which individuals are contributing additional offspring to the next generation, and thus, how the population might evolve.

There are several ways selection can affect population variation: stabilizing selection, directional selection, diversifying selection, frequency-dependent selection, and sexual selection. As natural selection influences the allele frequencies in a population, individuals can either become more or less genetically similar and the phenotypes displayed can become more similar or more disparate.

Inbreeding:

The process of mating of individuals which are more closely related than the average of the population to which they belong, is called inbreeding. For example, parthenogenesis in animals and apomixes and self-fertilization in plants are the most extreme types of inbreeding.

Inbreeding in self-fertilizing pea plants was a real advantage to Mendel in his studies which provided pure lines of pea plants for his hybridization experiments. The term 'pure line' was coined by W. Johannsen in 1903 for the true breeding, self-fertilized plants.

Methods of Inbreeding:

In plants ova fertilized by the pollen of either the same plants (in case of bisexual plants) or of the other plant of the same genotype (in case of unisexual as well as bisexual plants), is called self-fertilization. However, in bisexual plants numerous structural and functional adaptations have been recorded which help plants with bisexual or hermaphrodite flowers avoid self-fertilization.

Normally, inbreeding is affected by restrictions in population size or area which brings about the mating between relatives. Since close relatives have similar genes because of common heritage, inbreeding increases the frequency of homozygotes, but does not bring about a change in overall gene frequencies. Thus, a mating between two heterozygotes as regards two alleles, A and a will result in half of the population, homozygous for either gene A or a and half of the population heterozygous like the parent but the overall frequencies of A and a remain unchanged:

$$Aa \times Aa$$

$$1AA : 1Aa$$

Thus, inbreeding brings about the recessive gene to appear in a homozygous stale (aa). Once a recessive allele is in a homozygous state, natural selection can operate upon the rare recessives. Artificial selection is also possible as the homozygous recessives are phenotypically differentiated from the dominant population.

Assortative and Disassortative Mating:

In sexually reproducing organisms, the most rapid inbreeding system is that between brothers and sisters who share both parents in common. This type of mating is called full-sib mating and produces inbreeding coefficient of 25 per cent in the first generation of inbreeding (F_2 of Mendel).
This rate is reduced in succeeding generations since some of the alleles are now already identical. Within 10 generations, full-sib mating can produce an inbreeding coefficient of 90 per cent. The other inbreeding systems are half-sib mating, parent-offspring mating, third-cousin mating and so on.

All these inbreeding systems are called genetic assortative mating since the parents of each mating type are sorted and mated together on the basis of their genetic relationship. Such a breeding method tends to increase the inbreeding coefficient. The assortative mating is also of the phenotypic type, i.e., the mating between two like phenotypes, two like dominant phenotypes or between two like recessive phenotypes. If assortative selective mating is continued for many generations, the heterozygotes are eliminated and the resulting population consists of homozygous dominants and homozygous recessives. If more than one locus is considered at a time, the rate of homozygosity achievement will be slower than for one locus. This is so because now the kind of heterozygotes produced will be more combinations of different loci, e.g., Aa BB, AA Bb, …) and eliminating these will need more number of generations.

Disassortative mating refers to the mating of unlike phenotypes and genotypes and tends to maintain heterozygosity, as in the case of mating between unlike sexes. This preserves the dissimilarities both genetic as well as phenotypic.

In primitive organism, sexual differences arose at a single gene locus, i.e., one sex was homozygous and the other heterozygous for that locus, and the disassortative mating were the mating between an homozygous and an heterozygous individual for sex locus. Disassortative mating also results from dichogamy, (Dichogamy = producing mature male and female reproductive structures at different times); self-sterility in plants in which the mating of like phenotypes (inbreeding) is not possible and fertilization between plants with different genotype is favoured. This maintains heterozygosity within a diploid breeding population.

Genetic Effects of Inbreeding:

The continuous inbreeding results, genetically, in homozygosity. It produces homozygous stocks of dominant or recessive genes and eliminate heterozygosity from the inbred population.

For example, if we start with a population containing 100 heterozygous individuals (Aa) as shown in figure, the expected number of homozygous genotype increasing by 50% due to selfing or inbreeding in each generation. Thus, due to inbreeding in each generation the heterozygosity is reduced by 50% and after 10 generations we can expect the total elimination of heterozygosity from the inbred line and production of two homozygous or pure lines.

But, because a heterozygous individual possesses several heterozygous allelic pairs, we can conclude that inbreeding will operate on all genes loci to produce totally pure or homozygous offspring's. In human beings if inbreeding continued over a number of generations, it would results in increasing homozygosity, but somewhat slowly.

Inbreeding Depression:

In a heterozygote, the inbreeding increases the probability of homozygosity of deleterious recessive alleles in an inbred population. In other words, one of the consequence of inbreeding is a loss in vigour (i.e., less productive vegetatively and reproductively) which commonly accompanies an increase in homozygosity. This is called Inbreeding depression.

Inbreeding depression is found to occur due to following four features of inbreeding:

(1) Increase in frequency of homozygotes,

(2) Increase in variability between different inbred families,

(3) Reduction in value of quantitative character in the direction of recessive values, and

(4) The dependence of this reduction in value upon dominance.

If this inbreeding effect is multiplied for many genes at many loci, there may be a large reduction in value for many traits, including those that affect fitness and survival. In com (maize) for example, E.M. East (1908) and G. H. Shull (1909) studied the effects of inbreeding for 30 generations of inbreeding and found independently, that the yielding ability in these lines finally reduced to about one-third of the open-pollinated variety from which these samples were derived.

Both of these authors draw the following important conclusions:

(1) A number of lethal and sub-vital types appear in early generations of selfing.

(2) The material rapidly separates into distinct lines, which become increasingly uniform for differences in various morphological and functional characteristics.

(3) Many of the lines decrease in vigour and fecundity until they cannot be maintained even under the most favourable culture conditions.

(4) The lines that survive show a general decline in size and vigour.

Figure 52.4 shows the decline in size and vigour due to inbreeding in maize; here, the inheritance of two quantitative traits namely plant height and grain yield of three lines are shown for 30 generations of inbreeding. It can be noticed that fixation for plant height occurred after five generations of inbreeding. However, yield continued to decline for at least 20 generations until it reached one-third that of open-pollinated variety from which they were derived.

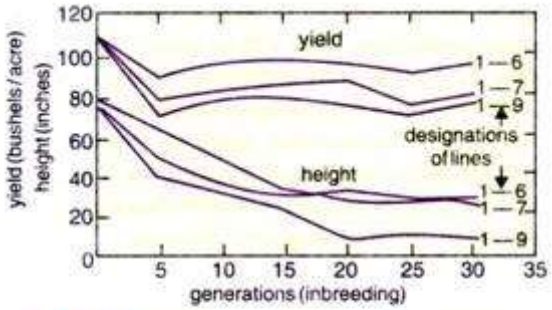


Fig. 52.4. A comparison of three lines of maize, derived from a variety, self-fertilized for 30 generations. Intitially, there were four lines, but it became impossible to maintain one of them beyond 20 generations of inbreeding.

Despite this conspicuous decline, maize was found more tolerant to inbreeding than some organisms where few strains survive two or three generations of inbreeding, e.g., alfalfa and onions.

In alfalfa, upon selfing many sub-vital and lethal types appear and the rate of decline of general vigour and productivity is alarming. The very small number of lines which survive give a greatly reduced forage yield. But onions (a normally cross- pollinated species) are quite tolerant to inbreeding, i.e., they show much less depression in vigour due to inbreeding than alfalfa and maize. Carrot is another cultivated species in which inbreeding leads to loss in vigour and production.

The following cross- pollinated plants are found to be fairly tolerant to inbreeding: sunflowers, rye, timothy, smooth broom-grass and orchard grass. In certain self- pollinated species and normally cross-fertilizing species such as cucurbits, inbreeding is found to be continued indefinitely without any ill effect. In most animals, inbreeding is found to have less remarkable effects on vigour. For example, in rats continuous brother-sister mating were performed for 25 generations, but no drastic deterioration was detected. In Drosophila, inbreeding usually results in a rapid loss of vigour, but some strains compare favourably with outbreed populations after long continued inbreeding.

However, in certain breeds of cattle, intensive inbreeding has led to an unfortunate condition; for example, exhaustive inbreeding and selection of beef cattle breed (Hereford) produced dwarf calves of low economic value. These calves show characteristic head and body features of the brachycephalic dwarfism (i.e., the characteristic short, broad head, extra long lower jaw, bulging forehead, out of proportion abdomen and short legs).Breeding data indicate that a basic recessive gene is necessary for dwarfing, but additional modifier genes have been postulated to account for the different types of dwarfs.

Practical Applications of Inbreeding:

The correlation of inbreeding and homozygosity exhibits how inbreeding may cause deleterious effects. As we already know that in a heterozygous individual, the harmful recessive alleles remain masked by their normal dominant alleles.

If a hetetozygous individual undergoes inbreeding for various generations, there will be equal chances of homozygosity for dominant as well as recessive alleles. In homozygous condition, recessive alleles will be able to express their deleterious phenotypic effects on an individual. On the other hand, the homozygosity for dominant alleles have equal opportunity to express their beneficial phenotypic effects on inbred races.

The practical applications of inbreeding:

1. Because inbreeding cause homozygosity of deleterious recessive genes which may result in defective phenotype, therefore, in human society, the religious ethics unknowingly and modem social norms consciously have condemned and banned the marriage of brothers and sisters. Further, the plant breeders and animal breeders too avoid inbreeding's in the individuals due to this reason.

2. The inbreeding because, results in the homozygosity of dominant allele, therefore, it is a best means of mating among hermaphrodites and self-pollinating plant species of several families. The animal breeder have employed the inbreeding to produce best races of horses, dogs, bulls, catties, etc.

The modern race horses, far example, are all descendants of three Arabian stallions imported into England between 1689 and 1730 and mated with several local mares of the slow, heavy type that had carried the medieval knights in heavy armour.

The fast runners of F_1 were selected and inbred and stallions of the F_2 appear as beginning points in the pedigrees of almost all modem race horses. This sort of inbreeding in also called line breeding which has been defined as the mating of animals in such a way that their descendants will be kept closely related to an unusually desirable individual.
Similarly, merino sheep are widely known as fine wool producers. They are the result of about 200 years of inbreeding. This strain was being developed in Spain in the 17th century by stock raisers.

They observed that the ancestors of the present day merino sheep had two coats of wool, one composed of long, coarse fibres arising from primary follicles, and a second coat composed of short fine wool arising from clusters of secondary follicles.

Intensive artificial selection was maintained for animals with more uniform production of fine wool and a lesser amount of coarse wool. For a time, Spain had a monopoly on the valuable merino sheep.

When France invaded Spain, merino sheep were moved to France where they were maintained and eventually distributed to other parts of the world. Merino sheep were taken to South Africa and in 1796 they were introduced into Australia which has since become the world's largest producer of fine wool.

Conclusion:

Unlike natural selection, genetic drift does not depend on an allele's beneficial or harmful effects. Instead, drift changes allele frequencies purely by chance, as random subsets of individuals (and the gametes of those individuals) are sampled to produce the next generation.

Every population experiences genetic drift, but small populations feel its effects more strongly. Genetic drift does not take into account an allele's adaptive value to a population, and it may result in

loss of a beneficial allele or fixation (rise to 100\%100%100, percent frequency) of a harmful allele in a population.

The founder effect and the bottleneck effect are cases in which a small population is formed from a larger population. These "sampled" populations often do not represent the genetic diversity of the original population, and their small size means they may experience strong drift for generations.

Suggested Readings:

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7. University of California Museum of Paleontology. (2016). Bottlenecks and founder effects. In Understanding evolution. http://evolution.berkeley.edu/evolibrary/article/bottlenecks_01.
8. University of California Museum of Paleontology. (2016) Genetic drift. In Understanding evolution. Retrieved from http://evolution.berkeley.edu/evolibrary/article/evo_24.

Probable Questions:

1. What is Genetic Drift? How it differs from Natural Selection?
2. Define and explain Founder Effect with examples.
3. Define and explain Bottle neck Effect with examples.
4. What is adaptive evolution?
5. What is inbreeding? Describe the mechanism.
6. What is Assortative and Disassortative Mating?
7. Define inbreeding depression.
8. What are the practical applications of inbreeding?
9. What are the genetic effects of inbreeding.

Unit-XI

Biodiversity Resource Management: values and uses of biological diversity, invertebrate diversity as bioindicator; putting a price on biological diversity ; pollinating insect diversity and their management and utilization in sustainable agriculture

Objective: In this unit you will learn about values and uses of biological diversity. You will also learn about bioindicator species and pollinating insect diversity and their management and utilization in sustainable agriculture

Values of Biodiversity:

Some of the major values of biodiversity are as follows: 1. Environmental Value 2. Social Value 3. Ecosystem Services 4. Economic Value 5. Consumptive use value 6. Productive Use Value 7. Ethical and Moral Value 8. Aesthetic Value.

Biodiversity is the most precious gift of nature mankind is blessed with. As all the organisms in an ecosystem are interlinked and interdependent, the value of biodiversity in the life of all the organisms including humans is enormous.

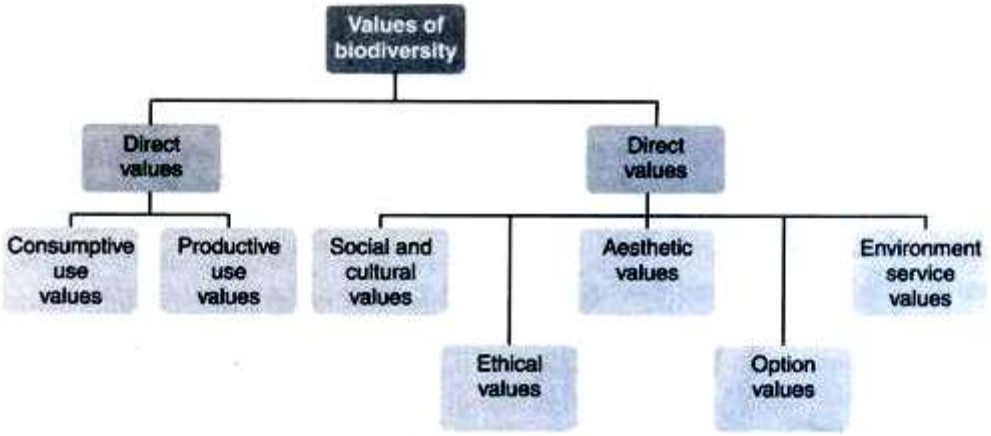
The role of biodiversity in providing ecosystem services is twofold.

Firstly, biodiversity is directly used as a source for food, fibre, fuel and other extractable resources. Secondly, biodiversity plays an important role in ecosystem processes providing the regulating, cultural and supporting services.

For example, vegetation cover protects the soil from erosion by binding soil particles and minimizing the effects of water runoff. Likewise, cultivation of crops is to a large extent dependent on the availability of pollinating insects. Biodiversity has a fundamental value to humans because we are so dependent on it for our cultural, economic, and environmental well-being. Elements of biodiversity can contribute to cultural identity, and many ecosystem characteristics are frequently incorporated into cultural traditions.

Other facts of human well-being, such as health and economic and political security, can influence the value of biodiversity. Many arguments to increase efforts to conserve diversity often emphasize the value of the “un-mined riches” that has yet to be discovered. These include potential sources of new foods, medicines, and energy which can further fuel economic activity, as well as a healthier population. Biodiversity has proven to hold enormous value when adapted for use in health, agricultural, or industrial applications.

In the field of medicine alone, approximately 50% of current prescription medicines are derived from or modelled on natural substances. The health and diversity of ecosystems can have a significant effect on the overall stability of nearby communities.



Values of Biodiversity

1. Environmental Value:

The environmental value of biodiversity can be found by examining each ecosystem process and identifying the ecosystem services that result. For instance, in wetlands the vegetation captures water-carried sediment and the soil organisms break down a range of nutrients and pollutants washed into the area.

These processes provide the ecosystem service of purifying water. Wetlands also act as spawning and nursery grounds for some fish and provide a refuge for animals in times of drought. Some ecosystem services are easy to overlook until the underlying process is impaired.

For instance, dry-land salinity has emerged as a problem following sustained clearance of deep rooted perennial plants over wide areas. Water tables have raised carrying dissolved salts which then concentrate in the soil. Forests regulate the amount of carbon dioxide in the air by releasing oxygen as a by-product during photosynthesis, and control rainfall and soil erosion.

2. Social Value:

The social value of biodiversity includes aesthetic, recreational, cultural and spiritual values. To this can be added health benefits resulting from recreational and other activities. While traditional societies which had a small population and required less resources had preserved their biodiversity as a life supporting resource, modern man has rapidly depleted it even to the extent of leading to the irrecoverable loss due to extinction of several species.

Thus apart from the local use or sale of products of biodiversity there is the social aspect in which more and more resources are used by affluent societies. The biodiversity has to a great extent been preserved by traditional societies that valued it as a resource and appreciated that its depletion would be a great loss to their society.There can be marked differences in landscape and biodiversity preferences according to age, socioeconomic factors and cultural influences. The lifestyle of the ancient people was closely interwoven with their surroundings.

The life of the indigenous people in many parts of the world still revolves around the forests and environment, even in these modern times, many of them still live in the forests and meet their daily requirements from their surroundings.

The biodiversity in different parts of the world has been largely preserved by the traditional societies. Since the indigenous people always protect the forests for their own benefit. In ancient times, especially in India, the environment in totally i.e., flora, fauna, etc., were held in high esteem.Trees like Peepal, Banyan and Tulsi are still worshipped. Ladies offering water to Tulsi daily is considered good and there are festivals when ladies tie sacred threads around Peepal and Banyan trees and pray for the welfare of their families.

3. Ecosystem Services:

These services also support human needs and activities such as intensely managed production ecosystems.

Ecosystem service includes:
a. The production of oxygen by land based plants and marine algae;

b. The maintenance of fresh water quality by vegetation slowing run off, trapping sediment and removing nutrients and by soil organisms breaking down pollutants;

c. The production and maintenance of fertile soil as a result of many interacting processes;

d. The provision of foods such as fish, pastures for cattle and sheep, timber, fire wood and harvested wildlife such as kangaroos and native cut flowers;

e. The provision of native species and genes used in industry research and development, for instance, in traditional breeding and biotechnology applications in agriculture, forestry, horticulture, mariculture, pharmacy, chemicals production and bioremediation;

f. Pollination of agricultural crops, forest trees and native flowering plants by native insects, birds and other creatures;

g. Pest control in agricultural land by beneficial native predators;

h. Flood mitigation by vegetation slowing run off and trapping sediment;

i. Breakdown of pollutants by micro-organisms in soil and aquatic ecosystems and sequestration of heavy metals in marine and fresh water sediments;

j. Greenhouse gas reduction by, for instance, sequestering atmospheric carbon in wood and marine calcium carbonate deposits;

k. Maintenance of habitats for native plants and animals; and

l. Maintenance of habitats that are attractive to humans for recreation, tourism and cultural activities and that has spiritual importance.

4. Economic Value:

The economic potential of biodiversity is immense in terms of food, fodder, medicinal, ethical and social values. Biodiversity forms the major resource for different industries, which govern the world economy.

The salient features regarding the economical potential of biodiversity are given below:

1. The major fuel sources of the world including wood and fossil fuels have their origin due to biodiversity.

2. It is the source of food for all animals and humans.

3. Many important chemicals have their origin from the diverse flora and fauna, used in various industries.

4. Diverse group of animals are used for medical research during the testing of new drugs.

5. Consumptive use value:

This is related to natural products that are used directly for food, fodder, timber, fuel wood etc. Humans use at least 40,000 species of plants and animals on a daily basis. Many people around the world still depend on wild species for most of their needs like food, shelter and clothing. The tribal people are completely dependent on the forests for their daily needs.

6. Productive Use Value:

This is assigned to products that are commercially harvested and marketed. Almost all the present date agricultural crops have originated from wild varieties. The biotechnologists continuously use the wild species of plants for developing new, better yielding and disease resistant varieties. Biodiversity represents the original stock from which new varieties are being developed.

7. Ethical and Moral Value:

It is based on the principle of 'live and let others live'. Ethical values related to biodiversity conservation are based on the importance of protecting all forms of life. All forms of life have the right to exist on earth. Man is only a small part of the Earth's great family of species.

Don't plants and animals have an equal right to live and exist on our planet which is like an inhabited spaceship? Morality and ethics teach us to preserve all forms of life and not to harm any organism unnecessarily. Some people take pleasure in the hunting of animals. People also sometimes degrade and pollute the environment by their unethical actions. Through proper education and awareness, the people's conscience against such practices must be raised.

8. Aesthetic Value:

The beauty of our planet is because of biodiversity, which otherwise would have resembled other barren planets dotted around the universe. Biological diversity adds to the quality of life and provides some of the most beautiful aspects of our existence. Biodiversity is responsible for the beauty of a landscape.

People go far off places to enjoy the natural surroundings and wildlife. This type of tourism is referred to as eco-tourism, which has now become a major source of income in many countries. In many societies, the diversity of flora and fauna has become a part of the traditions and culture of the region and has added to the aesthetic values of the place.

Bioindicators:

Bioindicators are living organisms such as plants, planktons, animals, and microbes, which are utilized to screen the health of the natural ecosystem in the environment. They are used for assessing environmental health and biogeographic changes taking place in the environment. Each organic entity inside a biological system provides an indication regarding the health of its surroundings such as plankton responding rapidly to changes taking place in the surrounding environment and serving as an important biomarker for assessing the quality of water as well as an indicator of water pollution. Even the health of aquatic flora is best reflected by plankton, which acts as an early warning signal. In this review we have tried to explain the concept behind Bioindicators and plankton, with particular emphasis on their potential to be used as Bioindicators for water quality assessment and outcomes relating to this.

Naturally occurring Bioindicators are used to assess the health of the environment and are also an important tool for detecting changes in the environment, either positive or negative, and their subsequent effects on human society. There are a certain factors which govern the presence of Bioindicators in environment such as transmission of light, water, temperature, and suspended solids. Through the application of Bioindicators we can predict the natural state of a certain region or the level/degree of contamination (Khatri & Tyagi 2015) The advantages associated with using Bioindicators are as follows: (Figure 1)

a. Biological impacts can be determined.

b. To monitor synergetic and antagonistic impacts of various pollutants on a creature.

c. Early stage diagnosis as well as harmful effects of toxins to plants, as well as human beings, can be monitored.

d. Can be easily counted, due to their prevalence.

e. Economically viable alternative when compared with other specialized measuring systems.

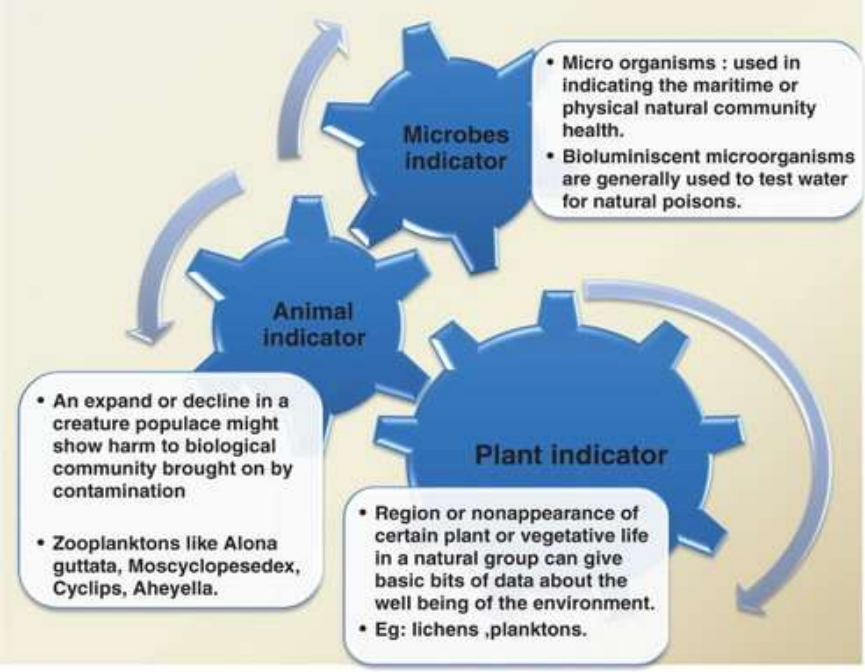


Figure 1. Types of Bioindicato

Utilization of Bioindicators:

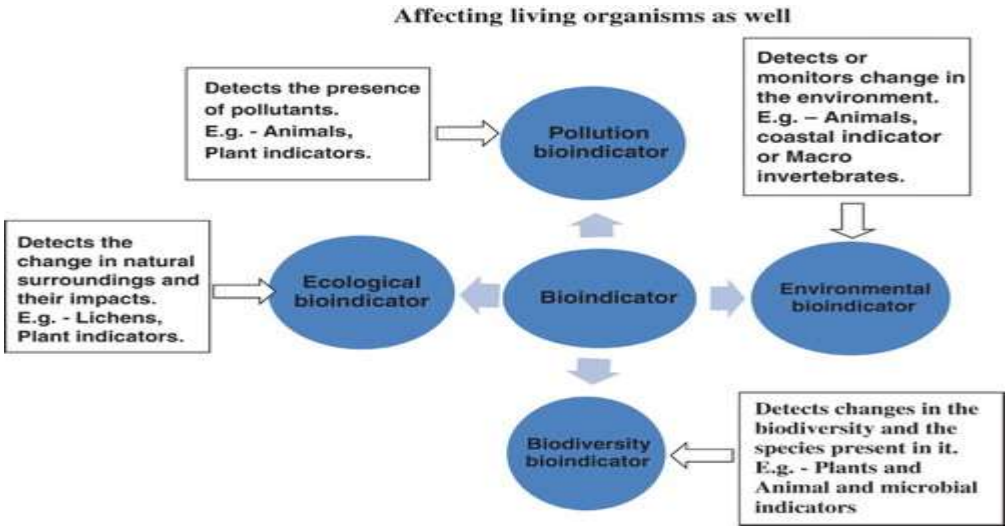
The expression 'Bioindicator' is used as an aggregate term referring to all sources of biotic and abiotic reactions to ecological changes. Instead of simply working as gauges of natural change, taxa are utilized to show the impacts of natural surrounding changes, or environmental change. They are used to detect changes in natural surroundings as well as to indicate negative or positive impacts. They can also detect changes in the environment due to the presence of pollutants which can affect the biodiversity of the environment, as well as species present in it

The condition of the environment is effectively monitored by the use of Bioindicator species due to their resistance to ecological variability. Hasselbach et al. utilized the moss i.e. *Hylocomium splendens* as a natural indicator of heavy metals in the remote tundra environment of north-western Alaska. Here, the ore of mineral is mined from Red Dog Mine, the world's largest creator of zinc (Zn), and is carried to a singular street (~75 km long) to storage spaces on the Chukchi Sea. Hasselbach and her partners inspected whether this overland transport was influencing the encompassing physical biota. The contents of heavy metals inside the moss tissue were analyzed at different distances from the street. The concentrations of metals in moss tissue were most prominently adjacent to the haul street and reduced with distance, therefore supporting the theory that overland transport was in fact modifying the encompassing environment. In this study, lichens were utilized as biomonitors by utilizing the quantitative estimation of metal concentrations inside individual lichen.

Natural, biological, and biodiversity markers can be found in various organisms occupying different types of environments. Lichens (a symbiosis among Cyano bacteria, algae, and/or fungi) and Bryophytes (liverworts) are frequently used to monitor air contamination. Both, Lichens and Bryophytes are powerful Bioindicators of air quality on the grounds that they have no roots, no fingernail skin, and acquire all their supplements from immediate introduction to the climate. Their high surface region to volume ratio further supports the theory of their use as a bioindicator, or supports their ability to capture contaminates from the air. Cynophyta, a type of phytoplankton, is one particularly powerful bioindicator which is known to indicate rapid eutrophication of water bodies such as reservoirs, lakes, etc. via the creation of bloom formations.

Types of bioindicators:

Bioindicators are presently utilized and promoted by various organizations (the World Conservation Union, International Union for Conservation of Nature), as a means to handle biomonitoring and evaluate human effects.



Animals as Bioindicators:

Variations in the populations of animals may indicate harmful changes caused due to pollution into the ecosystem. Changes in the population density may indicate negative impacts to the ecosystem. Changes in populations may be a result of the relationship between populations and food sources; if food resources become scarce and cannot provide for the population demand reduction of said population will follow (Plafkin et al. 1989; Phillips & Rainbow 1993; Jain et al. 2010). Animal indicators also help in detecting the amount of toxins present in the tissues of animals (Joanna 2006; Khatri & Tyagi 2015). Frogs are also Bioindicators of quality of environment and changes in environment. Frogs are basically influenced by changes that take place in their freshwater and terrestrial habitats. This makes them important Bioindicators of ecological quality and change. Zooplanktons like Alonaguttata, Mesocyclopsedax, Cyclops, Aheyella are zone-based indicators of pollution (Underwood & Shapiro 1999; Hans et al. 2003; Jha & Barat 2003; Ramchandra et al. 2006; Pradhan et al. 2008; Zannatul & Muktadir 2009; Jain et al. 2010; Nkwoji et al. 2010; Hosmani 2014). Invertebrates can also be Bioindicators; aquatic invertebrates tend to be bottom feeders (also known as Benthos or macro invertebrates), living near the bottom of water bodies. These types of Bioindicators may be particularly powerful indicators of watershed health as they are not difficult to distinguish in a lab, frequently live for more than one year, have restricted mobility, and are integrators of ecological condition (Plafkin et al. 1989; Khatri & Tyagi 2015).

Microbial indicators:

Microorganisms are often used as health indicators of aquatic and terrestrial ecosystems. Due to their abundance, they are easy to test and readily available. Some microorganisms when exposed to cadmium and benzene contaminants develops new proteins known as stress proteins which can be used as early warning signs (Khatri & Tyagi 2015). Microorganisms are an important part of oceanic biomass and are responsible for the majority of productivity and nutrient cycle in a marine ecosystem. Microorganisms have a rapid rate of growth, and react to even low levels of contaminants and other physicochemical and biological changes. From a research perspective they give important signs of environmental change (Underwood & Shapiro 1999; Gerhardt 2002; Hans et al. 2003; Jha & Barat 2003; Markert et al. 2003; Ramchandra et al. 2006; Pradhan et al. 2008; Zannatul & Muktadir 2009; Nkwoji et al. 2010; Hosmani 2014). Microbial indicators can be used in a variety of ways to detect environmental pollutants in water including the use of bioluminescent bacteria. The presence of toxins in waters can be easily monitored either by changes in the digestion system of microbes which is hindered or disturbed by the presence of toxins which may result in changes in the amount of light emitted by the bacteria (Arora 1966; Grizzle 1984; Butterworth et al. 2001; Uttah et al. 2008). In comparison to other available traditional tests, these tests are very quick to monitor; however, their limitation is they can only indicate the changes in the organisms due to presence of toxins (Malik & Bharti 2012; Khatri & Tyagi 2015). One such example is the bacterium *Vogesella indigofera* which reacts to heavy metals quantitatively. Under the influence no metal pollution, this bacterium produces blue pigmentation which is an important marker of morphological change that has taken place which can be effectively observed visually. Alternatively, under the vicinity of hexavalent chromium, the production of pigment is blocked. This pigment production can be attributed due to the relationship between concentration of chromium and the generation of blue pigmentation by the bacterium (Arora 1966; Grizzle 1984; Paoletti 1999; Oberholster et al. 2009; Jain et al. 2010; Aslam et al. 2012; Malik & Bharti 2012).

Biomonitoring:

Bio-organisms are basically used to define the characteristics of a biosphere. These organisms are known as Bioindicators or biomonitors, both of which may vary considerably (Purdy 1926; Mohapatra & Mohanty 1992; Gaston 2000; Lilian 2009; Offem et al. 2011). When studying the environment the quality of changes taking place can be determined by Bioindicators while

biomonitors are used to get quantitative information on the quality of the environment biological monitoring also incorporating data regarding past aggravations and the impacts of various variables (Noss 1990; Gaston 2000; Chakrabortty & Paratkar 2006). Monitoring can be done for various biological processes or systems with the objective of observing the temporal and spatial changes in health status, assessing the impacts of specific environment or anthropogenic stressors and assessing the viability of anthropogenic measures (e.g. reclamation, remediation, and reintroduction) (Lund 1969; Cairns et al. 1993; Niemi et al. 1997; Burger & Gochfeld 2001; Mahadev & Hosmani 2004; Pandey & Verma 2004; Hosmani 2013). The species diversity is used as a prime aspect in biological monitoring, which is considered to be a valuable parameter in determining the health of the environment (Marques 2001; Joanna 2006). Biomonitoring is one of the essential components for assessing the quality of water and has become an integral element of conducting studies on water pollution (Vitousek et al. 1997; Butterworth et al. 2001). Biomonitors are freely available all around the world. They fundamentally mirror the natural impact over creatures and can be used and understood with minimum preparation and training (Reynolds 1984; Burger 1993; Green 1993; Siddiqui & Chandrasekhar 1996; Chorus & Bertram 1999; Carignan & Villard 2001; Nájera et al. 2002; Kumari et al. 2007; Fadila et al. 2009). Despite the fact that all natural species can be considered biomonitors to some degree, the above focal points applies well to planktons and similar species type, when water pollution is considered (Singh et al. 2013).

Pollination by Insects:

Animal-mediated pollination plays an important functional role in most terrestrial ecosystems and provides a key ecosystem service vital to the maintenance of both wild and agricultural plant communities as most angiosperms are pollen-limited and rely on animals for sexual reproduction. A large proportion of the human diet depends directly or indirectly on animal pollination. Insects are the primary animal-mediated pollinators of 80% of all plant species in world, including most fruits, many vegetables and some biofuel crops and include diverse species of Hymenoptera (bees, solitary species, bumblebees, pollen wasps and ants), Diptera (bee flies, houseflies, hoverflies), Lepidoptera (butterflies and moths), Coleoptera (flower beetles), and other insects. Insects especially are of enormous importance in the pollinations of many agriculturally important crops. Although gravity, wind, water, molluscs, birds, bats and humans are agents of pollination, it is often possible to manipulate insects in their performance on high value cropping systems. Presently insect pollinators are considered to be in a state of decline due to a range of recent and projected environmental changes, such as habitat loss and climate change, with unknown consequences for pollination service delivery.

Insect-Pollinated Flowers Classified According to Insect Adaptation

a. Pollen Flowers (Group I):

Flowers such as rose, poppy, elderberry and potato, have no nectar but may be scented, they are generally conspicuous, simple, regular, with pollen freely exposed and usually abundant. A great variety of color types are included. Insects involved are usually Syrphidae flies, soldier flies and pollen feeding beetles. Many pollen gathering bees, including honeybees, usually frequent these flowers. They are generally unattractive to Colletiidae bees, male bees, bee flies, moths, butterflies and hummingbirds.

b. Flowers with Exposed Nectar (Group II):

Maple, carrot, some elderberries, Euphorbia, poison oak, grapes and saxifrage flowers are included here. Their sparce nectar is freely exposed as droplets, the flowers are simple, open and regular, and the inflorescence is usually inconspicuous and greenish-white. They attract many kinds of wasps and

short-tongued flies and bees. They are not very attractive to long-tongued bees or flies and Lepidoptera, but some are attractive to honeybees.

c. Flowers with Partly Concealed Nectar (Group III):

Examples are strawberry, cactus, raspberry, stone fruits, many cruciferous species and buttercups. Here the nectar is partly concealed by numerous stamens or hairs or overlapping petals. The flowers are usually completely open only in sunshine and may be moderately to quite conspicuous. White and yellow colours predominate, but pink can be common. Attracted insects are Syrphidae flies and short-tonged bees. Some Rosaceae are also attractive to long-tonged bees and honeybees. Sawflies are common on many species, and some beetles and butterflies may also be attracted.

d. Flowers with Concealed Nectar (but not sexual organs or deep narrow corollas) (Group IV):

Currant, onion, orange, mallow and blueberry are included here. The nectar is completely hidden in pouches or by hair tufts. The flowers usually have corolla tubes and may be somewhat irregular. They are generally conspicuous flowers with blue, red or violet predominating. Long-tongued bees and honeybees are attracted. Also some short-tonged bees, bee flies, long-tongued wasps, Lepidoptera. Rarely attracted are most wasps and short-tongued flies and beetles.

e. Social Flowers with Completely Concealed Nectar (Group V):

The Compositae such as dandelion, aster, sunflower and *Scabiosa* are included. The nectar is hidden in narrow but not deep corolla tubes, but access to nectar is blocked by the stigma and the cone of stamens. Pollen is very abundant. The inflorescence is conspicuous because of the grouping of flowers into heads. The colour groups white and yellow, and red and blue, are attractive. This group is very attractive to short and long-tongued bees, many butterflies and polleniferous beetles and Syrphidae flies. Insects that visit white and yellow flowers in this group are akin to those visiting flowers with partly concealed nectar, while those visiting red, blue and purple flowers are akin to those visiting flowers with concealed nectar.

f. Hymenoptera Flowers (Group VI):

Violets, legumes, sages, mints, monkshood, *Delphinium*, iris and some lilies are included here. The nectar is concealed in bilaterally symmetrical flowers with slightly long corolla tubes closed at the throat. The sexual organs are usually partially concealed by modified petals that require operation of a special mechanism to expose them. They are usually positioned horizontally, with special landing structures for the pollinator. These flowers are visited primarily by medium to long-tongued bees that can operate the mechanisms to get at the pollen and nectar. Lepidoptera that visit these flowers generally do not operate the mechanism exposing pollen so they do not accomplish pollination. They are visited in the same manner by long-tongued Conopidae flies and bee flies. Many have such deep nectarines as to be accessible only to bumble bees and a few other insects. Others have tough tripping mechanisms that require large, powerful bees for pollination. Other bees may bite holes in the corollas to rob the nectar without pollinating. One group of Hymenoptera flower might be called "wasp flower." It has a ventral pouch filled with nectar and a dull red colour.

g. Lepidoptera Flowers (Group VII):

This group includes such species as tobacco, trumpet flowers, honeysuckle, croc gentian, many orchids and some lilies. The flowers bear nectar at the base of long, narrow corolla tubes and

spurs. They are rather large and conspicuous with a strong scent. Mainly Lepidoptera pollinate these, but long-tongued Hymenoptera may frequent some species. In tropical areas stingless bees are able to crawl into the slender corollas and spurs. Long-tongued bee flies may also use them. Hummingbirds and honey birds are also important pollinators in tropical regions. Within the Lepidoptera butterfly and moth flowers differ. Butterfly flowers have variable colours and they usually open and are fragrant during daytime. On the other hand, moth flowers usually open and are fragrant only at night. They are generally white or pale coloured.

h. Special Types of Flowers (Group VIII):

Nauseous flowers that are attractive to flies include some umbellifera, calla lilies, skunk cabbage and many types of saxifrage. They may give off odours of faeces, carrion or ammonia. They are especially attractive to filth flies, dung beetles and others.

Pitfall flowers are also often nauseous. Included are Jack-in-the-pulpit, pitcher plants and Dutchman's pipe. They capture flies, holding them until they become covered with pollen, after which they are released before the stigma is receptive.

Pinch-trap flowers include the milkweeds and some orchids. The pollen born on "pollenia" fastens onto visitors and are later pulled off in stigmatic grooves of the pistil. These are attractive to flies, bees and wasps.

Syrphid fly flowers include *Veratrum* and *Veronica*. The flowers bear radiating streaks that lead to small, definite centers. Two long stamens are able to dehisce on the back of the syrphid fly when grasped at the base. Only syrphids are able to accomplish this.

Small insect flowers include some aquatic species and euphorbias and figs. There is an array of minute flowers that are attractive to tiny insects. The flowers may be clustered in a hollow receptacle (as in the fig) with an opening to the inflorescence that is just large enough to accommodate the tiny insect.

Importance of Pollination to Agriculture:

Insects in their pollination activities have a direct impact on the evolution of flora and fauna. It is believed that angiosperm plants and the more highly evolved insects evolved together. Primitive flowering plants are all insect pollinated. Therefore, grasses and all other angiosperms arose from plants dependent upon insects. Some beetles, most Hymenoptera, many Diptera and almost all Lepidoptera are dependent upon materials provided by flowers. Without angiosperms the evolution of mammals would certainly have been different. Rodents, herbivores and primates are especially dependent upon the products of flowering plants. Thus, angiosperms were a required forerunner to the stocks, which gave rise to humans, and insect pollination was necessary to the development of angiosperms.

There would be grave consequences for the flora and fauna were pollinating insects to disappear or cease pollinating. Many types of plants would most likely perish eventually because in time they would be dependent on insect pollination for competitive reproduction. These would embrace by far most of the angiosperms. Certain elements of flora would rapidly perish. Plants that are usually propagated by seed are dependent upon insects for adequate pollination. Included here would probably be over half of the existing species. Plants that usually propagate asexually could probably survive for many seasons or generations. But asexual propagants are very limited in powers of dissemination and those species would have a fixed genetics incapable of adjusting to changes, which would be expected to be rapid under such conditions. Self-fertile plants that are capable of auto-self pollination might be able to persist longer. However, most of these are dependent upon occasional crossing in order to retain vigor. All would require some crossing in order to retain the genetic plasticity necessary to adjust to changing environmental conditions.

Some plants might survive indefinitely without insect pollinators and some might increase in the absence of normal competition. These include many nut-bearing trees, grasses, all conifers, and various other wind pollinated plants such as poplars, birches, elms, alders, etc. Even so, many grasses and other plants most certainly depend upon the surrounding flora for their survival. Those plants that are produced as crops by humans and propagated by asexual means might also be unaffected. Breeding for disease resistance, for example, could be done with hand pollination. Nevertheless, there are many consequences of a drastic reduction and elimination of most floras. These include the loss of plants with nitrifying bacteria, soil erosion, a drastic curtailment of the human diet, loss in forage values for livestock, loss of many kinds of animals, loss of most kinds of wild flowers, and a general upset in the balance of nature, with unpredictable results.

Advanced agriculture manages the production of products that require pollination, which are primarily fruits and seeds. Seeds are used for general plant propagation and for bedded plants. Some plants like papaya require occasional seeding; alfalfa is seeded every few years and spinach is seeded annually. Alfalfa and forage grasses often require a large amount of seed, while tomatoes and melons need little seeding. Plant breeding by crossing, selfing and selecting is done with pollination and planting with seeds. Plant products that are consumed directly include cereals, beans, nuts, oils, fruits, preserves and many vegetables. Seeds such as grains, oilcake and peanuts are also used for livestock feed. Many seeds are used as medicines, spices and flavourings. Seeds, fruit oils and seed fibers are deployed in industry for soaps, paints, plastics, explosives, alcohol and textiles.

Biodiversity of insect pollinators:

a. Insect Pollinators Other Than Hymenoptera:

There are more insect species than all other animals and plants combined, the total number estimated to be over two million as of 2010. Joined appendages and an external skeleton characterize insects as part of the Arthropoda. Included are spiders, crustaceans, centipedes and scorpions. Insects are classified into 28 major orders, but seven comprise most of the species. These are, in order of increasing specialization and importance as pollinators, the Orthoptera (cockroaches, grasshoppers, crickets, walking sticks, praying mantis), Hemiptera (true bugs, cicadas, leafhoppers, scale insects, aphids), Thysanoptera (thrips), Coleoptera (beetles), Diptera (flies, gnats, mosquitoes), Lepidoptera(moths and butterflies), and Hymenoptera (ants, wasps, bees, sawflies, Ichneumon flies and chalcid flies). For the most part the Orthoptera of no importance as pollinators.

a. Hemiptera :

Only a few Hemiptera of value are Anthocoridae (minute pirate bugs), Phymatidae (ambush bugs) and Reduviidae (kissing bugs). The Anthocoride prey on thrips in flowers; a few Reduviidae prey on bees in flowers and most Phymatidae prey on bees and flies in flowers. Anthocoridae are found in almost any flowers that are visited by thrips. Phymartids and reduvids are found primarily on Compositae and flowers that are grouped into tight heads.

b. Coleoptera :

Except for a few flower-inhabiting forms, the Coleoptera are not as important pollinators as the Diptera, Lepidoptera and Hymenoptera. There are nine families of Coleoptera that are at times involved in the pollination of flowers. Most species of Cantharidae, the leather-winged beetles, that are predaceous as larvae occasionally pollinate.. Polleniferous species are also predaceous as adults. The majority of Meloidae, or blister beetles, occasionally are involved in pollination. The larvae of some species are parasitic in bee nests; others are parasitic on grasshopper egg masses. All

adult Meloidae feed on pollen or on both nectar and pollen. The larvae of some species of Cleridae are flower inhabiting. They are mainly parasites in the nests of wasps and bees. The adults are predaceous, but they also feed on pollen. Most Melyridaeare predaceous as larvae and both predaceous and polleniferous as adults. One genus of Buprestidae, *Acmaeodera* (flat-headed borer) is polleniferous. The larvae bore into wood and the adults feed on pollen. Many genera of Cerambycidae, or long-horned beetles and round-headed borers, can be involved as pollinators. The larvae bore into wood but the adults feed on pollen. Several genera of Scarabaeidae, or white grubs, visit flowers. They are primarily root-feeders as larvae, but they also feed on pollen as adults. Elateridae, or click beetles, are mostly root-feeders as larvae, but adults will feed on nectar and pollen. In the Dermestidae, the genus *Anthrenus* feed on decaying animal matter as larvae, but adults may also utilize pollen (especially *Anthrenus*). There are also other small families of Coleoptera, such as the Mordellidae, Oedemeridae, Lycidae and Rhipiphoridae, whose members have been observed to act as pollinators.

Most groups of flowers do not escape visits by beetles feeding on their petals as well as nectar and pollen. Some blister beetles will feed on legume petals in order to expose the pollen and nectar. Some very tiny flower-visiting beetles may crawl into the narrowest corollas or tightest keels. Nevertheless, only a few groups of flowers are visited regularly by a variety of beetles. Examples are flowers with abundant pollen, social flowers with concealed nectar, flowers with exposed nectar and flowers with partially concealed nectar.

c. Diptera:

The adults of several large families of Diptera feed frequently on nectar or pollen or both, but the larvae are usually harmful to plants. Examples are found in the Anthomyidae (hovering house flies), Bombyliidae (bee flies), Calliphoridae (blow flies & bottle flies), Ceratopogonidae (biting midges) Conopidae (thick-headedflies), Cyrtidae (small-headed flies), Empididae (dance flies), Muscidae (house flies), Sarcophagidae (flesh flies), Stratiomyidae (soldier flies), Syrphidae (flower flies, syrphid flies, hover flies), Tabanidae males (horse flies), Tachinidae (tachinid flies), Tephritidae (fruit flies). These families might be considered in the following order of decreasing importance: Syrphidae, Muscidae, Calliphoridae, Sarcophagidae, Bombyliidae, Conopidae, Tachinidae, Empididae, Stratiomyiidae, Tabanidae, Tephritidae, Ceratopogonidae and Cyrtidae. However, this order may differ for any one-plant species. A few of the more important pollinating Diptera are discussed in the following.

Syrphidae have larvae with a wide variety of habit. They occur under bark, manure and liquid and are predatory on small insects such as aphids. The adults re almost all flower visitors. Most species feed on nectar and pollen or only nectar. Nectar-feeding species have a long, slender proboscis and generally visit the same group of flowers as the long-tongued bees. Those syrphids with short or moderate tongue length visit predominantly flowers of Groups I, Group II and Group III. Some also consume pollen on flowers of Group V. Bombyliidae have larvae that either feed on grasshopper egg masses or those that feed on the larvae of wasps and wild bees. Adults of the latter group have a long, slender proboscis and visit flowers of Group III to Group VIII, but mostly Group III and Group IV. Although a few genera are intermediate, most have very a short proboscis and visit primarily flowers of Group II.

Muscidae have larvae with various habits. Some are internal parasites of other insects, while some feed on plant roots, and a great many feed on decaying animal and plant material. The adults of most species visit flowers and eat pollen and nectar. Flowers of Group II are favored, but a few others like onion in Group IV are also visited. Species in other families of Diptera will on rare occasions pollinate plants either directly or accidentally.

d. Lepidoptera :

Adults of most Lepidoptera feed mainly on nectar from flowers, while their larvae feed on herbage, some roots or stored food products and wool and are therefore pestiferous. Their preferred flowers are in Groups IV to VII. Encounters with Hymenopterid flowers (Group VI) often do not expose the pollen and therefore do not result in pollination.

The tongue lengths of Lepidoptera vary from 1 to 250 mm. Those with 4-10 mm. Tongues are most often seen on flower Groups IV & V, while those with longer tongues are most apt to be seen on Groups VI & VII.

Butterflies tend to frequent day-blooming flowers and moths visit constantly open or evening and night-blooming flowers. The entire suborder, Rhopalocera and 5 families of Heterocera that are numerous or specially adapted as pollinators are Arctiidae (tiger moths &wooly bears), Geometridae (loopers), Noctuidae (nun moths, cut worms), Pyralidae (snout moths), Rhopaloceridae (butterflies) and Sphingidae(hawk moths & horn worms).

Because investigations of visits to flowers have been made primarily in daylight, the value of moths as pollinators is probably underestimated. Butterflies often spend a lot of time on the same flowers and they are regularly less effective than bees in pollination. Haw moths that fly in the evening or at night are assiduous flower visitors by darting rapidly from plant to plant. Their very long proboscis seems to be especially suited for the most highly developed Lepidoptera flowers that have musky odors, long and narrow corolla tubes or long spurs that contain nectar. Butterflies tend to prefer red flowers while moths prefer white flowers. Nun moths are similar to haw moths in rapid flight and long tongues. They are usually more abundant also. Many flowers are sometimes referred to as haw moth flowers, and where the corolla tube exceeds 25 mm. the term is deserved. But, hummingbirds and honeybirds contribute more effectively to the pollination of such flowers in some areas.

e. Thysanoptera (Thrips):

A small order, Thysanoptera are tiny but individual species occur in large numbers. Adults and larvae feed either mostly on honey and pollen or are predators of other thrips in flowers. It has been suggested that few indigenous flowers in Europe escape from occasional or frequent visits by thrips. Even though individual thrips may only convey pollen accidentally, their great abundance enhances their value for pollination. Nevertheless, they are generally thought to be ineffective in the pollination of many flower species and consequently they are rarely credited with much influence. They rarely migrate from plant to plant so that their role would be primarily self-pollination.

Generalizations on the use of non-hymenoptera pollinators:

Among the Coleoptera, larvae of most species are destructive and not advisable for propagation. One genus of Cantharidae (*Chauliognathus*) are predators as larvae on aphids and as adults they feed on nectar and pollen. It is able to trip alfalfa and might be adaptable to mass production in insectaries and mass release in field crops.

The larvae of many genera of Diptera are destructive. Adults may pose a health hazard and are thus unsuitable for purposeful deployment. Muscidae may be useful in confinement for breeding work and small-scale increase of desirable plant stocks. There are may good pollinators among the Syrphidae, however. They could be increased rapidly and used as predaceous forms in insectaries. Although species may resemble bees and wasps, they are non-biting. Semi-aquatic species could be increased in field crops. The drone fly, e.g., is an efficient fruit pollinator and might be propagated in shallow tanks infused with organic material.

Most larvae of Lepidoptera are also destructive and thus the group is mostly unsuitable for deployment. There may be some exceptions, but any species considered would need to be carefully studied for any possible destructive tendency. *Vanessa cardin* & *V. atalanta* (L.) feed on thistles as larvae and might be considered for the pollination of some ornamentals. Sphinx moths are more destructive to weeds than crops (excluding grapes) and could be deployed to pollinate ornamental plants. Vanilla is usually hand-pollinated, and the search for a non-destructive Lepidoptera might be made. The possibilities for deploying Lepidoptera as pollinators are probably greatest for agriculture in tropical regions.

Pollinators enhance Crop Yield and Quality:

There are many examples of the major significance of pollinators to farmers, especially in terms of increasing the volume and quality of crops produced. One example is a project ran by CropLife India, which aimed to raise farmers' awareness of the importance of honey bees to their crop yields.31 The project used a number of approaches, such as providing subsidized honey bee hives and training farmers how to use them for managed crop pollination, as well as training in Integrated Pest Management, the responsible use of pesticides32 and practical beekeeping. Overall, the project helped 230 farmers in Maharashtra district to increase their onion seed and pomegranate yields by 17 percent and almost 35 percent, respectively. Importantly, the farmers also saw their incomes increase, by 19 percent on average for onion farmers and a staggering 42 percent for those growing pomegranates. These increased profits were not only due to higher yields but also improved quality: For example, the color and shape of the pomegranates were more attractive to consumers. Moreover, spending on pesticides was reduced as farmers were taught to use them selectively and in a more targeted way. It is not just managed bees that influence crop quality. High pollinator diversity can improve several commercially important crops.33 For example, strawberries that have not been pollinated by a diverse group of insects like flies, solitary bees and honey bees can be smaller and irregular in shape, whereas those which are pollinated by these diverse insects are larger and have fewer deformations34, enabling farmers to charge a higher price. Coffee is another globally important crop where it seems that a high diversity of pollinators can improve the quality.

Suggested Readings:

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4. Chumacero de Schawe, C. et al. 2016. Abundance and diversity of flower visitors on wild and cultivated cacao (Theobroma cacao L.) in Bolivia. Agroforest Syst. 92(1): 117–125. Doi: 10.1007/s10457-016-0019-8.

5. Corbet, S.A. and P.G. Willmer. 1980. Pollination of the yellow passion fruit: nectar, pollen and carpenter bees. J. Agric. Sci. 95(3): 655–666.

6. De Luca, P.A. and M. Vallejo-Marín. 2013. What's the 'buzz' about? The ecology and evolutionary significance of buzz-pollination. Curr. Opin. Plant Biol. 16(4): 429–435. Doi:10.1016/j.pbi.2013.05.002.

7. Evans, E. 2017. From humble bee to greenhouse pollination workhorse: can we mitigate risks for bumble bees? Bee World 94(2): 34–41. Doi:10.1080/0005772X.2017.129

Probable Questions:

1. How pollination occurs by Lepidopteran insects ? Cite suitable examples.
2. How pollination occurs by Hymenopteran insects ? Cite suitable examples.
3. How pollination occurs by Hemipteran insects ? Cite suitable examples
4. How pollination occurs by Coleopteran insects ? Cite suitable examples.
5. How pollination occurs by Dipteran insects ? Cite suitable examples.
6. How pollination occurs by Thysanoptera insects ? Cite suitable examples.
7. How Pollinators enhance Crop Yield and Quality ?
8. Write down the values of Biodiversity.
9. Define Bioindicators?
10. Write up about Biomonitoring.
11. Describe the role of animals as bioindicators.
12. Describe the role of microbes as bioindicators.

Unit-XII

Vermiculture: Types of earthworms and their utilization; use in sustainable agriculture

Objective: In this section you will learn about vermiculture. You will also learn about types of earthworms and their utilization and also use in sustainable agriculture**.**

Introduction:

Vermicomposting is the operation of composting process of organic materials by involving earthworms. It is a sustainable biofertilizer generated from organic wastes. Vermicompost is an excellent source of nutrients for vegetables, ornamentals, fruits and plantation crops. Using vermicompost one can get 10-15% more crop yield, besides improvement in quality of the products.

For the first time, in 1970, vermicomposting was started in Ontario (Canada). In recent years the USA, Japan and Philippines are the leaders of vermicompost producers. So far least attention has been paid in India. But in recent years, Government and nongovernment organisations (NGOs) are trying to popularise the vermicomposting process. Through an NGO, Pithoragarh Municipality (Uttaranchal) has started vermicomposting by using a thermotolerant earthworm *Eisenia foetida*. Vermicomposting is also being done at Shanti Kunj (Haridwar). One kg earthworm can consume 1 kg organic materials in a day.

They secrete as casting which are rich in Ca, Mg, K, N and available P. Depending on substrate quality, vermicompost consists of 2.5-3.% N,1-1.5% P and 1.5-2.0% K, useful microorganisms (bacteria, fungi, actinomycetes, protozoa), hormones, enzymes and vitamins. Earthworms make tunnels and mix soil. Thus they aerate the soil which promotes the growth of bacteria and actinomycetes. Consequently, microbial activity of soil is increased due to increase in enzymatic and biological activity of earthworms. About 500 species of earthworms are known in India and over 3,000 in the world. The most common members of the earthworm to be used in vermicomposting include: *Eisenia andrie. E. foetida, Dravidawillsii, Endriluseuginee, Lamitomauritii, Lubrieusrubellus and Perionyx excavatus*.

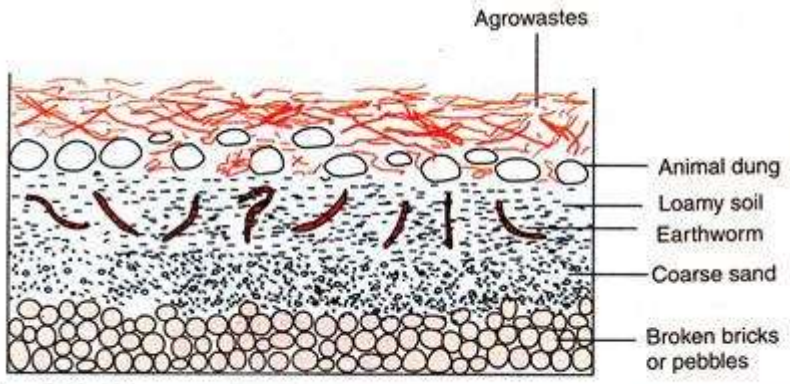

Fig. 33.1 : Diagram of a pit for operation of vermicomposting process.

Participating Organisms of the Vermicomposting:

Bacteria, micro-organisms and earthworms participate in the vermicomposting process, of which bacteria and micro-organisms are used as food for worms.

Throughout the world, 3 or 4 species are used in vermiculture but two species are used extensively in India. *Eisenia foetida* and *Eudrilus eugeniae* are used throughout India for vermiculture. A list of some earthworm species is given below with some features which are used in different parts of the country.

Family Lumbricidae:

Eisenia foetida:

Distribution:
They are found throughout the country in muddy areas, sewage damps and tanks. The species is used in vermiculture throughout the world.

Features:
The colour of the body is red or brown or purple. The dorsal surface bears coloured bands often two per segment and the ventral surface is paler in colour. The clitellum occurs over 7-9 segments which include 24, 25 or 26-32 body segments. The body weight is about 1.5 gm/matured worm. Maturity attains in about 50-55 days and during adult stage the body wall becomes ridged. The species is generally called red worm or tiger worm.

Bimastosparvus:

Distribution:
They occur in Kashmir, Himachal Pradesh, Punjab, Rajasthan and Uttar Pradesh

Features:
The body colour is brownish red. The clitellum is saddle-shaped and occurs over 6 or more segments which include 24 or 25-30. The body segments are usually 90.

Family Eudrilidae:

Eudriluseugeniae:

Distribution:
Though the species was first recorded in South-West India but at present it is widely used in South India in vermiculture. The species was originally distributed in equatorial West Africa but recently it is found in most areas of the world. It is called ‘night crawler’ in U.S.A.

Features:
The colour of the body is brown or red dark violet. The clitellum occurs over 5-6 segments and include 13 or 14-18. Female gonopores are on the 14th segment and male gonopore, a few segments behind them. The body segments usually range 145 to 196, and length of the body varies from 32 to 140 mm and diameter is about 5 to 8 mm. Maturity is attained within 40 days. The weight of the mature worms is about 4.3 mg per individual. The higher temperature tolerance is more higher than *E. foetida.*

Family Megascolecidae:

***Lampitomauritii (Megascoles mauritii*):**

Distribution:
They are found in Punjab, South Rajasthan, Gujarat, Maharashtra, Madhya Pradesh, Andhra Pradesh, Tamil Nadu, Kerala, Lakshadweep Islands and Andaman Island.

Features:
The body colour is dark yellowish except the anterior end which bears purplish tinge. The clitellum is ring-shaped and spreads over 4 segments that include 14- 17 body segments. The length of the body ranges from 80-210 mm, with diameter 3.5 to 5 mm. The total body segments vary 166-190.

Metaphireanomala (= Pheretimaanomala)

Distribution:
The species has recorded in Kokata (W. Bengal), Odisha, Bihar, Madhya Pradesh, Andhra Pradesh, Tamil Nadu, Karnataka and Kerala.

Features:
Total length of the body ranges from 80 to 90 mm, with diameter varies from 5 to 5.5 mm. The clitellum is ring-shaped which spreads over 3 body segments that include 13 to 16.

Pheretima posthuma:

Distribution:
It is the most common species in India and South-East Asia including Japan and Australia. They prefer to live the damp soil in the gardens and also in the dead organic matter.

Features:
Colouration of the dorsal side is brown and the ventral side is brightly coloured. The whole length of the full grown worm measures about 200 mm and 3 to 5 mm in diameter. The body segments about 100-120. The clitellum covers 3 segments on XIV to XVI. The use of the species in vermitechnology is not well studied.

Family Octochaetidae

Octochaetus surensis:

Distribution:
The species was recorded in Burkul and Sur Lake in Odisha, and has also been recorded across other parts of India.

Features:
Colouration of the body is greyish with dark tinge at the dorsal part of the anterior region. The total length of the body measures 75 mm and diameter is about 2 to 2.5 mm. The clitellum is ring-shaped and occurs over 5 segments which include 13^{th} to 18^{th}.

***Dichogaster affinis*:**

Distribution:
The species is found in Gujarat, Maharashtra and South India. Outside India it is recorded in Sri Lanka, East Africa, and Madagascar etc.

Features:
The colour of the body is pinkish brown. The worm measures 30-32 mm in length with diameter is about 1.2 to 1.5 mm. The total body bears 140 segments. The clitellum is saddle-shaped and 8-10 segments, namely 13^{th} or 14^{th} to 21^{th} or 22^{th}.

Dichogaster curgensis:

Distribution: South India.

Features:
The colour of the body is greyish. The length of the body measures 65-75 mm and diameter is 2 mm. The whole body contains 90-110 segments. The clitellum is ring-shaped and contains 8 segments over 13^{th} to 20^{th}.

Oenerodrilus occidentalis:

Distribution:
They are found in Rajasthan, Maharashtra, Karnataka and Andaman Islands.

Features:
The total body length measures 15 to 30 mm and diameter is about 1 mm. The body segments are 70. The clitellum is ring-shaped and contains 6-8 segments, namely 13^{th} or 14^{th} to 19^{th} or 20^{th}.

Family Moniligastridae:

Moniligaster perrieri:

Distribution: South India.

Features:
The body colour is generally blackish grey which is deeper on the dorsal side than the ventral side. The body length is about 210 mm and diameter is 5 mm. The total body segments are 175. The clitellum is ring-shaped and occurs over 5 segments from 9^{th} to 14^{th} segments.

Trophic Classification of Earthworms:

Based on the feeding habits, the earthworms are generally classified into detritivores and geophages. The detritivore earthworms feed mainly on plant litter, plan debris or mammalian dung. They collect the feed from the soil surface. These worms compose the epizeic forms. The detrivorous earthworms are *Eisenia foetida, Eudriluseugeniae, Lampitomauritii, Polypheretimaelongata, Octochaetona serrata, Octochaetonasurensis*, etc.

The geophagous earthworms live in underground burrows and eat large quanties of deeper organic rich soil which remains beneath the surface layer. These worms are called humus feeders. *Pheretimaposthuma and Octochaetonathurstoni* are the examples of geophagous earthworms.

Process of Vermicomposting:

It deals with the various aspects like:

(i) Types of container,

(ii) Filling it with moist bedding

(iii) Introducing worms and

(iv) Feeding the worms on a regular basis.

These types of aspects for vermicomposting are practised in small scale vermicomposting but in larger scale the basic processes are same excepting the composting containers.

1. Composting container:

The shape or size of the containers depends upon the amount of waste to be composted and the number of earthworms which we want to culture

In 1m × 1m × 0.5 m high container 2000 adult earthworms can be maintained which can convert 200 kg wastes into composting material per month. In 2.23 sq. metre container 10 kg earthworms can be maintained which can convert about 1 ton waste per month. The selected container should be cleaned before use.

The followings are considered as composting materials which are being listed below:

(i) The animal dungs such as cattle dung, goat dung, sheep dung and poultry dropping are used as the composting material. The preliminary testing and precautions are necessary for the pathogens which can be harmful to earthworms excepting cowdung.

(ii) The agricultural waste materials which include the discarded part after harvesting and threshing of the paddy, wheat, etc. The vegetable wastes, leaf litter and sugarcane trash are considered as the composting material. The various kinds of forestry wastes, such as peels, saw dust are also considered. The leaf litters, such as mango, guava and grasses are used as composting materials.

(iii) The garbage includes various kinds of biodegradable and non-degradable materials. The biodegradable materials, such as kitchen vegetable waste, paper, should be sorted, recycled or composted.

2. Bedding material:

At the bottom of the container, a 2-3 inches thick layer of biodegradable matter, e.g., husk, grasses, stem of crops and sugar cane trash, etc. which are used as the feed of earthworms, is laid. Next to this layer, another layer of 2″-3″ thick of powdered cowdung is put.

The whole material is maintained moistened and required number, of live earthworms are released. Above this a 7″-9″ thick layer of earthworm feed matter is put and watered. The whole material is covered with a moist gunny bag.

The earthworms would multiply by consuming the food and compost the matter from upper layers. The upper layer of vermicompost becomes ready within 60-70 days. Gradually the whole matter will be converted into vermicompost within 30-40 days with the bacterial decomposition.

Feed Materials of Earthworms:

Earthworms eat decomposed organic matters. So various kinds of food are prepared used in vermiculture.

A list of feeds is given below:

1. Old cow dung which is used after 7 days.

2. Cow dung is mixed with kitchen wastes in the ratio of 10:3.

3. Cow dung is mixed with agricultural wastes in the ratio of 10:3.

4. Cow dung is mixed with rice polish or wheat bran in the ratio of 10: 3.

5. Cow dung is mixed with sewage sludge in the ratio of 10: 3.

Only cow dung or mixed dung, such as goat dung, sheep dung or poultry dropping are used. Mixed dung is prepared in the ratio of 50: 50. Any of the above-mentioned mixed materials are mixed thoroughly. Heaps should be kept in shaddy places for 2-3 weeks and watered. Then the material is dried and beaten to make the material powdery which is used as feed for earthworms.

Required Physical Conditions for Vermicomposting:

During vermicomposting the following conditions, such as:

(i) Moisture content(ii) Oxygen,(iii) Temperature and(iv) pH of the substrate must be noticed for the vermiculture.

(i) Moisture content: The moisture content must be maintained between 30-40%.

(ii) Oxygen: The concentration of oxygen should be optimum.

(iii) Temperature: For better results, the growth or multiplication of worms takes place between 20-30 °C.

(iv) pH of the substrate: pH of the substrate would be between 6.8-7.5.

Process of Vermicomposting:

Process of vermicomposting can be done in pits or concrete tanks, wells or wooden crates. A pit of $2\times1\times1$ m^3 dimension (1 m maximum depth) is dug under a shade to prevent the entry of water during rain (Fig. 33.1). Wooden bricks or pebbles are spread on the bottom of pit followed by coarse sand to facilitate the drainage. It is covered by a layer of loamy soil which is moistened and inoculated by earthworms. It is covered by small lumps of fresh or dry cattle dung followed by a layer of hay or dry leaves or agro wastes. Every day for about 20-25 days water is sprinkled over it to keep the entire set up moist. Until the pit is full dry and green leaves are put into the pit in each week. Vermicompost is ready after 40-45 days. Vermicomposting appears soft, spongy, dark brown with sweet smelling. Then it is harvested and kept in dark. It is sieved and packed in polythene to retain 20% moisture content.

Vermicompost is ready for harvest when it contains few-to-no scraps of uneaten food or bedding. There are several methods of harvesting from small-scale systems: "dump and hand

sort", "let the worms do the sorting", "alternate containers" and "divide and dump." These differ on the amount of time and labour involved and whether the vermicomposter wants to save as many worms as possible from being trapped in the harvested compost.

The pyramid method of harvesting worm compost is commonly used in small-scale vermiculture, and is considered the simplest method for single layer bins. In this process, compost is separated into large clumps, which is placed back into composting for further breakdown, and lighter compost, with which the rest of the process continues. This lighter mix is placed into small piles on a tarp under the sunlight. The worms instinctively burrow to the bottom of the pile. After a few minutes, the top of the pyramid is removed repeatedly, until the worms are again visible. This repeats until the mound is composed mostly of worms.

When harvesting the compost, it is possible to separate eggs and cocoons and return them to the bin, thereby ensuring new worms are hatched. Cocoons are small, lemon-shaped yellowish objects that can usually be seen with the naked eye. The cocoons can hold up to 20 worms (though 2-3 is most common). Cocoons can lay dormant for as long as two years if conditions are not conducive for hatching.

Advantages of Vermicomposting:

Vermicomposting is the usage of earthworms to convert vegetable waste to a 100 percent natural plant fertilizer. Vermiculture is the side issue of the breeding of common earthworms for use in vermicomposting. The use of worm farms for vermicomposting is becoming a favourite way of converting waste to a valuable product while also growing more worms to increase the capacity of the worm farms.

1. Organic

The most important aspect of compost produced by earthworms is that it is 100 percent organic. There are no harmful chemicals and it does not need to be mixed with anything.

2. More Nutritious

Vermicomposting produces a product that is naturally designed to benefit plants in several different ways. The most significant benefit is that the nutrients in earthworm compost are very easily absorbed by the roots of plants. Unlike chemical fertilizers, vermicompost is not easily flushed from the soil because of the worm mucus that it contains. Plants have longer to obtain the nutrients and get the maximum benefit.

3. Micro-organisms

As the compost is passing through the body of the worms, it is enriched with bacteria and microbes. These help plants to become more disease resistant and also repel some plant pests. The presence of increased microbial activity can make the area much more attractive to birds which also help to remove plant pests.

4. Healthier Plants

As the compost works on the plants and they become healthier, the need for pesticides is reduced. The reduction in pesticides helps the area to recover faster and can start an improvement cycle that will run on. This compares with the typical cycle when chemical fertilizers are used. The chemical fertilizers might increase plant yields, but they do nothing for plant health. Continued use of chemical fertilizers inevitably leads to a breakdown in the soil. Ammonia and salts build up which attack the plants making them less able to withstand disease.

5. Plant Growth

Among the hormones that earthworm compost contains are hormones that help plants to grow. Germination of seeds is encouraged, the growth of the plant is stronger and the crop yield improved. This natural support for the plants is not available with chemical fertilizers. The distribution of the compost through the soil also helps to encourage healthy root growth.

6. Water Retention

Vermicompost is a colloid and holds up to nine times its own weight in water. This can make a huge difference when there is a dry spell. The water is held at an organic level so tends to evaporate slowly while still being available to the plants.

7. Slow Nutrition Release

Chemical fertilizers bombard plants with huge amounts of nutrients that are going to drain by and eventually be washed out of the soil by the rain. The chemicals can get into the aquifers and contaminate your drinking water. This can be avoided by using a compost which is lower in nutrient content but which does not get washed out of the soil. The nutrients are held in place and released slowly so that the plants receive what they need over a prolonged period.

Using earthworms creates a product that is natural and behaves naturally. The cycle of regularly over-dosing the soil is broken. Plant health is promoted by long-term exposure to nutrients and the soil condition will continue to improve.

Suggested readings:

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3. Deo Kumar.2008Analyzing impact of Vermicompost in various combinations on physio-chemical properties of soil and wheat crop(Ph.D Thesis)

4. Richard Tom, Nancy Troutmann C/M Ration Corwell composting. Composting in school. Corwell University College of Agriculture and life Sciences.2007

Probable questions:

1. What is vermicompost?

2. Name three species which are used in vermicomposting. Describe their features and

distributions.

3. How feeding material is prepared in vermicomposting.

4. Briefly describe the process of vermicomposting.

5. What are the advantages of vermicomposting?

6. What are the required physical conditions required for vermicomposting?

Disclaimer:

The study materials of this book have been collected from various books, e-books, journals and other e sources.

Post-Graduate Degree Programme (CBCS)

in

ZOOLOGY

SEMESTER-III

HARD CORE THEORY PAPER

ENVIRONMENTAL TOXICOLOGY AND ENDOCRINOLOGY

ZHT-310

SELF LEARNING MATERIAL

DIRECTORATE OFOPEN AND DISTANCE LEARNING

UNIVERSITY OF KALYANI

KALYANI, NADIA,

W.B., INDIA

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Director's Message

Satisfying the varied needs of distance learners, overcoming the obstacle of distance and reaching the unreached students are the threefold functions catered by Open and Distance Learning (ODL) systems. The onus lies on writers, editors, production professionals and other personnel involved in the process to overcome the challenges inherent to curriculum design and production of relevant Self Learning Materials (SLMs). At the University of Kalyani a dedicated team under the able guidance of the Hon'ble Vice-Chancellor has invested its best efforts, professionally and in keeping with the demands of Post Graduate CBCS Programmes in Distance Mode to devise a self-sufficient curriculum for each course offered by the Directorate of Open and Distance Learning (DODL), University of Kalyani.

Development of printed SLMs for students admitted to the DODL within a limited time to cater to the academic requirements of the Course as per standards set by Distance Education Bureau of the University Grants Commission, New Delhi, India under Open and Distance Mode UGC Regulations, 2017 had been our endeavour. We are happy to have achieved our goal.

Utmost care and precision have been ensured in the development of the SLMs, making them useful to the learners, besides avoiding errors as far as practicable. Further suggestions from the stakeholders in this would be welcome.

During the production-process of the SLMs, the team continuously received positive stimulations and feedback from Professor (Dr.) Sankar Kumar Ghosh, Hon'ble Vice-Chancellor, University of Kalyani, who kindly accorded directions, encouragements and suggestions, offered constructive criticism to develop it within proper requirements. We gracefully, acknowledge his inspiration and guidance.

Sincere gratitude is due to the respective chairpersons as well as each and every member of PGBOS (DODL), University of Kalyani. Heartfelt thanks is also due to the Course Writers-faculty members at the DODL, subject-experts serving at University Post Graduate departments and also to the authors and academicians whose academic contributions have enriched the SLMs. We humbly acknowledge their valuable academic contributions. I would especially like to convey gratitude to all other University dignitaries and personnel involved either at the conceptual or operational level of the DODL of University of Kalyani.

Their persistent and co-ordinated efforts have resulted in the compilation of comprehensive, learner-friendly, flexible texts that meet the curriculum requirements of the Post Graduate Programme through Distance Mode.

All the Self Learning Materials are self writing and collected from e-book, journals and websites.

Prof Manas Mohan Adhikary
Director
Directorate of Open and Distance Learning
University of Kalyani

HARD CORE THEORY PAPER (ZHT- 310)

Environmental Toxicology and Endocrinology

Group A: Environmental Toxicology

Module	Unit	Content	Credit	Class	Time (h)	Page No.
ZHT - 310 (Environmental Toxicology and Endocrinology)	I	Basic concept of toxicology : Scope, division, toxicants and toxicity, factors, dose- response relationship.	2.0	1	1	
	II	Toxicity testing : Bioassays, LC50, LD50, ED50, Synergism, Antagonism, Additive Effect		1	1	
	III	Toxicants of public health hazards: Pesticides, Heavy metals, Radiation, food and additives		1	1	
	IV	Toxicokinetics: Absorption, distribution, elimination.		1	1	
	V	Organ toxicity: Hepato, Nephro, Respiratory, Reproductive.		1	1	
	VI	Plant Allelochemicals .types and its role in insect-plant interaction.		1	1	
	VII	Plant signalling chemicals, insect response.		1	1	

Unit-I

Basic concept of toxicology: Scope, division, toxicants and toxicity, factors, dose- response relationship.

Objective: In this unit you will learn about basic concept of toxicology. You will also learn about scope and division of toxicology and dose response relationship.

Introduction to Toxicology:

Science of Toxicology, deals with the study of interactions between chemicals and biological systems in order to quantitatively determine the adverse effects in living organisms and investigate the nature, incidence, mechanism of production, factors influencing their development and reversibility of such adverse effects. In formal terms, it appears to be a young science, however, it was conceptualized by a physician known as Paracelsus. Borzellaca (2000) honoured Paracelsus as herald of modern toxicology. Paracelsus discounted the humoral theory of Galen who postulated that balance amongst four humors in the body (blood, phlegm, yellow and black bile) is essential for health. Paracelsus believed in three humors- salt (representing stability), sulfur (representing combustibility) and mercury (representing liquidity).

He defined disease as a separation of one humor from the other two. He propounded the principle of simplitude meaning that "a poison in the body will be cured by a similar poison". He introduced chemistry into medicine. He extended his interest in chemistry and biology to what we now consider toxicology. Basic tenets of Paracelsianism were summed up by Temkin and Coworkers (1996).Since then, toxicological developments have witnessed new heights. War and prospects of war played a great role in the development of toxicology. In "world war I", a variety of chemicals were used in the battlefields of France. Occupational toxicology originated in 19^{th} century as a product of industrial revolution.

Development of chemical and pharmaceutical industries in 19^{th} and 20^{th} century gave birth to regulatory toxicology. Increasing concerns for consumer and environmental health during last three decades brought toxicology to the age of Science. World War II offered stimulus to the evolution of Environmental Toxicology.

Definition of Toxicology:

Toxicology can be defined quite simply as the branch of science dealing with poisons. Broadly speaking, a poison is any substance causing harmful effects in an organism to which it is administered, either deliberately or by accident. Clearly, this effect is closely related since any substance, at a low enough dose, is without effect, while many, if not most, substances have deleterious effects at some higher dose.

Much of toxicology deals with compounds exogenous to the normal metabolism of the organism. Such compounds are referred to as foreign compounds or more recently, as xenobiotics. However, many compounds endogenous to the organism e.g., metabolic intermediates such as glutamate, and hormones such as thyroxine, are toxic when administered in unnaturally high doses.

Similarly, trace elements such as selenium, which are essential in the diet in low concentrations, are frequently toxic at higher levels. Whether the harmful effects of physical phenomena such as irradiation, sound, temperature, and humidity are included in toxicology, appear to be largely dependent on the preference of the writer, it is convenient, however, to include them under the broad

definition of toxicology. The method of assessing toxic effects is another parameter of considerable complexity. Acute toxicity, usually measured as mortality and expressed as the LD_{50}– the dose required to kill 50% of a population of the organism in question under specified conditions- is probably the simplest measure of toxicity.
Even so, reproducibility of LD_{50} values is highly dependent upon the extent to which many variables are controlled. These include the age, sex, and physiological condition of the animals, their diet, the environmental temperature and humidity, and the method of administering the toxicant.
Chronic toxicity may be manifested in a variety of ways – carcinomas, cataracts, peptic ulcers, and reproductive effects, to name only a few. Furthermore, compounds may have different effects at different doses. Vinyl chloride, a potent hepatotoxic at high doses, is a carcinogen with a very long latent period at low doses. Most drugs have therapeutic effects at low doses but are toxic at higher levels. The relatively nontoxic acetylsalicylic acid (aspirin) is a useful analgesic at low doses, is toxic at high doses, and may cause peptic ulcers with chronic use.

Considerable variation also exists in the toxic effects of the same compound administered to different animals, or even to the same animal by different routes. The insecticide Malathion has a low toxicity to mammals, whereas it is toxic enough to insects to be a widely used commercial insecticide. The route of entry of toxicants into the animal body is frequently oral, in the food or drinking water in the case of many chronic environmental contaminants such as lead or insecticide residues, or directly as in the case of accidental or deliberate acute poisoning. Other routes for non-experimental poisoning include dermal absorption and pulmonary absorption. The above routes of administration are all used experimentally, and in addition, several types of injection are also common -intravenous, intraperitoneal, intramuscular, and subcutaneous. The toxicity of many compounds varies tenfold or greater depending upon the method of administration.

Earlier Developments in Toxicology:

Soon the nature and magnitude of toxic effects were studied. Factors viz.- physicochemical properties of the substance, its bioconversion, the conditions of exposure, and the presence of bio-protective mechanisms subsequently dominated the scene.

Morphological and biochemical injury produced by a toxin was classified as inflammation, necrosis, enzyme inhibition, biochemical uncoupling, lethal synthesis, lipid peroxidation, covalent binding, receptive interaction, immune mediated hypersensitive reactions, genotoxicity, developmental and reproductive toxicity and pharmacological effects.

In 1848, Blake in the United States published his opinion that the biological activity of a salt was due to its basic or its acidic component and not to be whole salt; as with lead nitrate it was the lead moiety and not the acetate or nitrate part. This was, for 1st time, a daring thought, because it was not until 1884 Arrhenius introduced his theory of electrolytic dissociation.

The Scottish authors Crum Brown and Fraser (1869) made a major discovery. They wrote, “there can be no reasonable doubt that a relation exists between the physiological action of a substance and its chemical composition and constitution”, understanding by the latter term, the mutual relations of the atoms in the substance. This discovery was the first to show structure-action relationship at the turn of present century. Ernest Overton and Hans Meyer independently put forward a, “Lipoid theory of cellular Depression”. This stated that chemically inert substances exert depressant properties on cells (particularly those of central nervous system) that are rich in lipids and that higher the partition coefficients, the greater the depressant action.

The idea that drugs act upon receptors began with John Langley in (1878) in Cambridge. Later, Langley coined the term ‘receptive substances’. Paul Ehrlich was already using the term receptor in Germany. In his Noble prize address Ehrlich outlined the receptor as a small chemically defined area,

which was normally occupied with the cell's nutrition and metabolism but which could take up specific antigens or drugs instead.

First the idea of receptor was received with skepticism because of repeated failure to isolate any such substance. However, the idea of receptors became more firmly established by the work of Alfred Clark who showed that combination of drug with a receptor quantitatively followed the law of mass action. He summed up his work in a monograph, a few years before his death in 1941.The period of Second World War (1939-1944) was a turning point in the study of structure action relationship.
There was a period when dose response relationships were highly predominant. Development of physiology and biochemistry also influenced the growth of toxicology. Metabolism of substances was conveniently classified as phase – I and phase – II reactions.

Concept of QSAR:

The concept of QSAR (quantitative structure activity relationships) was applied to study the toxicity of inorganic cations. While molecules of organic compounds reflect their properties as a whole, the inorganic compounds dissociate in various degrees and properties have thus to be attributed to anions, cations or un-dissociated molecules.

Inorganic cations can form complexes with inorganic or organic ligands contributing new properties to the complex. Components of this system (cations, anions, un-dissociated molecules) could mutually influence each other depending upon the ratio amongst components. Quantitative relationships between a chemical structure of the complex and the biological activity formed a new line of action in toxicology.

QSAR studies generated the concept of molecular connectivity in order to characterize the organic biologically effective substances. The index of connectivity is deduced from the numeric evaluation of the extent of branching of chemical bonds in the section of the molecule. There exists a correlation between the connectivity index and toxicity of cations.

Relationship of Toxicology to other Sciences:

Toxicology is frequently said to be a branch of pharmacology, a science that deals primarily with the therapeutic effects of exogenous substances and with all the chemical and biochemical ramifications involved in those effects. Since the therapeutic dose range of pharmacological compounds is usually quite small, and most of these compounds are toxic at higher doses, it may be more appropriate to consider pharmacology a branch of toxicology.
Toxicology is clearly related to the two applied biology-medicine and agriculture. In the former, clinical diagnosis and treatment of poisoning as well as the management of toxic side effects are areas of significance, while in the latter the development of agricultural biocides such as insecticides, herbicides, nematicides, and fungicides is of great importance.

The detection and management of the off-target effects of such compounds is also an area of increasing importance that is essential to their continued use. Toxicology may also be considered an area of fundamental biology since the adaptation of organisms to toxic environments has important implications for ecology and evolution.

The tools of chemistry and chemical biology since the adaptation of organisms to and progress in toxicology are closely related to the development of new methodology. Those of chemistry provide analytical methods for toxic compounds, particularly for forensic toxicology and residue analysis, and those of biochemistry provide the techniques to investigate the metabolism and mode of action of toxic compounds.

On the other hand, studies of the chemistry of toxic compounds have contributed to fundamental organic chemistry, and studies of the enzymes involved in detoxication and toxic action have contributed to our basic knowledge of biochemistry.

Scope of Toxicology:

Toxicology in the most general sense may be one of the oldest practised sciences. From his earliest beginnings, man must have been aware of numerous toxins such as snake venoms and those of poisonous plants. From the earliest written records it is clear that the ancients had considerable knowledge of poisons.

The Greeks made use of hemlock as a method of execution, more particularly, the Romans made much use of poisons for political and other assassinations. Indeed, it was Dioscorides, a Greek at the court of Nero, who made the earliest known attempt to classify poisons.

Although poisoning has enjoyed a considerable vogue at many times and places, the scientific study of toxicology can probably be dated from Paracelsus, who in the sixteenth century, put forward the necessity for experimentation and included much in his range of interests that would today be classified as toxicology.

The modem study of toxicology is usually dated from the Spaniard, Orfila (1787- 1853), who, at the University of Paris, identified toxicology as a separate science. Among his many contributions, he devised chemical methods for the detection of poisons and stressed the value of chemical analysis to provide legal evidence. He was also the author, in 1815, of the first book devoted entirely to the toxic effects of chemicals.

Toxicology can be subdivided in a variety of ways. Loomis refers to the three “basic” subdivisions as environmental, economic, and forensic. Environmental toxicology is further divided into such areas as pollution, residues, and industrial hygiene; economic toxicology is said to be devoted to the development of drugs, food additives, and pesticides; and forensic toxicology is concerned with diagnosis, therapy, and medicolegal considerations. Clearly, these categories are not mutually exclusive; for example, the off target effects of pesticides are considered to be environmental, while the development of pesticides is economic.

Environmental Toxicology:

Environmental toxicology is the most rapidly growing branch of science. Public concern over environmental pollutants and their possible chronic effects, particularly carcinogenicity, has given rise, in the United States, to new research and regulatory agencies and recently to the Toxic Substances Control Act.

Similar developments are also taking place in many other countries. The range of environmental-pollutants is enormous, including industrial and domestic effluents, combustion products of fossil fuels, agricultural chemicals, and many other compounds that may be found in food, air, and water. Such compounds as food additives and cosmetics are also being subjected to the same scrutiny.
Other sub-specialties are frequently mentioned that do not fit into the above divisions. Behavioural toxicology, an area of increasing importance, could be involved in any of these and is usually treated as a separate sub-speciality. Analytical toxicology provides the methods used in essentially every branch of the subject, while biochemical toxicology, provides the fundamental basis for all branches of toxicology.

Language of Toxicology:

Like any other specialized field, environmental health has its own language. Some of the terms may need a few words of introduction. Toxic, a central concept simply means capable of causing illness. The types of illnesses caused by environmental toxins are conventionally divided into acute and chronic. Acute illness are those which appear soon after exposure to a toxic compound, last for a relatively short time, and then resolve themselves, even if the resolution is in death.The term sub-acute is also occasionally used to describe disorders with subtle symptoms that are not immediately obvious without special tests. Lead workers, for example, often appear to be much healthier than a thorough medical examination reveals them to be Chronic illnesses, by contrast, that may appear years or even decades after exposure, and which may "remain", unresolved, for the victim's lifetime.
There are three special kinds of toxic hazards that have special relevance to environmental health: carcinogens, mutagens and teratogens. As most of us know, a carcinogenic substance is one that causes cancer. A mutagenic substance is one that causes changes in the genetic material of a cell. Spontaneous, natural mutations occur in our body cells all the time; the vast majority of them cause no damage, and even when they do it is usually limited to the lifetime of the cell they occur in.But in rare cases the cell may continue to grow and divide after a mutagen has altered its basic genetic structure, and if this mutation is passed on to succeeding generations via egg or sperm, it may cause birth defects, inherited diseases, mental deficiency, increased susceptibility to disease, and a host of other abnormalities and disorders. If the mutated genes are recessive, it may take more than one generation for these effects to show up.

Mutagenicity and carcinogenicity are related in some way, but were not yet sure just how. Radiation is probably the best-known example of a mutagenic environmental hazard.Contamination is measured in terms of the concentration of a substance in the environment, and there are a number of different conventions governing the measurement of concentration. The most common system makes use of metric units, particularly the milligram (one-thousandth of a gram, abbreviated mg) and the microgram (one million of a gram, abbreviated Ì g). Occasionally, in very refined, ultrasensitive measurements, nanograms (one-billionth of a gram), picograms (one- trillionth of a gram), and even smaller units may be used.

Obviously, a contaminant concentration of one milligram per kilogram is the same as one part of contaminant per million parts of non-contaminant, or 1 ppm, and this alternative method of indicating relative concentrations is also widely used, particularly for air pollutants, food additives, and pesticides residues. The terms ppm and ppb (parts per billion) are common- ppt (parts per trillion) appears only rarely.Exposure is a way of saying that the contamination in the environment has passed into an organism; a human being is exposed to a toxic compound if some amount of it has entered his or her body. Exposure does not mean that a person has merely been in the proximity of a toxic substance.

For example, if you walk past a drum bearing a warning label and containing a toxin, you are not necessarily exposed to whatever it contains. But if the drum leaks its contents into the air or soil, and pollutes the air you breathe or the water you drink, you probably will be exposed to its contents. Like illnesses, exposures may also be subdivided into acute and chronic types. Acute exposures are those that occur over short periods of time, often to high concentrations of a hazardous substance. Chronic exposures, which are much more common among the general public, involve longer periods of time and for the most part, lower concentrations. Dose is the term for measuring exposures. Basically, the dose a person exposed to a toxic substance receives is dependent on its concentration in the immediate environment and the duration of the exposure. However, because the interaction of human beings and the environment is a complex, constantly changing process, numerous other factors may also play a part in determining dose.

Dose can be a function of weather conditions, the persistence and solubility of the toxic substance in the biosphere, the size of its molecules or particulates, the presence of other compounds in the environment that it may react with, the age and overall health of the exposed individual, whether the

substance is inhaled, swallowed, or absorbed through the skin, and the effectiveness of the body's natural defences in detoxifying the substance and eliminating it from the body.Some substances, like asbestos, become virtually permanent contaminants in the body once they have penetrated far enough into the lungs or other organs. Others, such as methanol, are metabolized and excreted from the body in a matter of hours at most.

The tendency for some substances to collect in the tissues of a living organism and stay there is known as bioaccumulation. Their tendency to move up the food chain as one species consumes another, becoming ever more concentrated as they go, is called biomagnification.

Traditionally a threshold was a measurable level of exposure to a toxic substance below which there would probably be no adverse health effect and above which there probably would be. The setting of safety standards for the work site as well as the general environment often involves the assumption that approximate thresholds can be determined, monitored and enforced for the toxin in question.But this assumption has been subjected to various criticisms in the past few decades. First, it is often pointed out that, whether we can measure it or not, it is entirely possible that every molecule of every substance we take into our bodies has some effect on us. It may not be a detectable effect and it may not be harmful or long lasting, but it is an effect.

Thus, this argument goes, the concept of a specific cutoff point below which a substance is treated as though it didn't exist and above which it is considered harmful is misleading. Far more appropriate, proponents of this view argue, is the assumption that these substances have a range of effects, beginning at once end with those that are imperceptibly molecular and extending to the catastrophically toxic, ultimately fatal effect at the other end of the spectrum. An LD_{50} is customarily expressed in terms of milligrams per kilogram of body weight. Thus, a substance whose LD_{50} is 2 mg/kg is five times as toxic as one whose LD_{50} is 10 mg/kg. In general, substances with LD_{50} values below 50 mg/kg are considered highly toxic. Those with values between 50 and 500 mg/kg are considered moderately toxic, and those with values above 500 mg/kg are regarded as less toxic.

Distribution of a Toxin:

Various factors responsible for the distribution of toxicants throughout the body need discussion. This is primarily concerned with the binding of toxicants to blood proteins particularly lipoproteins. Lipoproteins are an important class of protein, particularly in the vascular fluids.

They vary in molecular weight from 200,000 to 10,000,00 and the lipid content varies from 4% to 95%, being composed of triglycerides, phospholipids, and free and esterified cholesterol. Although they are classified into groups based on their flotation constants, each group is, in fact, a mixture of many similar lipoproteins.

The nature and importance of various types of ligand-protein interactions are assessed, including covalent binding, ionic binding, hydrogen bonding, Vander Waals forces, and hydrophobic interactions. Many of the same binding forces are also important in toxicant receptor interactions. It deals with the mathematical approach to the distribution of toxicants, or toxicokinetics. It provides a simplified, but still mathematically rigorous, treatment of distribution data, including both analysis and the formulation of mathematical models.

Metabolism of a Toxin:

The majority of xenobiotics that enter the body do so because they are lipophilic. The metabolism of xenobiotics, which is carried out by a wide range of relatively nonspecific enzymes, serves to increase their water solubility and make possible their elimination from the body. This process consists of two

phases. In phase I, a reactive polar group is introduced into the molecule, rendering it a suitable substrate for phase II reactions.

Phase I reactions include the well-known cytochrome P_{450} -dependent mixed-function oxidations as well as reductions, hydrolyses, etc. Phase II reactions include all the conjugation reactions in which a polar group on the toxicant is combined with an endogenous compound such as glucuronic acid, glutathione etc. to form a highly water- soluble conjugate that can be eliminated from the body.It should be pointed out at this early stage that these metabolic reactions are not all detoxications since many foreign compounds are metabolized to highly reactive products that are responsible for their toxic effect. These include the activation of carcinogens and hepatotoxic cants.

Although the liver is the most studied organ with regard to xenobiotic metabolism, several other organs are known to be active in this respect, although neither the specific activity nor the range of substrates metabolized is as large as in the liver. These organs include the lungs and the gastrointestinal tract, as one might expect of organs that are important site for the entry of xenobiotic into the body, and to a lesser extent, the other important portal of entry, the skin. Other organs, such as the kidney, may also be important sites for xenobiotic metabolism.

Because toxicants are both activated and inactivated metabolically, physiological factors affecting metabolic rates can have dramatic effects on the expression of toxicity. These effects, including age, sex, pregnancy, and diet need to be considered.Comparative toxicology is of considerable importance from the point of view of selectivity, resistance to toxic action, and environmental studies of toxicants, as well as of some academic importance from the evolutionary viewpoint.Although only a few generalizations can be made on the basis of phylogenetic relationships, there have been many comparisons between species of toxicological interest.Foreign compounds can be substrates, inhibitors, inducers of the enzymes that metabolize them and, not infrequently, serve in more than one of these roles. Since the enzymes in question are nonspecific, numerous interactions between foreign compounds are possible.
These may be synergistic or antagonistic and may have a profound effect on the expression of toxicity. Depending upon the compounds and the enzymes involved in a particular interaction, the effect can be an increase or a decrease in either acute or chronic toxicity. The basic principles of such interactions are summarized.

The cell type that has been studied most intensively in biochemical toxicology is the hepatocyte, the cell that forms the bulk of the liver. These cells are highly active metabolically, both in normal intermediary metabolism and in reactions involving xenobiotics. The principal cell organelles shown in the diagram play of important sole in biochemical toxicology. The nucleus, the chromosomes that contain DNA responsible for most of the proteins synthesized in the cell, is the site for the primary reaction of carcinogenesis, since carcinogens react with DNA. Depending upon the toxicant, the organ, and the cell type involved, similar reactions are involved in mutagenesis and teratogenesis. The nuclear envelope has recently been shown to have an active aryl hydrocarbon hydroxylase system.Mitochondria are the site of electron transport and oxidative phosphorylation pathways that provide sites for the action of many acute toxicants.

The endoplasmic reticulum exists in two forms- rough, which is associated with protein synthesis, and while both rough and smooth are active in the oxidation of xenobiotics, the latter usually has the highest specific activity. After, disruption of the cells, followed by differential centrifugation, the two types are isolated as rough and smooth microsomes.

Sites of Action of a Toxin:

Compounds of intrinsic toxicity and active metabolites produced in the body ultimately arrive either at a site of action or an excretory organ. Although almost any organ can show the effects of toxicity, some are more easily affected than others by particular classes of toxicants, and some have been

studied in greater detail than others. In all cases, however, toxicant-receptor interactions are important. Acute toxicants tend to affect either oxidative metabolism, the synapses of the nervous system, or the neuromuscular junction. Toxic effects on the central nervous have been widely studied. The commonest modes of chronic toxicity involve interaction with nucleic acids, causing carcinogenesis or reproductive effects. Although specific organ damage is known for several toxicants, the central role of the liver in studies of toxic action is acknowledged.While toxicants can be classified in many ways, based either on natural distribution, commercial use patterns, or chemistry, only two such groups viz. metals and pesticides.

Many metabolic pathways are affected by toxicants. They include glycolysis, the tricarboxylic acid cycle, the pentose cycle, the electron transport system and oxidative phosphorylation, nucleic acid synthesis, protein synthesis, and many others, as well as such specialized systems as photosynthesis in plants. In vivo testing for chronic toxicity in animals and short-term mutagenicity tests are both somewhat remote from a strictly biochemical treatment of the mechanisms involved in toxicology.

Excretion of Toxin:

Either the un-metabolized toxicants or their metabolic products are ultimately excreted, the latter usually as conjugated products resulting from phase II reactions. The two primary routes of excretion (the urinary system and the biliary system), minor routes also (such as the lungs, sweat glands, sebaceous glands, hair, feathers, and nails) and sex related routes (such as milk, eggs, and foetus) constitute the routes of excretion.

Nature of Toxic Effects:

The nature and magnitude of a toxic effect depend on many factors, amongst which are the physicochemical properties of the substance, its bioconversion, the conditions of exposure, and the presence of bio-protective mechanisms. The last factor includes physiological mechanisms such as adaptive enzyme – induction, DNA repair mechanisms and phagocytosis.Some of the frequently encountered types of morphological and biochemical injury constituting a toxic response are listed below. They may take the form of tissue pathology, aberrant growth processes, altered or aberrant biochemical pathways or extreme physiological responses.

Inflammation is a frequent local response to irritant chemicals or may be a component of systemic tissue injury. The inflammatory response may be acute with irritant or tissue damaging materials, or chronic with repetitive exposure to irritants or the presence of insoluble particulate material. Fibrosis may occur as a consequence of the inflammatory process.

Necrosis, used to describe circumscribed death of tissues or cells, may result from a variety of pathological processes induced by chemical injury, e.g. corrosion, severe hypoxia, membrane damage, reactive metabolite binding, inhibition of protein synthesis and chromosome injury.With certain substances, differing patterns of zonal necrosis may be seen. In the liver, for example, galactosamine produces diffuse necrosis of the lobules, acetominophen (paracetamol) mainly centrilobular necrosis and certain organic, arsenicals peripheral lobular necrosis.

Enzyme Inhibition by chemicals may inhibit biologically vital pathways, producing impairment of normal function. The induction of toxicity may be due to accumulation of substrate or to deficiency of product or function.For example, organophosphate anticholinesterases produce toxicity by – accumulation of acetylcholine at cholinergic synapses and neuromuscular junctions. Cyanide inhibits cytochrome oxidase and interferes with mitochondrial oxygen transport, producing cytotoxic hypoxia. Biochemical uncoupling agents interfere with the synthesis of high- enephosphate molecules, but electron transport continues resulting in excess liberation of energy a heat. Thus, uncoupling produces increased oxygen consumption and hyperthermia. Examples of uncoupling agents are dinitrophenol and pentachlorophenol.

Lethal synthesis occurs when foreign substances of close structural similarity to normal biological substrates become incorporated into biochemical pathways and are then metabolized to a toxic product. A classical example is fluoroacetate, which becomes incorporated in the Kreb's cycle as fluoroacetyl coenzyme A, which combines with oxaloacetate to form fluorocitrate. The latter inhibits aconitase, blocking the tricarboxylic acid various system toxicity.

Lipid peroxidation in biological membranes by free radicals starts a chain of events causing cellular dysfunction and death. The complex series of events includes oxidation of fatty acids to lipid hydro-peroxides which undergo degradation to various products, including toxic aldehydes. The generation of organic radicals during peroxidation results in a self-propagating reaction.

Carbontetrachloride, for example, is activated by a hepatic cytochrome P_{450}-dependent mono-oxygenase system to the trichloromethyl and trichloromethyl peroxy radicals; that covalently bind with macromolcules and the latter initiates the process of lipid peroxidation leading to hepatic centrilobular necrosis. The zonal necrosis is possibly related to high cytochrome P_{450} activity in centrilobular hepatocytes.

Covalent binding of electrophilic reactive metabolites to nucleophilic macromolecules may have a role in certain genotoxic, carcinogenic, teratogenic and immunosuppressive events. Important cellular defence mechanisms exist to moderate these reactions, and toxicity may not be initiated.Receptor interaction at a cellular or macromolecular level, with specific chemical structures may modify the normal biological effect mediated by the receptor, these may be excitatory or inhibitory. An important example is effects on Ca channels.Immune-mediated hypersensitivity reactions by antigenic materials are particularly important considerations for skin and lung resulting in allergic contact dermatitis and asthma, respectively.
Immunosuppression by xenobiotics may have important repercussions in increased susceptibility to infective agents and certain aspects of tumorigenesis.Neoplasia, resulting from aberration of tissue growth and control mechanisms of cell division, and resulting in abnormal proliferation and growth, is a major consideration in repeated exposure to xenobiotics.

The terms tumorigenesis and oncogenesis are general words used to describe the development of neoplasms; the word carcinogenesis should be restricted specifically to malignant neoplasms. In experimental and epidemiological situations, oncogenesis may be exhibited as an increase in specific types of neoplasm, the occurrence of rare, or unique neoplasms or a decreased latency to detection of neoplasm.The preceding description of the nature and scope of biochemical toxicology should make it clear that the biochemistry of toxic action is a many-faceted subject, covering, all aspects from the initial environmental contact with a toxicant to its ultimate excretion back into the environment.

Modern Toxicology:

In recent years, toxicology has developed from an activity relying principally on the tools of classical pathology to observe and classify harmful effects so as to become a discipline of increasing ability to explain the effects of toxic compounds in molecular and mechanistic terms.Over the last several years, it has become apparent that many environmental toxicants exert their effects by the action or disruption of specific signalling pathways, ultimately resulting in alterations in gene expression. With the completion of the human genome project and the advent of many powerful new technologies, there has been a revolution in our understanding of these mechanisms at molecular level.

Toxicant-induced alterations in gene expression depend on receptors. Four receptors namely the Ah receptor (AhR, the constitutive Androstone Receptor (CAR), the Pregnane X Receptor (PXR), and the peroxisome Proliferator Activated Receptor (PPAR) mediate the toxicity of four broad classes of chemicals.In contrast to these specific receptor mechanisms, metals exert their toxicity through both stress response pathways and specific metal responsive transcription factors. Role of tissue selective

transcription factors on the expression of xenobiotic metabolizing enzymes is now being investigated in several laboratories.

Recent developments show that toxicology is not merely a study of the effects of a variety of poisons in animals, plants and man but a multidisciplinary science embracing pathology, pharmacology, cell biology, biochemistry and public health.While dwelling with the subject for about three decades, I could witness the sustained progress made by science of toxicology. Earlier developments got smoothly integrated into modern concepts of toxicology. This communication is an attempt to review the present status of toxicology.

i. Toxicogenomics:

It is broadly defined as gene and protein expression technology that addresses pertinent issues of toxicology. The term genome has been traditionally used to define the haploid set of chromosomes in the nuclei of multicellular organisms. The study of genome is referred as genomics. The patterns by which genes and their protein products act in concert to affect function is known as functional genomics. Certain environmental stimuli will perturb the normal cellular functions of proteins and cause changes in gene expression. These kinds of environmental factors can also lead to the pathology of disease. Often the development of disease will be the result of complex mix of factors including inherent genetic susceptibility and a series of environmental changes or challenges.

The term proteome was coined in 1994 by Mark Wilkins. It refers to total protein repertoire able to be expressed from a given genome. A proteome of a cell, tissue, or organ is not only different, it can express differently under particular set of conditions. Thus toxicogenomics appear challenging.

ii. Metabonomic Technology:

Toxicants disrupt the normal composition and flux of endogenous biochemical in, or through, key intermediate cellular metabolic pathways. These disruptions, either directly or indirectly, alter the blood that percolates through the target tissues. The diagnostic utility of any one trace biomolecule is limited by the number of variables affecting its concentration in situ and by the common biochemical processes disrupted by toxicants. However, if a significant member of trace molecules is monitored, the overall pattern or “fingerprint” produced may be more consistent and protective than any other marker. This comprehensive information can be obtained from high field nuclear magnetic resonance (NMR) spectroscopy coupled with pattern recognition technology.

Magic angle spinning NMR technology enables similar information to be garnered from tissues as well. Temporal evaluation of metabolic consequences of toxicity, coupled with genomic and proteomic technologies and metabolomics permit complete assessment of toxicity from genotype through phenotype.

iii. Pharmacogenetics:

Pharmacogenetics, a term originally coined in the 1950s may now be viewed as the study of correlations between an individual’s genotype and the same individuals’ ability to metabolize an administered drug or compound. Genotypic variations, often in the form of single nucleotide polymorphism (SNPs), exist for many of the enzymes that metabolize drugs/chemicals. Extensive metabolism of a drug is a general characteristic of the normal population.

Poor metabolism which typically is associated with excessive accumulation of specific drugs or active metabolic products is a recessive trait requiring a functional change, such as frame shift or splicing defect in both copies of the relevant gene. Ultra-extensive metabolism which may have the effect of diminishing a drug, apparent efficacy in an individual, is generally an autosomal dominant state derived from a gene duplication or amplification. For example in cancer chemotherapy, several

common drugs show wide polymorphism related metabolic variations with 30 fold or greater inter-individual variability.

iv. Molecular Toxicology:

Apoptosis is a natural consequence in vivo and there is now substantial evidence that apoptosis plays an important role in the toxic effects of a number of drugs and chemicals. Numerous coherent pathways regulate cell suicidal process. Target organ toxicities, target organ apoptogenic drugs and chemicals, regulation of apoptosis at organs, cellular and subcellular and molecular levels emerges a new discipline. Since oxidative stress, caspases, caspase activated DNase, reactive oxygen species, mitochondrial and cell cycle related events are known to modulate this process, their respective roles are under investigation in several laboratories.

In-excitable and non-excitable tissues are the direct and indirect targets of many xenobiotics that produce apoptotic and necrotic cell death. Determination of the temporal and sequential relationships between the opening of the mitochondrial permeability transition (PT) pore, mitochondrial depolarization and swelling, cytochrome C release and caspase activation during cell death are critically important.

In toxicology, understanding the role and molecular mechanisms of PT pore opening will allow the development of pharmacological and genetic strategies to prevent inappropriate apoptosis as well as to initiate and control the apoptotic process for therapeutic purposes. The current evidence suggests that the PT pore is a complex of the voltage dependent anion channel (VDAC), adenine nucleotide translocase (ANT) and cyclophilin-D (CyP-D), formed at connect sites between the inner and outer mitochondrial membranes.

v. Concept of Biomarkers:

The emergence of specific biomarkers offer the promise of being able to measure signals and/or events that reflected more accurately the biology associated with exposure, effects, and susceptibility. The 1983 NRC publication formalized human health risk assessment into a four component process namely exposure, assessment, hazard identification, dose response assessment and risk characterization.

vi. Chronotoxicology:

Biological rhythms are toxicologically important because they have a positive or negative effect on all measures of normal physiological functions, and health of the individual. Circadian patterns affect the absorption of drug/toxin. Once the drug/toxin has been absorbed, it is transported to its tissue/target sites and its elimination sites. Large circadian variations have been shown in humans and rats in plasma proteins binding of a variety of drugs. In addition, drug/toxin transport can occur by their binding to red blood cells. In general, lipophilic materials pass into blood cells more rapidly than hydrophilic materials. Circadian variation with respect to drug permeability of the blood-brain barrier is of interest. Circadian rhythms in heavy metal toxicology have been described for mercury and cadmium. The level of liver microsomal benzene hydroxylase activity is highest at a particular time of the day. Carcinogenicity and teratogenicity of chemicals have also been found to be affected by circadian rhythms.

Circadian time structure is not routinely considered in toxicity testing in human or preclinical (rodent) models. Actually, they are not the only rhythms which modulate the outcome following drug or chemical exposure. Other cycles, such as fertility cycle and seasonal cycles markedly and reproducibly alter toxicity profiles. In summary, considering toxicology in the absence of these three

factors within the biological time structure of living animals seems to be uneconomical, misleading and unwise.

Superinteractions:

It is now well recognized that human environmental exposures are not to single chemical. Rather humans are exposed concurrently or sequentially to multiple chemicals, by various routes of exposure and from a variety of sources. The process of carcinogenesis can be modified significantly by other chemicals. The term co-carcinogenesis was initially defined as the enhancement of neoplasm induction brought about by new carcinogenic factors, which act in conjunction with an initiating carcinogen.

Whereas the additive or synergistic effects of two or more carcinogens in neoplasm production has been defined as syn-carcinogenesis. When the toxic responses grossly exceed the expected response after an ideal substantial, the process is called as superinteraction. An important example of an environmental 'superinteraction' is that of chlordecone (CD) and CCl_4.

In this case a prior 15 days exposure to CD enhanced the acute toxicity of CCl_4 in male rats by 67-fold. This physiological/biochemical framework within which extremely potent interactions could occur is important in planning screening programmes or to predict superinteractions in toxicology/pharmacology.A brief review of studies made by the science of Toxicology from the times of its founder Paracelsus to modern times as presented in this communication might attract young workers to this wonderful discipline of science.

Possible Questions:

1. Define toxicology. How toxicology was developed.
2. State interrelationship between toxicology and other science disciplines.
3. What are scopes of toxicology.
4. How toxins are metabolized?
5. Briefly state the nature of toxic effects.
6. What is toxicogenomics?
7. What is pharmacogenetics?
8. Define molecular toxicology.
9. What is chronotoxicology?
10. How toxic products are eliminated?

Suggested Readings:

1. Principles of Toxicology by Stephen Roberts.
2. Toxicology Handbook by Lindsay Murray
3. Principles of Ecotoxicology by C.H. Walker
4. Casarett&Doull's Toxicology: The Basic Science by Curtis D. Klaa

Unit-II

Toxicity testing: Bioassays, LC50, LD50, ED50, Synergism, Antagonism, Additive Effect

Objective: In this unit you will learn about different toxicity testing procedures. You will also know about LC50, LD50, ED50, synergism, antagonism and additive Effect.

Introduction:

The purpose of toxicity testing is to generate information about a substance's toxic properties so that the health and environmental risks it poses can be adequately evaluated. Federal agencies use information from toxicity testing to establish acceptable concentrations of environmental agents in drinking water, to set permissible exposure limits for workers, to establish tolerances for pesticide residues on food, to register and re-register pesticides, and ultimately to protect public health and the environment. As reflected in new directives and initiatives for toxicity testing in the United States and Europe, the demand for toxicity information to provide a rational basis for regulating environmental agents has increased. At the same time, testing technologies and methods have continued to emerge. Thus, the U.S. Environmental Protection Agency (EPA) recognized the need for a comprehensive review of established and emerging toxicity-testing methods and strategies and asked the National Research Council (NRC) to conduct such a review and to develop a long-range vision and strategy for toxicity testing. In response to EPA's request, the NRC convened the Committee on Toxicity Testing and Assessment of Environmental Agents, which prepared this report.

Toxicity tests:

Toxicity tests have taken on increased importance after scientists realized that many substances are toxic to living things at levels below chemical detection limits and that there are no methods to analyze for many toxic substances. Toxicity of chemicals is determined in the laboratory. The normal procedure is to expose the test animals to the concerned chemical and measure the effect.

Route ofexposure:

By ingestion (oral), application to the skin (dermal), by inhalation, gavage, or some other method which introduces the material into the body or by placing the test material in the water or air of the test animals' environment

Duration ofexposure:

Acute: short-term exposure (hours or days) of higher doses of toxicant in a single event or in multiple events over the time period and usually produce immediate effects, depending on absorption time of the toxicant. These tests are generally conducted on organisms during a specific time period of the organism's life cycle, and are considered partial life cycle tests. Acute tests are not valid if mortality in the control sample is greater than 10%. Generally it use lethal endpoints and results are reported inEC50.

Chronic: long-term exposure (weeks, months years) of low, continuous doses of a toxicant, relative to the test organism's life span (>10% of life span), and generally use sub-lethal endpoints. Usually, slowly effects are developed in test organism. Chronic

tests are generally considered full life cycle tests and cover an entire generation time or reproductive life cycle ("egg to egg"). Chronic tests are not considered valid if mortality in the control sample is greater than 20%. These results are generally reported in NOECs (No observed effects level) and LOECs (Lowest observed effectslevel).

Sub-chronic: chronic exposure during early, sensitive life stages of an organism that arelessthanacompletereproductivelifecycle.Itisalsocalledasearlylifestage tests, critical life stage, embryo-larval, or egg-fry tests. Early life stage tests are not considered valid if mortality in the control sample is greater than 30%.

Toxicity Endpoints: Toxicity is measured as clinical "endpoints" which include behavioral, physiological, biochemical, histological changes, as follows,

a. Mortality (death)

b. Teratogenicity (ability to cause birthdefects)

c. Carcinogenicity (ability to cause cancer), and

d. Mutagenicity or Genotoxicity (ability to cause heritable change in theDNA).

Measures of Toxicity:

- **Median Lethal Concentration (LC50)**: The concentration of a chemical in an environment (generally air or water) which produces death in 50% of an exposed population of test animals in a specified time frame. It is normally expressed as milligrams of substance per litter of air or water (mg/L) or as ppm.

- **The Median Lethal Dose (LD50):** The concentration of a chemical that is expected to kill 50% of a group of organisms to which it is administered by any of a variety of methods. It is normally expressed as milligrams of substance per kilogram of animal body weight (mg/kg). One of the more commonly used measures of toxicity is the LD50. Example: LD_{50} of sugar and ethanol are 30,000 mg/kg and 13,700mg/kg.

- **Median Effective Concentration (EC_{50})**: The concentration of a chemical that is expected to have one or more specified effects in 50% of a group of organisms after a specified exposure time.

- **Median Effective Dose (ED_{50}):** The dose level at which 50 percent of the test organism have turned over is known as the ED_{50}, which means effective dose for 50 percent of the organism tested. The ED_{50} of any toxicant varies depending on the effect measured. In general, the less severe the effect measured, the lower the ED_{50} for that particular effect
- **Lowest Observed Effect Concentration (LOEC)**: The lowest test concentration that has a statistically significant effect over a specified exposure time.

- **No Observed Effect Concentration (NOEC)**: The highest test concentration for which no effect is observed relative to a control over a specified exposure time. In toxicology, residue tolerance levels of poisons that are permitted in food or in drinking water, for instance, are usually set from 100 to 1,000 times less than the NOEL to provide a wide margin of safety for humans.

- **Maximum Acceptable Toxicant Concentration (MATC)**: An estimated value that represents the highest "no-effect" concentration of a specific substance within the range including the NOEC and LOEC.

- **Application Factor (AF)**: An empirically derived "safe" concentration of a chemical.

- **TLV (threshold limit value)**: The TLV for a chemical is the airborne concentration of the chemical (expressed in ppm) that produces no adverse effects in organism exposed for eight hours per day to five days per week. The TLV is usually set to prevent minor toxic effects like skin or eye irritation.

- **Model animals:** Obviously toxicity is not tested in humans. Instead, animals are used to predict the toxicity that may occur in humans. Common standard aquatic test species are the fathead minnow (*Pimephalespromelas*), daphnids (*Daphnia magna*, *D. pulex*, *D. pulicaria*, *Ceriodaphniadubia*), midge (*Chironomus tentans*, C. *ruparius*), rainbow trout (*Oncorhynchus mykiss*), sheepshead minnow (*Cyprinodonvariegatu*), mysids (Mysidopsis), oyster (Crassotreas), scud (*Hyalalla Azteca*), grass shrimp (*Palaemonetes pugio*), mussels *(Mytilus*). Common standard mammalian test species include rat and dog. These species are routinely selected on the basis of availability, commercial, recreational, and ecological importance, past successful use, and regulatoryuse.
- **Factors:** Toxicity assessment is quite complex, many factors can affect the results of toxicity tests. Some of these factors include variables like temperature, food, light, and stressful environmental conditions. Other factors related to the animal itself include age, sex, health, and hormonalstatus.

- **Application:** Toxicity tests are used to,

  i. provide qualitative and quantitative data on adverse (deleterious) effects on organisms from a toxicant, and
  ii. assess the potential for damage and the risk associated with in a situation for a specific toxicant.

Bioassay:

The foundation of bioassays was laid down by a German physician, Paul Ehrlich. His bioassay on diphtheria antitoxin was the first bioassay to receive recognition.

Definition: A bioassay is an analytical method to determine concentration or potency of a substance by its effect on living cells or tissues.

Principle: Bioassay is a biochemical test to estimate the relative potency of a sample compound to a standard compound. Typical bioassay involves a *stimulus* (ex. drugs) applied to a *subject* (ex. animals, tissues, plants) and a *response* (ex. death) of the subject is triggered and measured. The intensity of stimulus is varied by doses and depending on this intensity of stimulus, a change/response will be followed by a subject.

Classifications:

I. ***In vivo* bioassay**: if assays are used to estimate the potency of agents by observing their effects on living animals, it is called in vivo bioassay. In vivo studies are very important both in the field and laboratory (for validation). They are based on a wide variety of end points, including cell differentiation and enzyme activities. However, it is not possible to use *in vivo* methods for routine or monitoring studies due to ethical problems, expensive, time consuming, and big installations (aquariums etc.)

are needed.

II. ***In vitro* bioassays:** if assays are used to estimate the potency of agents by observing their effects on tissues (in vitro), it is called in vivo bioassay. It can be performed more quickly, and much more cost-effectives than *in vivo* assays. However, *in vitro* assays are not able to explain all the mechanisms.

III. **Direct assay:** The stimulus/standard sufficiently produces measurable and specific response. The response must be clear, easily recognized, and directly measured.

IV. **Qualitative bioassay:** If the measured response is binary, the assay is qualitative, if not, it is quantitative.

V. **Indirect assay based on quantitative response**: The relationship between the dose and the response is first ascertained. Then the dose corresponding to a givenresponse is obtained from the relation for each preparation separately.

VI. **Indirect assay based on quantal response:** The assay involves 'all or none' response (ex. life or death). The response is produced by thresholdeffect.

Examples:

1. **Plant and algae bioassay:** Test species, such as marine unicellular algae *Selenastrumcapricornutum*or *Dunaliellatertiolecta*are used as indicator species. Inhibition of algal growth is used as the indicator of toxicity. The main disadvantage of algal methods is a lack of reproducibility between consecutive assays.

2. **Invertebrate bioassays:** Chronic toxicity test using macro invertebrates have been extensively used in aquatic risks assessment studies. The parameters measured are mortality or reproduction. One of the most common invertebrate toxicity tests uses *Daphnia* and *Ceriodaphnia*, both freshwater species pertaining to *Cladocera*. Tests are carried out by exposing the test organisms to toxic substances under control conditions. Acute lethality tests with Daphnia conducted for 21 days are well established and standardized.

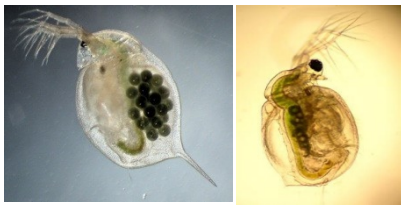


Fig: *Daphnia magna* and *Ceriodaphnia*sp. from left to right.

3. **"In vivo" Fish toxicity bioassays:** Zebra fish, medka, rainbow trout and fathead minnow are generally used in toxicological study. End Points of test includes, mortality (routinely used, 96 hr exposure), larval growth, larval survival, and reproduction. In vivo assays for estrogenicity are widely used. They are based on a wide variety of end points, including cell differentiation and enzyme activities. Vitellogenin (VTG) analysis is done by means of Immunoassay or any other analytical approach.

4. **"In vitro" Recombinant yeast assay:** This assay is based on the evaluation of the potential of a compound to interact with oestrogen receptor and activate hormone-regulated gene promoters. Yeast reporter assay is based on a two-hybrid system. Beta-galactosidase, has been used as the most common reporter enzyme. Novel yeast reporter assay are more suitable for high-throughput analysis, employing in the reporter assay luciferase, named CLuc, as a reporter enzyme.

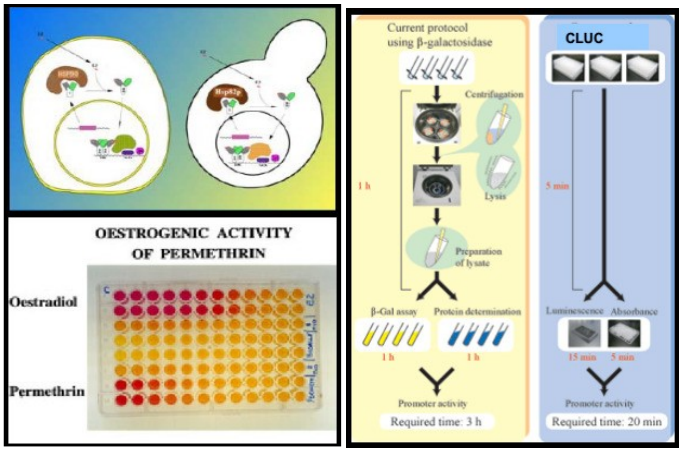


Fig: "In vitro" Recombinant yeast assay.

5. Bacterial toxicity assays:

a) Bioluminescence inhibition: The more widely used bioassays in routine laboratories for evaluating toxicity of wastewater effluents and industrial discharges are based on inhibition of the bioluminescence of marine bacteria. The better-known species of luminescent marine bacteria are *Vibrio fischeri* and *Photobacterium phosphoreum*, which naturally emit light due to an enzyme, the bacterial luciferase. This technique allows the easy screening of large numbers of aqueous samples in a quick, reliable, and inexpensive way.

Fig: Bacterial toxicity assays: Bioluminescence inhibition

b) Genotoxicity Ames Test and umu test*:* Genotoxicity is associated with different structures, such as phenols, chlorophenols, polychlorinated biphenyls (PCBs), or polyaromatic hydrocarbons (PAHs), and constitutes an early screening for possible cancer inducing activity of pollution. The most widespread is the Ames test that is based on the retromutation of *Salmonella typhimurium* TA98 (histidine dependent). The umu test is also based on genetically engineered bacteria *Salmonella thyphimurium*TA 1535 pSK1002 (gram negative, facultative anaerobic enterobacteriaceae) and the genotoxicity is detected measuring the activation of the bacterial SOS repair response of genetic damage in the bacterium, through measuring b-galactosidaseactivity.

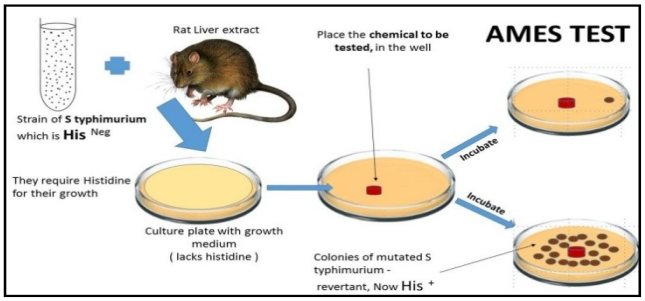


Fig: Bacterial toxicity assays: Genotoxicity Ames Test.

6. **ELISA (Enzyme-linked immunosorbent assay)**: quantitative analytical method that measures absorbance of colour change from antigen-antibody reaction (ex. Direct,indirect, sandwich, competitive). ELISA is used to measure variety of substances in human body from cortisol levels for stress to glucose level fordiabetes.

Uses: Bioassay is used for

1. To test carcinogenicity of chemicals.
2. To test toxicity and safety of drugs, food additives and pesticides.
3. To detect biological hazards.
4. Give a quality assessment of a mixture.
5. To monitor water quality and also sewage discharge and its impact on surrounding.
6. To assess the environmental impact and safety of new technologies and facilities.

Reliance on bioassay increased as the public concern for occupational and environmental hazards increased.

Determination of LC_{50}:
Different procedures are adopted depending on the size of the organisms subjected to a toxicant.

Mosquito Larvae (Test Animal):

Four 1000 ml glass beakers are half-filled with tap water/tank water. A measured amount of toxicant-liquid/solid—is thoroughly mixed with water in three beakers—1, 2, 3 to prepare an aquatic medium of toxicant of three known concentrations. The concentration of the toxicant in the three beakers should be in a gradually increasing order from a lower to higher level. The fourth beaker containing only water is used as a control. Properly label the beakers with necessary information.

A batch of 10 mosquito larvae of same age and size are released in each beaker.

Observe the behaviour—movement, irritability, etc. of the larvae both in test and control beakers and record those with reference to time of exposure.

Continue observation, count the dead larvae in each beaker and record the time of death till the mortality reaches 50%.

The period of 50% mortality is dependent on the concentration of the toxicant.

A dose-response curve for each dose may be prepared by plotting the period of exposure on x-axis (abscissa) and the number of dead larvae on the y-axis (ordinate).A dose — 50% mortality time curve may be prepared by plotting dose (concentration of toxicant) on x-axis and time for 50% mortality on y-axis.

Major Carp Fingerlings (Test Animal):

Four aquaria measuring 75 x 30 x 50 cm each are filled with tank water, preferably from a fish fingerling culture pond up to a height of 45 cm. A measured quantity of toxicant—liquid/ solid is thoroughly mixed with water in three aquaria — 1, 2, 3 to prepare an aquatic medium of toxicant of known concentration.

The concentration of the toxicant in the aquaria should be in a gradually increasing order from a lower to higher level. The fourth aquarium containing only water is used as a control. Properly label the aquaria with necessary information. All the aquaria are covered with mosquito net to prevent jumping out of fingerlings.

A batch of 10 healthy fingerlings — size 75 mm — are released in each aquarium. Regular food supply and aeration of aquarium water is maintained.

Observe the behaviour—movements irritability, loss of balance, etc. of the fingerlings — both in test and control aquaria and record these with reference to the time of exposure.

Continue observation, count the dead fingerlings in each aquarium and record the time of death till the mortality reaches 50%.

The period of 50% mortality is dependent on the concentration of the toxicant.

A dose-response curve for each dose may be prepared by plotting the period of exposure on x-axis and number of dead fingerlings on y-axis.

Determination of LD_{50}:

Albino Mice (Test Animal):

Four batches of healthy albino mice, each consists of six individuals of almost same age group and weight are taken. The toxicant may be used in solid/liquid state. The doses are prepared by mixing the toxicant with food at the ratio mg (toxicant)/kg (weight of mice) — 1 mg/kg, 2 mg/kg, 3 mg/kg.

The total amount of food for each concentration of toxicant should be such that it can be consumed by 6 mice of a batch in one meal. Six baits are prepared for each concentration. The total number of baits will be 3 x 6 = 18. Six baits are prepared without toxicant for the control batch.

The animals are not given food for about 12 hours prior to commencement of the experiment. The control group is treated in the same way.

The fasting animals are given the bait—one for each mice and water. The control batch is given bait without toxicant and water.

Observe the behaviour—movement, irritability, lack of activity, balance, etc. in both the test and control mices and record those with reference to the period of exposure.

Continue observations, count the dead mices in each batch and record the time of death, till the mortality reaches 50%.

The period of 50% mortality is dependent on concentration of the toxicant.

A dose-response curve for each dose may be prepared by plotting the period of exposure on x-axis and the number of dead mices in y-axis.[Dose-50% mortality time curve]

Types of Interactions among toxicants:

When toxic chemicals and substances come in contact with each other, chemical reactions occur. These reactions can be divided into one of four categories: additive, synergistic, antagonistic, and

potentiating.

I. Additive effects: The sum of the effects of the chemicals involved in the reaction. This usually occurs with chemicals that are similar in structure, so they work well as a team. The sum of the additive effects is sum of the effects exposed to each chemicalindividually.

Example: If you take aspirin and acetaminophen both together, you get the total effect of both pain-killing drugs on your body. Aspirin and acetaminophen, are the active ingredient in drugs like Tylenol.

II. Synergistic effects are when the sum of the effects is more than each chemical individually. This can create dangerous situations because each chemical is designed to work well on its own.
Example: Alcohol and acetaminophen are a dangerous combination for your body. This is because both are processed in your liver, and each puts a lot of strain on this small but powerful organ. If you put both drugs into your body at the same time, it can overwhelm the liver, sending it into failure.

III. Antagonistic effects are when the net effect of the chemical reaction is zero. If one is positive and another is negative, both neutralize each other's effect. Antagonistic effects are important because this is where we get antidotes for poisons.

Example: Anti-venom for snakebites and combination of caffeine and alcohol show antagonistic effect.

IV. Potentiating effects: This is when one chemical enhances the effect of another chemical. Some chemicals are not toxic on their own, but when they are in the presence of some other chemicals, they become toxic. This is one more less-common type of interaction.

Table 1. Types of interactions between toxic chemicals and substances.

additivity	a combination of two or more chemicals is the sum of the expected individual responses
antagonism	exposure to one chemical results in a reduction in the effect of the other chemical
potentiation	exposure to one chemical results in the other chemical producing an effect greater than if given alone
synergism	exposure to one chemical causes a dramatic increase in the effect of another chemical

Other Types of Toxicity Tests:

1. **Bioaccumulation tests** are toxicity tests that can be used for hydrophobic chemicals that may accumulate in the fatty tissue of organisms. Toxicants with low solubility in water generally can be stored in the fatty tissue due to the high lipid content in this tissue. The storage of these toxicants within the organism may lead to cumulative toxicity. Bioaccumulation tests use bioconcentration factors (BCF) to predict concentrations of hydrophobic contaminants in organisms. The BCF is the ratio of the average concentration of test chemical accumulated in the tissue of the test organism (under steady state conditions) to the average measured concentration in the water.

2. Effluent toxicity tests are conducted under the Clean Water Act, National Pollutant Discharge Elimination System (NPDES) permit program and are used by wastewater dischargers. Acute Effluent Toxicity Tests are used to monitor the quality of industrial effluent monthly using acute toxicity tests. Effluent is used to perform static-acute multi concentration toxicity tests with *Ceriodaphniadubia* and *Pimephalespromelas*. The test organisms are exposed for 48 hours under static conditions with five concentrations of the effluent. Short-term Chronic Effluent Toxicity Tests are used to monitor the quality of municipal waste water treatment plants effluent quarterly using short-term chronic toxicity tests. It lasts forseven days. The goal of this test is to ensure that the wastewater is not chronically toxic. Short term sublethal tests are used to evaluate the toxicity of effluents to aquatic organisms. Thesemethods are developed by the EPA (Environmental Protection Agency), and only focus on themost sensitive life stages. Endpoints for these tests include changes in growth, reproduction andsurvival and results are reported in NOECs, LOECs and EC50s.

Possible Questions:

1. What is acute, chronic and sub chronic toxicity ?
2. How toxicity is measured ?
3. Define bioassay? State the classification of bioassay.
4. How bacterial toxicity is assessed ?
5. What is in vitro recombinant yeast assay ?
6. What is in vivo fish toxicity bioassay ?
7. What are the uses of bioassay ?
8. State an experiment on fish by which you can determine LC_{50}
9. State an experiment on fish by which you can determine LD_{50}
10. What is additive effect of toxicity ? Give examples.
11. What is synergistic effect of toxicity ? Give examples.
12. What is antagonistic effect of toxicity ? Give examples.
13. What is Potentiating effectsof toxicity ? Give examples.
14. What is bioaccumulation test?
15. What is Effluent toxicity tests ?

Suggested Readings:

1. Principles of Toxicology by Stephen Roberts.
2. Toxicology Handbook by Lindsay Murray
3. Principles of Ecotoxicology by C.H. Walker
4. Casarett&Doull's Toxicology: The Basic Science by Curtis D. Klaassen

Unit-III

Toxicants of public health hazards: Pesticides, Heavy Metals, Radiation, Food and Additives

Objective: In this unit you will learn about toxicants related to public health hazards such as pesticides, heavy metals, radiation, food and additives.

Introduction:

Environmental toxicology is a multidisciplinary field of science concerned with the study of the harmful effects of various chemical, biological and physical agents on living organisms. Ecotoxicology is a sub discipline of environmental toxicology concerned with studying the harmful effects of toxicants at the population and ecosystem levels. Rachel Carson is considered the mother of environmental toxicology, as she made it a distinct field within toxicology in 1962 with the publication of her book *Silent Spring*, which covered the effects of uncontrolled pesticide use. Carson's book was based extensively on a series of reports by Lucille Farrier Stickel on the ecological effects of the pesticide DDT.

Organisms can be exposed to various kinds of toxicants at any life cycle stage, some of which are more sensitive than others. Toxicity can also vary with the organism's placement within its food web. Bioaccumulation occurs when an organism stores toxicants in fatty tissues, which may eventually establish a trophic cascade and the biomagnification of specific toxicants. Biodegradation releases carbon dioxide and water as by-products into the environment. This process is typically limited in areas affected by environmental toxicants.

I. PESTICIDES:

According to World Health Organization (WHO), Pesticides are chemical compounds that are used to kill pests, including insects, rodents, fungi and unwanted plants (weeds). Pesticides are used in public health to kill vectors of disease, such as mosquitoes, and in agriculture, to kill pests that damage crops. By their nature, pesticides are potentially toxic to other organisms, including humans, and need to be used safely and disposed of properly. In general, a pesticide is a chemical or biological agent (such as a virus, bacterium, or fungus) that deters, incapacitates, kills, or otherwise discourages pests. Target pests can include insects, plant pathogens, weeds, molluscs, birds, mammals, fish, nematodes (roundworms), and microbes that destroy property, cause nuisance, or spread disease, or are disease vectors. Although pesticides have benefits, some also have drawbacks, such as potential toxicity to humans and other species.

A. Classification of pesticides by target organisms:

There are many different types of pesticides, each is meant to be effective against specific pests. The term "-cide" comes from the Latin word "to kill."

a. Algaecidesare used for killing and/or slowing the growth of algae.

b. Antimicrobials control germs and microbes such as bacteria and viruses.

c. Acaricides are used against mites and ticks, members of Acaridae.

d. Herbicides kill or inhibit the growth of unwanted plants, aka weeds.

e. Fungicidesare used to control fungal problems like molds, mildew, and rust.

f. *Insecticides* are used to control insects.

g. Molluscicides are designed to control slugs, snails and other mollusks.

h. Rodenticides are used to kills rodents like mice, rats, and gophers.

i. Ovicides are used to control eggs of insects and mites.

j. Pisicides used to reduce the population of rough fish in a water body.

k. Nematicides are used against nematodes.

l. Mothballs are insecticides used to kill fabric pests by fumigation in sealed containers.

B. Classification of pesticides by mode of action:

Pesticides can be classify on the basis of the following ways-

a. Cell toxicants : inhibit different important steps of cell metabolism.

b. Neurotoxicants or nerve Poison : interferes with nervous system function.

c. Chemosterilants: sterilize males of insects or pest vertebrates, classical mode of biological pest control.

d. Disinfectant (Eradicant)*:* effective against pathogen that has already infected the crop.

e. Defoliants : removes the leaves of plants.

f. Germination Inhibitor: inhibits germination of weed seeds, fungus spores and bacterial spores.

g. Nonselective:kills broad range of pests and/or crop plants, usually used in reference to herbicides.

h. Protectants: protects crop if applied before pathogens infect the crop.

i. Repellents: deters or repels pest from crop or interferes with pest's ability to locate crop.

j. Systemic pesticides: absorbed and translocated throughout the plant to provide protection.

k.Stomach Poison: kills after ingestion by an animal.

l. Pheromones: are biologically active chemicals used to attract insects or disrupt their mating behavior.

Benefits of Pesticides:

Pesticides are used for the following purposes:

1. In agriculture, the protection of crops from variouspests.

2. In public health programmes for the control of vectors of variousdiseases.
3. To control household and garden pests.
4. In control of ectoparasites of domestic animals and even the humanbeings.
5. In industry and commercial establishment.
6. In weed control.
7. Invasive species control.

Effects of Pesticides:

a. Health Effects:

Pesticides may cause acute and delayed health effects in people who are exposed. Pesticide exposure can cause a variety of adverse health effects, ranging from simple irritation of the skin and eyes to more severe effects such as affecting the nervous system, mimicking hormones causing reproductive problems, and also causing cancer.

A 2007 systematic review found that "most studies on non-Hodgkin lymphoma and leukemia showed positive associations with pesticide exposure" and thus concluded that cosmetic use of pesticides should be decreased. There is substantial evidence of associations between organophosphate insecticide exposures and neurobehavioral alterations. Limited evidence also exists for other negative outcomes from pesticide exposure including neurological, birth defects, and fetal death.

b. Environmental Effects :

Pesticide use raises a number of environmental concerns. Over 98% of sprayed insecticides and 95% of herbicides reach a destination other than their target species, including non-target species, air, water and soil. Pesticide drift occurs when pesticides suspended in the air as particles are carried by wind to other areas, potentially contaminating them. Pesticides are one of the causes of water pollution, and some pesticides are persistent organic pollutants and contribute to soil contamination.

In addition, pesticide use reduces biodiversity, contributes to pollinator decline, destroys habitat (especially for birds), and threatens endangered species. Pests can develop a resistance to the pesticide (pesticide resistance), necessitating a new pesticide. Alternatively a greater dose of the pesticide can be used to counteract the resistance, although this will cause a worsening of the ambient pollution problem.

c. Economic aspects:

In one study, the human health and environmental costs due to pesticides in the United States was estimated to be $9.6 billion: offset by about $40 billion in increased agricultural production.

Additional costs include the registration process and the cost of purchasing pesticides: which are typically borne by agrichemical companies and farmers respectively. The registration process can take several years to complete (there are 70 different types of field test) and can cost $50–70 million for a single pesticide. At the beginning of the 21st century, the United States spent approximately $10 billion on pesticides annually.

Control of Pesticide Pollution:

1. The non-selective persistent pesticides such as DDT must be phased out of use.
2. Only selective pesticides must be used.
3. Measurement of pesticides to be applied is so important.
4. Repeated pesticides application should be stopped.
5. Proper knowledge about pesticides should be given to public and farmers.
6. Research on pesticides should progress.

2. HEAVY METALS

Metals are natural constituents that exist in the ecosystem. They are substances with high electrical conductivity which voluntarily lose their electrons to form cations. Metals are found all over the earth including the atmosphere, earth crust, water bodies, and can also accumulate in biological organisms including plants and animals. Among the 35 natural existing metals, 23 possess high specific density above 5 g/cm3 with atomic weight greater than 40.04 and are generally termed heavy metals. Theses metals generally termed heavy metals include: antimony, tellurium, bismuth, tin, thallium, gold, arsenic, cerium, gallium, cadmium, chromium, cobalt, copper, iron, lead, mercury, manganese, nickel, platinum, silver, uranium, vanadium, and zinc. This category of metals termed heavy metals have not only been known for their high density but most importantly for their adverse effects to the ecosystem and living organisms. Some of these heavy metals such as cobalt, chromium, copper, magnesium, iron, molybdenum, manganese, selenium, nickel and zinc are essential nutrients that are required for various physiological and biochemical functions in the body and may result to deficiency diseases or syndromes if not in adequate amounts but in large doses they may cause acute or chronic toxicities.

These heavy metals are distributed in the environment through several natural processes such as volcanic eruptions, spring waters, erosion, and bacterial activity, and through anthropogenic activities which include fossil fuel combustion, industrial processes, agricultural activities as well as feeding. These heavy metals do bioaccumulate in living organisms and the human body through various processes causing adverse effects. In the human body, these heavy metals are transported and compartmentalized into body cells and tissues binding to proteins, nucleic acids destroying these macromolecules and disrupting their cellular functions. As such, heavy metal toxicity can have several consequences in the human body. It can affect the central nervous function leading to mental disorder, damage the blood constituents and may damage the lungs, liver, kidneys and other vital organs promoting several disease conditions. Also, long term accumulation of heavy metals in the body may result in slowing the progression of physical, muscular and neurological degenerative processes that mimic certain diseases such as Parkinson's disease and Alzheimer's disease. More so, repeated long-term contact with some heavy metals or their compounds may even damage nucleic acids, cause mutation, mimic hormones thereby disrupting the endocrine and reproductive system and eventually lead to cancer.

This chapter will highlight on the various sources of heavy metals and the processes that promote their exposure and bioaccumulation in the human body. More focus will be laid on the various mechanisms that lead to heavy metal toxicity with emphasis on macromolecule and cellular damages, carcinogenesis, neurotoxicity and the molecular basis for their noxious effects.

Sources of heavy metal exposure to humans

Heavy metals are naturally present in our environment. They are present in the atmosphere, lithosphere, hydrosphere and biosphere. Although these heavy metals are present in the ecosystem, their exposure to humans is through various anthropogenic activities of man. In the earth crust, these

heavy metals are present in ores which are recovered during mining activities as minerals. In most ores heavy metals such as arsenic, iron, lead, zinc, gold, nickel, silver and cobalt exist as sulfides while others such as manganese, aluminum, selenium gold, and antimony exist as oxides. Certain heavy metals such as copper, iron and cobalt can exist both as sulfide and oxide ores. Some sulfides may contain two or more heavy metals together such as chalcopyrite, ($CuFeS_2$) which contains both copper and iron. During these mining activities, heavy metals are released from the ore and scattered in open in the environment; left in the soil, transported by air and water to other areas. Furthermore, when these heavy metals are used in the industries for various industrial purposes, some of these elements are released into the air during combustion or into the soil or water bodies as effluents. More so, the industrial products such as paints, cosmetics, pesticides, and herbicides also serve as sources of heavy metals. Heavy metals may be transported through erosion, run-off or acid rain to different locations on soils and water bodies. As reviewed from, the sources of specific heavy metals are described below.

a. Arsenic:

Arsenic is the 20th most abundant element on earth and the 33rd on the periodic table. The inorganic forms such as arsenite and arsenate compounds are lethal to humans and other organisms in the environment. Humans get in contact with arsenic through several means which include industrial sources such as smelting and microelectronic industries. Drinking water may be contaminated with arsenic which is present in wood preservatives, herbicides, pesticides, fungicides and paints.

b. Lead:

Lead is a slightly bluish, bright silvery metal in a dry atmosphere. The main sources of lead exposure include drinking water, food, cigarette, industrial processes and domestic sources. The industrial sources of lead include gasoline, house paint, plumbing pipes, lead bullets, storage batteries, pewter pitchers, toys and faucets. Lead is released into the atmosphere from industrial processes as well as from vehicle exhausts. Therefore, it may get into the soil and flow into water bodies which can be taken up by plants and hence human exposure of lead may also be through food or drinking water.

c. Mercury:

The metallic mercury is a shiny silver-white, odorless liquid metal which becomes colorless and odorless gas upon heating. Mercury is used in producing dental amalgams, thermometers and some batteries. Also, it can be found in some chemical, electrical-equipment, automotive, metal-processing, and building industries. Mercury can exist in a gaseous form thus it can be inhaled. Other forms of mercury contamination in humans may be through anthropogenic activities such as municipal wastewater discharges, agriculture, incineration, mining, and discharges of industrial wastewater.

d. Cadmium:

This metal is mostly used in industries for the production of paints, pigments alloys, coatings, batteries as well as plastics. Majority of cadmium, about three-fourths is used as electrode component in producing alkaline batteries. Cadmium is emitted through industrial processes and from cadmium smelters into sewage sludge, fertilizers, and groundwater which can remain in soils and sediments for several decades and taken up by plants. Therefore, significant human exposure to cadmium can be by the ingestion of contaminated foodstuffs especially cereals, grains, fruits and leafy vegetables as well as contaminated beverages. Also, humans may get exposed to cadmium by inhalation through incineration of municipal waste.

e. Chromium:

Chromium is a metal that is present in petroleum and coal, chromium steel, pigment oxidants,

fertilizers, catalyst, oil well drilling and metal plating tanneries. Chromium is extensively used in industries such as wood preservation, electroplating, metallurgy, production of paints and pigments, chemical production, tanning, and pulp and paper production. These industries play a major role in chromium pollution with an adverse effect on biological and ecological species. Following the anthropogenic activities by humans, disposal of sewage and use of fertilizers may lead to the release of chromium into the environment. Therefore, these industrial and agricultural practices increase the environmental contamination of chromium. Environmental pollution by chromium has been mostly by the hexavalent chromium in recent years.

f. Copper:

This is a heavy metal which is used in industries to produce copper pipes, cables, wires, copper cookware, etc. It is also used to make copper intrauterine devices and birth control pills. Copper in the form of copper sulfate is added to drinking water and swimming pools. Due to man's anthropogenic and industrial activities, it can accumulate in the soil and up taken by plants. As such, copper is present in some nuts, avocado, wheat germ and bran etc.

g. Manganese:

This metal is added to gasoline as methyl cyclopentadienyl manganese tricarbonyl (MMT) and thus, gasoline fumes contain a very toxic form of manganese.

h. Nickel:

It is used in the production of batteries, nickel-plated jewelry, machine parts, nickel plating on metallic objects, manufacture of steel, cigarette smoking, wire, electrical parts, etc. Also, it can be found in food stuff such as imitation whip cream, unrefined grains and cereals, commercial peanut butter, hydrogenated vegetable oils, as well as contaminated alcoholic beverages [19]. The various sources of heavy metals are summarized in Figure 1.

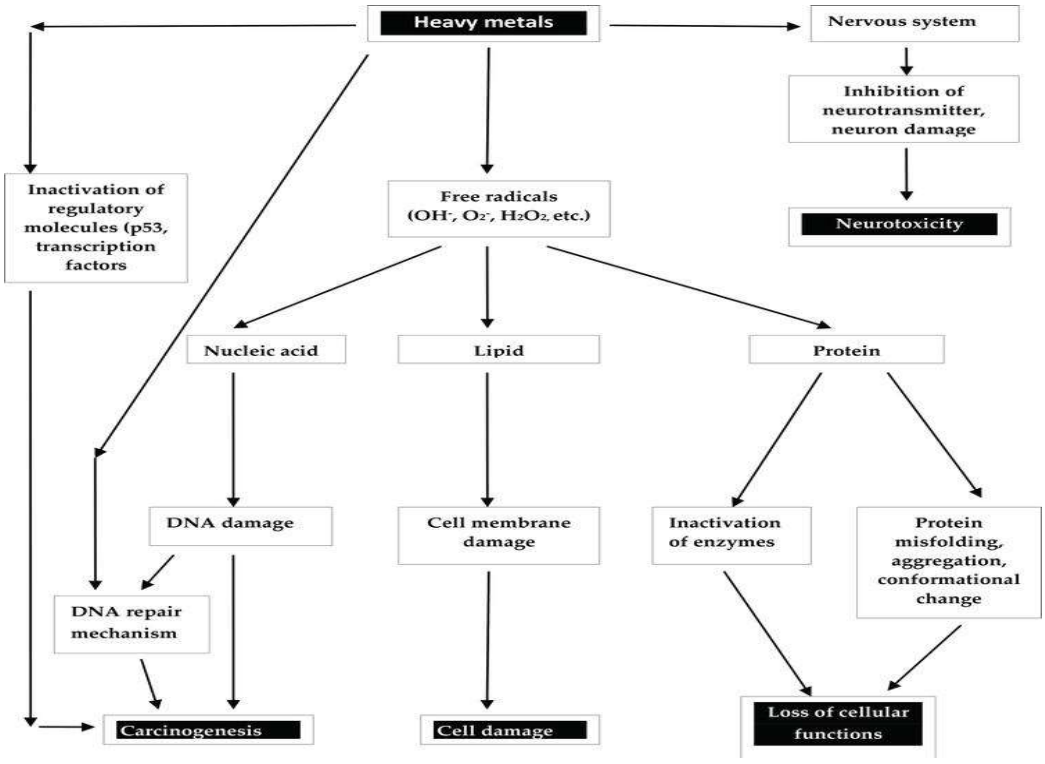


Figure 1. Pathway of heavy metals sources and exposure to human

Heavy Metals	Acute exposure	Chronic exposure
Cadmium	**Pneumonitis (lung inflammation)**	**Lung cancer; Osteomalacia (bones dysfunction); Proteinuria (excess protein in urine).**
Mercury	**Diarrhea; Fever; Vomiting.**	**Stomatitis (inflammation of gums and mouth); Nausea; Nephritic syndrome (nonspecific kidney disorder); Paraguesia (metallic disorder); Neurasthenia (neurotic disorder); Pink disease (pink coloration and pain of hands and feets).**
Lead	**Encephalopathy(brain dysfunction); Nausea;Vomiting.**	**Anemia; Foot drop/wrist drop (palsy); Neuropathy (kidney disease).**
Chromium	**Gastrointestinal hemorrhage; Hemolysis; renalfailure.**	**Pulmonary fibrosis; Lung cancer.**
Arsenic	**Arrhythmia; Nausea; Vomiting; painful neuropathy.**	**Diabetes; Hypopigmentation; Cancer.**

3. Route of exposure, bio-uptake and bioaccumulation of heavy metals in humans :

Humans may directly get in contact with heavy metals by consuming contaminated food stuffs, sea animals, and drinking of water, through inhalation of polluted air as dust fumes, or through occupational exposure at workplace. The contamination chain of heavy metals almost usually follows this cyclic order: from industry, to the atmosphere, soil, water and foods then human. These heavy metals can be taken up through several routes. Some heavy metals such as lead, cadmium, manganese, arsenic can enter the body through the gastrointestinal route; that is, through the mouth when eating food, fruits, vegetables or drinking water or other beverages. Others can enter the body by inhalation while others such as lead can be absorbed through the skin.

Most heavy metals are distributed in the body through blood to tissues. Lead is carried by red blood cells to the liver and kidney and subsequently redistributed to the teeth, bone and hair mostly as phosphate salt. Cadmium initially binds to blood cells and albumin, and subsequently binds to metallothionein in kidney and liver tissue. Following its distribution from blood to the lungs, manganese vapor diffuses across the lung membrane to the Central nervous system (CNS). Organic salts of manganese which are lipid soluble are distributed in the intestine for fecal elimination while inorganic manganese salts which are water soluble are distributed in plasma and kidney for renal elimination. Arsenic is distributed in blood and accumulates in heart, lung, liver, kidney, muscle and neural tissues and also in the skin, nails and hair.

A. Mechanism of heavy metal toxicity:

a. Iron:

Iron is a useful heavy metal in the human body as it is a constituent of certain biological molecules like the hemoglobin and involved in various physiological activities. However, in its free state, iron is one of the heavy metals generally known to generate hydroxyl radical (OH•) as shown below by the Fenton reaction.

$$Fe3{+}{+}O2{-}{\rightarrow}Fe2{+}{+}O2$$
$$Fe2{+}{+}H2O2{\rightarrow}Fe3{+}{+}OH{\cdot}{+}OH{-}(\text{Fenton reaction})$$

Net reaction (Haber-Weiss reaction):

$$O2{-}{+}H2O2{\rightarrow}OH{-}{+}OH{\cdot}{+}O2$$

In addition to the above reactions, the following reactions below can also occur:

$$OH{\cdot}H2O2{\rightarrow}H2O{+}H{+}{+}O2{\cdot}{-}$$
$$OH{\cdot}{+}Fe2{+}{\rightarrow}Fe3{+}{+}OH{-}$$
$$LOOH{+}Fe2{+}{\rightarrow}Fe3{+}{+}LO{-}{+}OH{\cdot}$$

Hydroxyl radical (OH•) is the most common free radical generated by the oxidation of iron. OH• is capable of reacting with biological molecules such as proteins, lipids and DNA damaging them. When OH• reacts with guanine, a nitrogenous base of nucleic acids, it leads to the generation of 8-oxo-7,8-dihydro-20-deoxyguanosine (8-oxo-dG) and 2,6-diamino-5-formamido-4-hydroxypyrimidine (FAPy-G), in which the former is a good marker for oxidative damage.

It is well documented that metal-induced generation of oxygen reactive species can attack polyunsaturated fatty acid such as phospholipids. The first of such observation was first presented by Bucher et al. who showed that iron-generated OH• can oxidize lipid membranes through a process known as lipid peroxidation. Following his experimental observations, he proposed the following mechanism:

Steps of lipid peroxidation:

$$Initiation{:}Lipid{+}R{\cdot}/OH{\cdot}{\rightarrow}Lipid{\cdot}$$
$$Propagation{:}Lipid{\cdot}{+}O2{\rightarrow}Lipid{-}OO{\cdot}$$
$$Lipid{-}OO{+}Lipid{\cdot}{\rightarrow}Lipid{-}OOH{+}Lipid{\cdot}$$
$$Termination{:}Lipid{\cdot}{+}Lipid{\cdot}{\rightarrow}Lipid{-}Lipid$$
$$Lipid{-}OO{\cdot}{+}Lipid{\cdot}{\rightarrow}Lipid{-}OO{-}Lipid$$

At the initiation stage, the radical (R•)/OH• attacks the lipid membrane to form a radial lipid. This radical lipid further propagates the formation of peroxyl lipid radical by reacting with dioxygen molecule or with a lipid. This reaction further promotes damage of the lipid molecule. At the termination stage, two radical lipid molecules and/or with a peroxyl lipid radical reacts to form a stable lipid molecule. The major aldehyde product of lipid peroxidation is malondialdehyde and it serves as a marker for lipid peroxidation.

Generally, proteins are not easily damaged by H2O2 and other simple oxidants unless transition metals are present. Thus, protein damaged are usually metal-catalyzed and involves oxidative scission, bityrosine cross links, loss of histidine residues, the introduction of carbonyl groups, and the formation of protein-centered alkyl (R•), alkoxyl (RO•) and alkylperoxyl (ROO•) radicals.

b. Copper :

Copper ions have been identified to participate in the formation of reactive oxygen species (ROS) as cupric (Cu2+) and cuprous (Cu1+) which can participate in oxidation and reduction reactions. The Cu2+ in the presence of biological reductants such as glutathione (GSH) or ascorbic acid can be reduced to Cu+ which is capable of catalyzing the decomposition of H2O2 to form OH• via the Fenton reaction as shown below.

$$Cu{+}{+}H2O2 \rightarrow Cu2{+}{+}OH\cdot{+}OH{-}$$

The OH• radical formed is capable of reacting with several biomolecules. Experimental studies confirmed that copper is also capable of inducing DNA strand breaks and oxidation of bases via oxygen free radicals. Though in vivo studies have not revealed copper-induced oxidation of low density lipoprotein (LDL), in vitro studies clearly demonstrated LDL oxidation induced by copper.

c. Chromium :

Chromium (Cr), particularly Cr4+ has been shown in in vitro studies to generate free radicals from H2O2. Also, in vivo studies were able to show the detection of free radicals due to chromium in the liver and blood of animals. It was observed that Cr5+ intermediates were generated as a result of one-electron reduction.

d. Cobalt :

Cobalt (Co), particularly Co2+ has been shown to generate superoxide (•O2−) from the decomposition of H2O2.

$$Co2{+}{+}O2 \rightarrow Co{+}{+}O2\cdot{-} \longrightarrow Co{+}{-}OO\cdot$$

e. Vanadium :

Vanadium is a heavy metal that occurs in various oxidative states and has been shown to generate free radical. In the plasma, vanadium (V) is rapidly reduced to vanadium (IV) by NADPH and ascorbic acid antioxidants which bind to plasma proteins for transportation.

$$V5{+}{+}NADPH \rightarrow V4{+}{+}NADP{+}{+}H{+}$$
$$V4{+}{+}O2 \rightarrow V5{+}{+}O2\cdot{-}$$
$$V5{+}{+}O2\cdot{-} \longrightarrow [V5{+}{-}OO\cdot]$$

More so under physiological conditions at approximately pH of 7, V(IV) can generate OH• from the decomposition of H2O2 according to the Fenton reaction.

$$V4{+}{+}H2O2 \rightarrow V5{+}{+}OH{-}{+}\cdot OH$$

f. Arsenic:

Arsenic has also been shown to generate free radicals such as superoxide (O2•–), singlet oxygen (1O2), nitric oxide (NO•), hydrogen peroxide (H2O2), the peroxyl radical (ROO•), dimethylarsinic peroxyl radicals ((CH3)2AsOO•) and also the dimethylarsinic radical ((CH3)2As•) in some studies though the mechanism for the generation of all these reactive species remains unclear.

B. Heavy metal-induced carcinogenesis:

Some heavy metals are known to have carcinogenic effect. Several signaling proteins or cellular regulatory proteins that participate in apoptosis, cell cycle regulation, DNA repair, DNA methylation, cell growth and differentiation are targets of heavy metals. Thus, heavy metals may induce carcinogenic effect by targeting a number of these proteins. More so, the carcinogenic effects of certain heavy metals have been related to the activation of redox-sensitive transcription factors such as AP-1, NF-κB and p53 through the recycling of electrons by antioxidant network. These transcription factors control the expression of protective genes that induce apoptosis, arrest the proliferation of damaged cells, repair damaged DNA and power the immune system. Metal signalization of transcription factor AP-1 and NF-κB has been observed in the mitogen-activated protein (MAP) kinase pathways where the nuclear transcription factor NF-κB, is involved in controlling inflammatory responses while AP-1 is involved in cell growth and differentiation . The p53 protein is an important protein in cell division as it guards a cell-cycle checkpoint and control cell division. Inactivation of p53 allows uncontrolled cell division and thus p53 gene disruption has been associated with most human cancers. Also, AP-1 and NF-κB family of transcription factors are involved in both cell proliferation and apoptosis, and also regulate p53. Heavy metals generated free radicals inside the cell selectively activates these transcription factors and thus, may suggest that cell proliferation or cell death may be related to the exposure to carcinogenic metals. There exist various mechanisms of heavy metal-induced carcinogenesis.

a. Arsenic:

Arsenic-induced carcinogenic mechanisms include epigenetic alterations, damage to the dynamic DNA maintenance system and generation of ROS. Alterations of histones, DNA methylation, and miRNA are the key epigenetic changes induced by arsenic which have shown to possess potentials to cause malignant growth. In vitro studies have shown arsenic to alter the expression of p53 protein which also led to decreased expression of p21, one downstream target. Arsenic compounds have been shown in an in vitro cell line study to promote genotoxicity in humans and mice leucocytes. Also, a methylated form of arsenic was shown to inhibit DNA repair processes and also generate ROS in liver and spleen as metabolic products. Arsenic can bind DNA-binding proteins and disrupt the DNA repair processes thereby increasing the risk of carcinogenesis. For example, the tumor suppressor gene-coded DNA was suppressed when arsenic was bound to methyl-transferase. Also, cancers of the liver, skin, prostate and Kupffer cell were associated with Arsenic poisoning.

b. Lead:

The mechanism of lead-induced carcinogenic process is postulated to induce DNA damage, disrupt DNA repair system and cellular tumor regulatory genes through the generation of ROS. Studies have supported with evidence that ROS generation by lead is key in altering chromosomal structure and sequence. Lead can disrupt transcription processes by replacing zinc in certain regulatory proteins.

c. Mercury:

Little is known on the potential of mercury to act as a mutagen or carcinogen. However, the proposed mechanism of mercury-induced cancer is through the generation of free radicals inducing oxidative stress thereby damaging biomolecules. Mercury has been shown to induce malignant growth through the generation of free radicals as well as disruption of DNA molecular structure, the repair and maintenance system.

d. Nickel:

Nickel has an extensive range of carcinogenic mechanisms which include regulation of transcription factors, controlled expression of certain genes and generation of free radicals. Nickel has been shown

to be implicated in regulating the expression of specific long non-coding RNAs, certain mRNAs and microRNAs. Nickel can promote methylation of promoter and induce the down regulation of maternally expressed gene 3 (MEG3) thereby upregulating hypoxia-inducible factor-1α, two proteins which are known to be implicated in carcinogenesis. It has also been demonstrated that nickel can generate free radicals, which contributes to carcinogenic processes.

e. Cadmium:

Cadmium has been implicated in promoting apoptosis, oxidative stress, DNA methylation and DNA damage.

f. Iron :

The main cause of cancer due to iron intoxication is through the generation of free radicals. A school of thought produced a mechanism for iron-induced cancer whereby bile acids (deoxycholic acid), iron(II) complexes, vitamins K and oxygen interact to generate free radicals which induced oncogenic effect in the colon.

C. Heavy metal-induced neurotoxicity:

Some heavy metals such as lead and manganese may affect the brain and cause neurological toxicity as reviewed from.

a. Lead:

Lead toxicity is targeted towards the memory and learning processes of the brain and can be mediated through three processes. Lead can impair learning and memory in the brain by inhibiting the N-methyl-d-aspartate receptor (NMDAR) and can block neurotransmission by inhibit neurotransmitter release, block the neuronal voltage-gated calcium (Ca2+) channels (VGCCs) and reduce the expression of brain-derived neurotrophic factor (BDNF).

b. Manganese:

Manganese is known to accumulate in the mitochondria of neurons, astrocytes and oligodendrocytes cells and disrupts ATP synthesis by inhibiting the F1/F0 ATP synthase or complex 1 (NADH dehydrogenase) of the mitochondrial respiration chain. More so, it has recently been shown that manganese inhibits ATP synthesis at two sites in the brain mitochondria which are either the glutamate/aspartate exchanger or the complex II (succinate dehydrogenase) depending on the mitochondrial energy source. The disruption of ATP synthesis by manganese leads to decreased intracellular ATP levels and generation of free radicals thereby increasing oxidative stress which may contribute to manganese cellular toxicity. Furthermore, manganese can oxidize dopamine (DA) to react with quinone species thereby disrupting the dopaminergic system. This has been shown in animal studies were manganese exposure has led to specific deficits in the dopaminergic system. The DA reactive species are taken up by the dopamine transporter (DAT1) thus causing dopaminergic neurotoxicity.

Biochemical mechanism of heavy metal toxicity:

When heavy metals are ingested through food or water into the body, they are acidified by the acid medium of the stomach. In this acidic medium, they are oxidized to their various oxidative states (Zn2+, Cd2+, Pb2+, As2+, As3+, Ag+, Hg2+, etc.) which can readily bind to biological molecules

such as proteins and enzymes to form stable and strong bonds. The most common functional group that heavy metals bind is the thio groups (SH group of cysteine and SCH3 group of methionine). Cadmium has been shown to inhibit human thiol transferases such as thioredoxin reductase, glutathione reductase, thioredoxin in vitro by binding to cysteine residues in their active sites. The equations of these reactions are shown below (Figure 2).

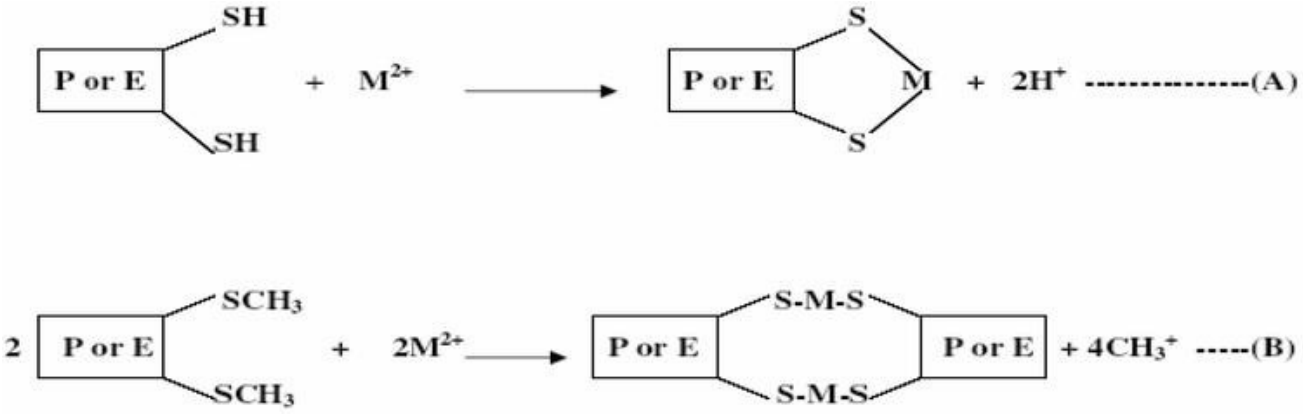


Figure 2.Reactions of Heavy metals with sulphydryl groups of proteins or enzymes (A) = Intramolecular bonding; (B) = Intermolecular bonding; P = Protein; E = Enzyme; M = Metal.

In the above reaction, the oxidized heavy metal replaces the hydrogen of the SH group and the methyl of the SCH3 group thereby inhibiting the function of the protein or activity of the enzyme. For example, methylmercury (MeHg) strongly inhibits the activity of l-glutamine d-fructose-6-phosphate amidotransferase in yeast.

Heavy metal-bound proteins may be a substrate for certain enzymes. In such situations, the heavy metal-bound protein fits into an enzyme in a highly specific pattern to form an enzyme-substrate complex and thus cannot accommodate any other substrate until it is freed. As such, the product of the substrate is not formed as the enzyme is blocked and therefore, the heavy metal remains embedded in the tissue leading to dysfunctions, abnormalities and damages in the body. Inhibition of thiol transferases lead to increased oxidative stress and cell damage. For example, toxic arsenic present in fungicides, herbicides and insecticides can attack –SH groups in enzymes to inhibit their catalytic activities as shown in Figure 3.

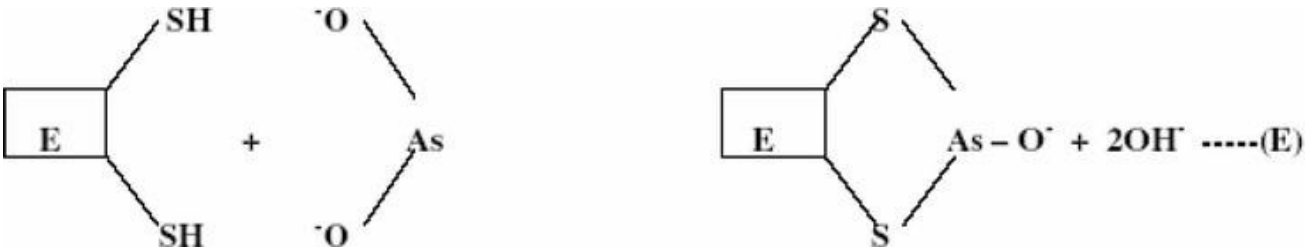


Figure 3.Reaction of arsenic with the thio group of enzymes.

Also, heavy metal toxicity may be induced by the replacement of a metallo-enzyme by another metal ion of similar size. Cadmium displaces zinc and calcium ions from zinc finger proteins and metalloproteins. For instance, cadmium can replace zinc in certain dehydrogenating enzymes, leading to cadmium toxicity. Such replacement can convert the enzyme structurally to an inactive form and completely alter its activity. These heavy metals in their ionic species such as Pb2+, Cd2+, Ag+ Hg2+ and As3+ form very stable biotoxic compounds with proteins and enzymes and are difficult to be

dissociated.

Heavy metals may also inhibit protein folding. This was first observed when heavy metals such as cadmium, lead, mercury and arsenite were shown to effectively interfere with the refolding of chemically denatured proteins. It was also observed that when protein misfolded in the presence of heavy metals, the misfolded protein could not be rescued in the presence of reduced glutathione or EDTA chelator. The order of heavy metal in terms of their efficacy in folding inhibition is mercury > cadmium > lead and correlates with the relative stability of their monodentate complexes with imidazole, thiol and carboxylate groups in proteins.
Heavy metal may cause proteins to aggregate as arsenite-induced protein aggregation was observed and shown to be concentration-dependent. Also, the aggregates contained a wide variety of proteins enriched in functions related to metabolism, protein folding, protein synthesis and stabilization. Saccharomyces cerevisiae (budding yeast) cells was shown to accumulate aggregated proteins after it was exposed to equi-toxic concentrations of cadmium, arsenite and chromium (Cr(VI)) and the effect of protein aggregation was influenced by heavy metals in this order: arsenic > cadmium > chromium [80]. The in vivo potency of these agents to trigger protein aggregation probably depends on the efficiency of their cellular uptake/export and on their distinct modes of biological action. Summarized in Figure 4 is the various mechanisms of heavy metal intoxication.

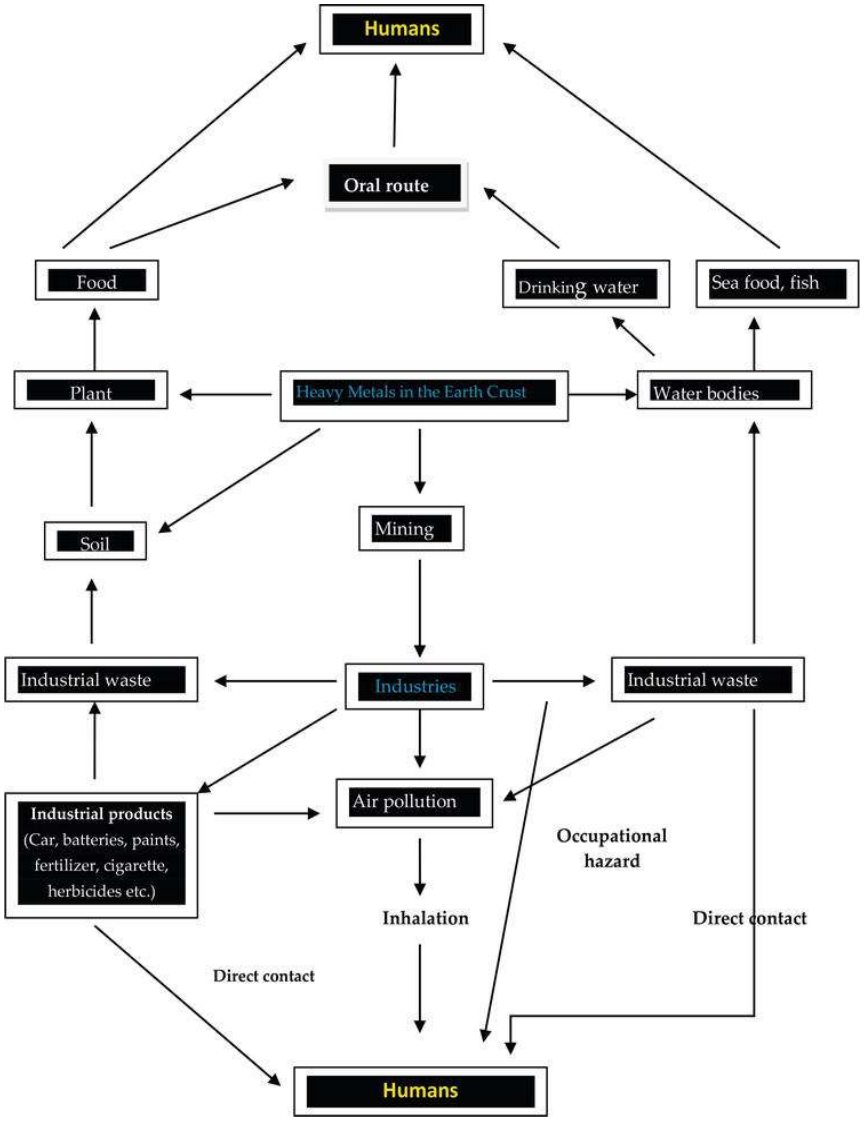


Figure 4. Mechanisms of heavy metal intoxication in humans.

Health effects of heavy metal toxicity in humans:

Heavy metal toxicity can have several health effects in the body. Heavy metals can damage and alter the functioning of organs such as the brain, kidney, lungs, liver, and blood. Heavy metal toxicity can either be acute or chronic effects. Long-term exposure of the body to heavy metal can progressively lead to muscular, physical and neurological degenerative processes that are similar to diseases such as Parkinson's disease, multiple sclerosis, muscular dystrophy and Alzheimer's disease. Also, chronic long-term exposure of some heavy metals may cause cancer. The various health effects of some heavy metals will be highlighted below.

a. Arsenic :

Arsenic exposure can lead to either acute or chronic toxicity. Acute arsenic poisoning can lead to the destruction of blood vessels, gastrointestinal tissue and can affect the heart and brain. Chronic arsenic toxicity which is termed arsenicosis usually focus on skin manifestations such as pigmentation and keratosis. Lower level exposure to arsenic can cause nausea and vomiting, reduced production of erythrocytes and leukocytes and damage blood vessels, cause abnormal heart beat and pricking sensation in hands and legs. Long-term exposure can lead to the formation of skin lesions, pulmonary disease, neurological problems, peripheral vascular disease, diabetes mellitus, hypertension and cardiovascular disease. Chronic arsenicosis may results to irreversible changes in the vital organs and possibly lead to death. Also, chronic arsenic exposure can promote the development of a number of cancers which include skin cancer, cancers of the bladder, lung, liver (angiosarcoma), and possibly the colon and kidney cancers. Recently in the United States, the tolerable amount of arsenic in drinking water is 50 μg/liter but there is much concern of lowering this standard dose of population exposures to arsenic as the present dose is believed to increase the risk for cancer. Most environmental scientists studying this problem are of the view that the current tolerable limit of arsenic in drinking water or food be reduced.

b. Lead:

Toxicity due to lead exposure is called lead poisoning. Lead poisoning is mostly related to the gastrointestinal tract and central nervous system in children and adults. Lead poisoning can be either acute or chronic. Acute exposure of lead can cause headache, loss of appetite, abdominal pain, fatigue, sleeplessness, hallucinations, vertigo, renal dysfunction, hypertension and arthritis while chronic exposure can result in birth defects, mental retardation, autism, psychosis, allergies, paralysis, weight loss, dyslexia, hyperactivity, muscular weakness, kidney damage, brain damage, coma and may even cause death. Although lead poisoning is preventable, it still remains a dangerous disease as it can affect most of the organs of the body. Exposure to elevated levels of lead can cause the plasma membrane of the blood brain barrier to move into the interstitial spaces leading to edema. Also, lead exposure can disrupt the intracellular second messenger systems and alter the functioning of the central nervous system. Developing fetuses and children are most vulnerable to neurotoxic effects due to lead exposure. A number of prospective epidemiologic studies in children less than 5 years of age have shown that low-level of lead exposure (5–25 μg/dL in blood) resulted to the impairment of intellectual development which was manifested by the lost of intelligence quotient points [85]. As such, the Centers for Disease Control (CDC) in the United States has reduced the tolerable amount of lead in children's blood from 25 to 10 μg/dL and recommended universal screening of blood lead for all children.

c. Mercury:

Mercury is an element that can easily combine with other elements to form inorganic and organic mercury. Exposure to elevated levels of metallic, inorganic and organic mercury can damage the kidney, brain and developing fetus while methyl mercury is highly carcinogenic. Organic mercury is lipophilic in nature and thus can easily penetrate cell membranes. Mercury and its compound affects

the nervous system and thus increased exposure of mercury can alter brain functions and lead to tremors, shyness, irritability, memory problems and changes in hearing or vision. Short-term exposure to metallic mercury vapors at higher levels can lead to vomiting, nausea, skin rashes, diarrhea, lung damage, high blood pressure, etc. while short-term exposure to organic mercury poisoning can lead to depression, tremors, headache, fatigue, memory problems, hair loss, etc. Since these symptoms are also common in other illness or disease conditions, diagnosis of mercury poisoning may be difficult in such cases. Chronic levels of mercury exposure can lead to erethism, a disease condition characterized by excitability, tremor of the hands, memory loss, timidity, and insomnia. Also, occupational exposure to mercury as observed by researchers has been associated with measurable declines in performance on neurobehavioral tests of motor speed, visual scanning, visuomotor coordination, verbal and visual memory. Dimethylmercury is a very toxic compound that can penetrate the skin through latex gloves and its exposure at very low dose can cause the degeneration of the central nervous system and death. Mercury exposure to pregnant women can affect the fetus and offspring may suffer from mental retardation, cerebellar symptoms, retention of primitive reflexes, malformation and other abnormalities. This has been confirmed in recent studies in which pregnant women exposed to mercury through dietary intake of whale meat and fish showed reduce motor neuron function, loss of memory, impaired speech and neural transmission in their offspring.

d. Cadmium:

Cadmium and its compounds have several health effects in humans. The health effects of cadmium exposure are exacerbated due to the inability of the human body to excrete cadmium. In fact, cadmium is re-absorbed by the kidney thereby limiting its excretion. Short-term exposure to inhalation of cadmium can cause severe damages to the lungs and respiratory irritation while its ingestion in higher dose can cause stomach irritation resulting to vomiting and diarrhea. Long-term exposure to cadmium leads to its deposition in bones and lungs. As such, cadmium exposure can cause bone and lung damage. Cadmium can cause bone mineralization as studies on animals and humans have revealed osteoporosis (skeletal damage) due to cadmium. It has been observed that “Itai-itai” disease, an epidemic of bone fractures in Japan is due to cadmium contamination. Increased cadmium toxicity in this population was found to be associated with increased risk of bone fractures in women, as well as decreased bone density and height loss in males and females. Cadmium is highly toxic to the kidney and it accumulates in the proximal tubular cells in higher concentrations. Thus, cadmium exposure can cause renal dysfunction and kidney disease. Also, cadmium exposure can cause disturbances in calcium metabolism, formation of renal stones and hypercalciuria. Cadmium is also classified as group 1 carcinogens for humans by the International Agency for Research on Cancer. Tobacco is the main source of cadmium uptake in smokers and thus, smokers are more susceptible to cadmium intoxication than non-smokers. Also, cadmium can cause testicular degeneration and a potential risk factor for prostate cancer.

e. Chromium:

Chromium, in its hexavalent form, is the most toxic species of chromium though some other species such as Chromium (III) compounds are much less toxic and cause little or no health problems. Chromium (VI) has the tendency to be corrosive and also to cause allergic reactions to the body. Therefore, breathing high levels of chromium (VI) can cause irritation to the lining of the nose and nose ulcers. It can also cause anemia, irritations and ulcers in the small intestine and stomach, damage sperm and male reproductive system. The allergic reactions due to chromium include severe redness and swelling of the skin. Exposure of extremely high doses of chromium (VI) compounds to humans can result in severe cardiovascular, respiratory, hematological, gastrointestinal, renal, hepatic, and neurological effects and possibly death. Exposure to chromium compounds can result in the formation of ulcers such as nasal septum ulcer which are very common in chromate workers. Exposure to higher amounts of chromium compounds in humans can lead to the inhibition of erythrocyte glutathione reductase, which in turn lowers the capacity to reduce methemoglobin to hemoglobin. In vivo and in vitro experiments have shown chromate compounds to induce DNA damage in many different ways

and can lead to the formation of DNA adducts, chromosomal aberrations, alterations in replication sister chromatid exchanges, and transcription of DNA. Thus, there are substantial evidence of chromium to promote carcinogenicity of humans as increase stomach tumors have been observed in animals and humans who were exposed to chromium(VI) in drinking water.

f. Iron :

Iron salts such as iron sulfate, iron sulfate heptahydrate and iron sulfate monohydrate are of low acute toxicity when exposure is through dermal, oral and inhalation routes. However, other forms of iron are of serious health problems. Iron toxicity occurs in four stages. The first stage which commences 6 h after iron overdose is marked by gastrointestinal effects such as vomiting, diarrhea and gastro-intestinal bleeding. The progression to the second stage occurs 6–24 h after an overdose and it is considered as a latent period of apparent medical recovery. The third stage commences between 12 and 96 h after the onset of clinical symptoms and is characterized by hypotension, shocks, lethargy, hepatic necrosis, tachycardia, metabolic acidosis and may sometimes lead to death. The fourth and final stage usually occurs within 2–6 weeks of iron overdose. This stage is marked by the development of strictures and formation of gastrointestinal ulcerations. Meat is rich in iron and thus meat eating countries are at risk of cancer as excess iron uptake increases the risk of cancer. Asbestos contains about 30% of iron and thus workers who are highly exposed to asbestos are at high risk of asbestosis, a condition which is known to cause lung cancer. Iron is known to generate free radicals which are suggested to be responsible for asbestos related cancer. Iron-induced free radicals can initiate cancer by the oxidation of DNA leading to DNA damage.

g. Manganese :

Although manganese is an essential metal for the body, it recently became a metal of global concern when methylcyclopentadienyl manganese tricarbonyl (MMT), which was known to be toxic was introduced as a gasoline additive. MMT has been claimed to be an occupational manganese hazard and linked with the development of Parkinson's disease-like syndrome of tremour, gait disorder, postural instability, and cognitive disorder. Exposure to elevated levels of manganese can result in neurotoxicity. Manganism is a neurological disease due to manganese characterized by rigidity, action tremour, a mask-like expression, gait disturbances, bradykinesia, micrographia, memory and cognitive dysfunction, and mood disorder. The symptoms of manganism are very similar to that of Parkinson disease. However, the main differences between manganism and Parkinson disease is the insensitivity of manganism to levodopa (l-DOPA) administration and also the differences in the symptoms and progression of the disease.

Remediation

In humans, heavy metal poisoning is generally treated by the administration of chelating agents. These are chemical compounds, such as CaNa2 EDTA (calcium disodium ethylene diamine tetra acetate) that convert heavy metals to chemically inert forms that can be excreted without further interaction with the body. Chelates are not without side effects and can also remove beneficial metals from the body. Vitamin and mineral supplements are sometimes co-administered for this reason.

Soils contaminated by heavy metals can be remediated by one or more of the following technologies: isolation; immobilization; toxicity reduction; physical separation; or extraction. *Isolation* involves the use of caps, membranes or below-ground barriers in an attempt to quarantine the contaminated soil. *Immobilization* aims to alter the properties of the soil so as to hinder the mobility of the heavy contaminants. *Toxicity reduction* attempts to oxidize or reduce the toxic heavy metal ions, via chemical or biological means into less toxic or mobile forms. *Physical separation* involves the removal of the contaminated soil and the separation of the metal contaminants by mechanical means. *Extraction* is an on or off-site process that uses chemicals, high-temperature

volatization, or electrolysis to extract contaminants from soils. The process or processes used will vary according to contaminant and the characteristics of thesite.

III. RADIATION HAZARD:

The term 'radiation' can refer to a wide variety of forms of energy moving around as waves or particles. It can mean x-rays, or it can mean microwaves. It can also refer to infrared light and even visible light. But when we say 'radioactive pollution,' we're being more specific. **Radioactive pollution** refers to the release of ionizing radiation into the environment as a result of human activity.

Type of radiation

1. Ionizing radiation:

It is the form of radiation that has a short wavelength and a high frequency. In short, it's the form of radiation that's commonly thought of as being high energy and thus harmful to living things. Ionizing radiation includes x-rays and gamma rays. Typical ionizing subatomic particles from radioactivity include alpha particles, beta particles and neutrons. Almost all products of radioactive decay are ionizing because the energy of radioactive decay is typically far higher than that required to ionize. Other subatomic ionizing particles which occur naturally are mesons, positrons, and other particles that constitute the secondary cosmic rays that are produced after primary cosmic rays interact with Earth's atmosphere. Cosmic rays are generated by stars and certain celestial events such as supernova explosions. Cosmic rays may also produce radioisotopes on Earth (for example, carbon-14), which in turn decay and produce ionizing radiation. Cosmic rays and the decay of radioactive isotopes are the primary sources of natural ionizing radiation on Earth referred to as background radiation. Ionizing radiation can also be generated artificially by X-ray tubes, particle accelerators, and any of the various methods that produce radioisotopes artificially. The ionizing radiations are further categorized into two types such as electromagnetic radiation and particulate radiation.

Measuring Ionising Radiation:

Radiation is measured in terms of an ionisation unit called roentgen or r unit, one r being equal to 1.8×10^9 ion pairs per cubic cm of air. In tissue which is ten times as dense as air, a high energy radiation produces about 1000 times the number of ion pairs per cubic cm as it does in air. Another unit called rad measures the total amount of radiant energy absorbed by the medium. One rad equals 100 ergs per gram of tissue. Another unit called gray is equivalent to 100 rads.

In the case of X-rays about 90 % of the energy left in the tissue is used to produce ions, the rest produces heat and excitation. Ultraviolet (UV) is a non-ionising type of radiation and is measured in rads instead of r units. When ionisation is caused by subatomic particles, the doses are measured in different units called rem and sievert. One rem is defined as the amount of any radiation that produces a biological effect equivalent to that resulting from one rad of gamma rays. A sievert is equal to 100 rems. For detecting radiation the Geiger-Muller tube is used. The tube contains a gas which is ionised by radiation. The amount of radiation is gauged from suitable amplifiers and counters.

2. Non-ionic radiation:

Non-ionizing radiation refers to any type of electromagnetic radiation that does not carry enough

energy per quantum (photon energy) to ionize atoms or molecules—that is, to completely remove an electron from an atom or molecule. Instead of producing charged ions when passing through matter, non-ionizing electromagnetic radiation has sufficient energy only for excitation, the movement of an electron to a higher energy state. *Ionizing radiation* which has a higher frequency and shorter wavelength than nonionizing radiation, has many uses but can be a health hazard; exposure to it can cause burns, radiation sickness, cancer, and genetic damage. Using ionizing radiation requires elaborate radiological protection measures which in general are not required with nonionizing radiation.

Alpha (α), beta (β), and gamma (γ) radiations are mainly responsible for radiation pollution. Alpha radiation contains energetic alpha particles. Each alpha particle carries two units of positive charges and interacts strongly with living tissues. Beta, radiation is made up of energetics electrons. Each beta particle carries one unit of negative charge and interacts strongly with matter. Gamma radiations are made up of high energy photons. Photons bring about strong electro-magnetic interaction with matter.

Sources of Radiation Pollution:

a. Natural sources of radiation:

1. Radioactive minerals:

The minerals containing Uranium- 235 (U^{235}), Uranium-238 (U^{238}), Thorium-232 (Th^{232}), Plutonium- 239 (Pu^{239}) etc. are capable of emitting energetic radiations causing pollution.

2. Cosmic rays:

The cosmic rays containing highly energetic particles reach the surface of the earth causing pollution. The intensity of cosmic rays depends on latitudes and altitude of the place. The intensity is maximum at the poles and minimum at the equator.

3. Radionuclides:

The unstable radio-nuclides in the atmosphere can be splitted up into smaller parts emitting energetic radiation. The smaller radio-nuclides enter into the body of organism along with air during respiration.

b. Anthropogenic or Man-made radiation:

1. Nuclear power plants:

Nuclear power plants emit radiation to a very smaller extent except accidental leaks (Chernobyl accident of undivided USSR).

2. Radio-activeWastes:

The nuclear power plants produce a lot of nuclear radio-active wastes. The disposal of these wastes has become a global problem. Some countries producing large quantity of nuclear wastes dump them in ocean near other countries.

3. Nuclear Explosion:

During nuclear explosion, a large number of radio-nuclides are generated in the atmosphere.

The radio nuclides settle down with rain contaminating the soil and water bodies. Finally, these enter into food chain causing serious problem to the living organisms.

4. Radio-isotopes:

Radio-isotopes are also prepared artificially either by nuclear fusion or by nuclear fission. If these radio-isotopes are not properly handled, these emit radiations causing pollution.

5. TelevisionSet:

Television sets produce radiations which can also cause cancer.

Mechanism of Radiation Toxicity:

Ionizing radiation comprising alpha and beta particles and gamma rays loses energy when passing through organic matter by releasing ion pairs of an electron and a positively charged atoms. Ionization can break the bonds in DNA and subsequently damage DNA.

These ion pairs rapidly interact with organic molecules in the tissue and produce free highly reactive oxidative species (ROS) radicals by forming super oxide O_2" anion which subsequently converted to a strong oxidizing agent hydrogen peroxide.

Free radicals or H_2O_2 cause cellular damage by interacting and disrupting structure and function of proteins, amino acids, carbohydrates, nucleic acids, lipids, thiols etc. Damage to DNA results in mutation, chromosomal aberrations and loss of genes and subsequently leading to cell death. The extent and rate of chromosomal aberrations is directly related to radiation dose.

Radiation Toxicity:

There is a variation in susceptibility among different species of organism and also their organs to radiation toxicity as donkeys, rabbits, and poultry are less susceptible than man, dogs, pigs and goats and the organs with fast proliferating cells like skin, gastrointestinal tract and haematopoietic system are most affected with the exception of human lymphocytes. Young animals and foetus are more radiosensitive than adults. Depending upon dose and exposure of duration radiation may cause acute, sub-acute and chronic toxicity in man and animals.

a. Acute radiation toxicity:

Exposure to high doses of irradiation results in acute toxicity and is characterized by severe irritation of GIT resulting in intense and refractory diarrhoea, dehydration, redness of skin, thirst, weakness, recumbency, rapid respiration, panting, profuse and blood stained nasal discharge.

If animal survive, there may be severe depression of bone marrow manifested by anaemia, lymphopenia, agranulocytosis, thrombocytopenia, impaired blood clotting and antibody production, and necrosis of mucosa of GIT, loss of hair and ulceration of skin followed by secondary infections, degenerative changes in lens of eye (cataract), high rate of mutations, tumours mostly of haemopoietic system, particularly leukaemia may be observed.Death may occur due to dehydration and salt depletion few days or weeks post exposure. Most of the deaths in acute and sub-acute cases occur in 1-4 weeks of irradiation.

b. Sub-acute Radiation Toxicity:

Sub-acute toxicity occurs as result of low level radiation continuously for few weeks and is characterized by anorexia, vomiting, depression and weakness followed by fever, knuckling at the fetlock, swelling of legs, diarrhoea, dysentery, polydipsia, recumbency and hyperirritability and severe anaemia and septicaemia in terminal stages leading to death 3-4 weeks post exposure.

c. Chronic Radiation Toxicity:

Prolong exposure of animals as result of ingestion of contaminated pasture may result in chronic toxicity. Consumption of milk, vegetables food grains etc. contaminated with radioactive material may be the source of chronic toxicity and is manifested by retarded growth alopecia, sterility, mutational changes, cancer of blood (leukaemia), thyroid, breast, lungs, colon, stomach, liver, urinary bladder and other tissues and teratogenesis.

d. Pathological Lesions:

Oedema of the dermis, swelling and ulceration of mucosa of gastrointestinal tract, severe congestion and fibrosis in lungs, hypertrophy of adrenals, atrophy and degenerative changes in bone marrow, lymphoid organ, testicles and hepatomegaly, ascites and jaundice are the major pathological lesion.

Diagnosis:

Diagnosis is made on the basis of history, clinical signs and pathological lesions.

Effect of Radiation Pollution:

When radiation passes through different living organisms the following disorders takes place:

1. Radiation splits the molecules of the tissues into ions and free radicals and causes mutation by breaking DNA (Deoxy ribonucleic acid) molecules in the nucleus.

2. Radiation in bone marrow may cause leukaemia.

3. Radiation may cause skin burns which may lead to skin cancer.

4. Radiation at pelvic regions of pregnant ladies, cause damage to the foetus.

Effects of Ionising Radiation on DNA:

Zirkle in 1930 showed that in plants the nucleus is more sensitive to ionising radiation than the cytoplasm. It is now known with certainty that many molecules including DNA are affected by ionising radiation. The purines are less sensitive to radiation than pyrimidines. Out of the pyrimidines, thymine is most sensitive. Large doses of ionising radiation destroy thymine, uracil and cytosine in aqueous solutions. By depolymerizing DNA, ionising radiations prevent DNA replication and stop cell division.

Several mechanisms have been proposed to explain the effects of X-rays and gamma rays. They can break different kinds of chemical linkages and damage genetic material in a variety of ways. Figure 20.2 shows that the effect may be direct or indirect. When a hydrogen atom consisting of one proton and one electron is ionised, the free electron may directly interact with DNA. Or the electron may interact with a molecule of water to produce OH, a free radical which can cause damage to DNA in

the same way as the free electron. The following types of destruction of DNA are possible; hydrogen bonds may break between chains; a base may be changed or deleted; a single or double chain fracture may occur; cross linking might take place within the double helix; a deoxyribose may become oxidised.

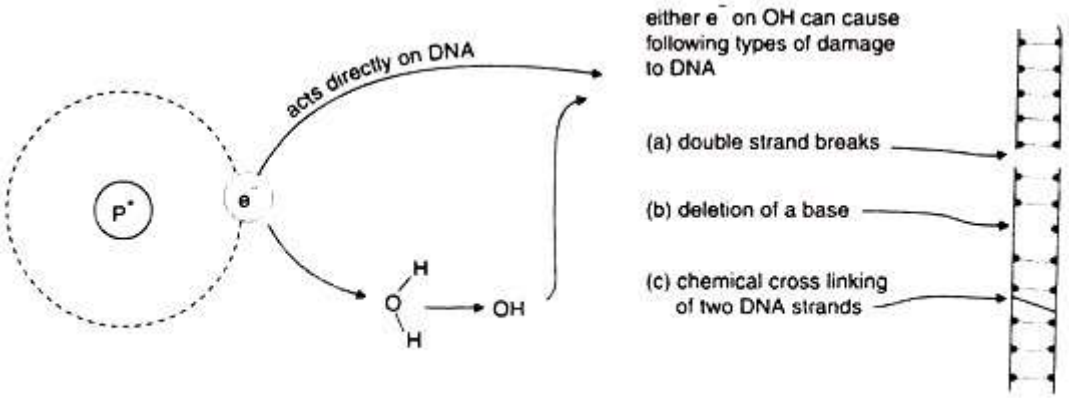


Fig. 20.2 Diagram showing types of damage to DNA by ionising radiation.

If a cell is irradiated in the S phase, DNA replication is inhibited resulting in failure of cell division and cell death. But if the cell is irradiated during mitosis or in G1, in that case DNA replicates normally but mitosis is delayed. Ionising radiation causes breakage and rearrangements in chromosomes which may interfere with normal segregation of chromosomes during cell division. When breaks in two different chromosomes in a cell occur close together in time and space they can join to produce chromosomal aberrations such as inversions, translocations and deletions.

Micro-organisms are more resistant to ionising radiation than higher organisms. It is found that D_{37} dose, that is the radiation dose to a cell population with 37% survival is about 2000 to 30000 rads in bacteria. In human cells D_{37} is about 120 rads. Some chemicals have a protective effect on the cell in reducing the effect of a radiation dose. Aminothiols which have an – SH and – NH_2 group separated by two carbon atoms are most powerful in reducing the effect. The protective effect is expressed as dose reduction factor (DRF).DRF is the ratio of $LD_{50(30)}$ for protected animals to $LD_{50(30)}$ for unprotected animals. LD is the lethal dose or the amount of radiation that kills all individuals in a large group of organisms. $LD_{50(30)}$ is the dose which kills 50 % of organisms within 30 days of exposure. LD_{50} for dog is estimated to be 350 rads, for mouse 550, goldfish 2300.Whether the natural background radiation, though small in amount is dangerous for human beings or not has been questioned. The background radiation consists mainly of cosmic rays, emissions from radioactive elements in the earth such as uranium, radium and thorium, as well as emissions from radioactive isotopes (carbon 14, potassium 40) occurring naturally in the body.

People living at sea level receive an average dose of about 0.8 millisievert of radiation per year. A study of the coastal area of Kerala in South India, a region having high background radiation, has revealed a high incidence of Down's syndrome in the population. Radiation-induced genetic and chromosomal anomalies were also observed.

Control of Radiation Pollution:

Radiation pollution can be controlled in the following ways:

1. Care should be taken to check manmade radiation pollution at source.

2. Nuclear reactor should be perfectly maintained to avoid accidental leakage.

3. Nuclear tests should be banned.

IV. FOOD AND ADDITIVES

Substances that are added to food to maintain or improve the safety, freshness, taste, texture, or appearance of food are known as food additives. Some food additives have been in use for centuries for preservation – such as salt (in meats such as bacon or dried fish), sugar (in marmalade), or sulfur dioxide (in wine).Many different food additives have been developed over time to meet the needs of food production, as making food on a large scale is very different from making them on a small scale at home. Additives are needed to ensure processed food remains safe and in good condition throughout its journey from factories or industrial kitchens, during transportation to warehouses and shops, and finally to consumers.

The use of food additives is only justified when their use has a technological need, does not mislead consumers, and serves a well-defined technological function, such as to preserve the nutritional quality of the food or enhance the stability of the food.Food additives can be derived from plants, animals, or minerals, or they can be synthetic. They are added intentionally to food to perform certain technological purposes which consumers often take for granted. There are several thousand food additives used, all of which are designed to do a specific job in making food safer or more appealing. WHO, together with FAO, groups food additives into 3 broad categories based on their function.

Types of Food Additives

Food additives can be divided into several groups, although there is some overlap because some additives exert more than one effect. For example, salt is both a preservative as well as a flavor.

1. **Acidulants :** Acidulants confer sour or acid taste. Common acidulants include vinegar, citric acid, tartaric acid, malic acid, fumaric acid, and lactic acid.
2. **Acidity regulators :** Acidity regulators are used for controlling the pH of foods for stability or to affect activity of enzymes.
3. **Anticaking agents:** Anticaking agents keep powders such as milk powder from caking or sticking.
4. **Antifoaming and foaming agents:** Antifoaming agents reduce or prevent foaming in foods. Foaming agents do the reverse.
5. **Antioxidants:** Antioxidants such as vitamin C are preservatives by inhibiting the degradation of food by oxygen.
6. **Bulking agents** : Bulking agents such as starch are additives that increase the bulk of a food without affecting its taste.
7. **Food colouring agents** : Colourings are added to food to replace colors lost during preparation or to make food look more attractive.
8. **Fortifying agents** :Vitamins, minerals, and dietary supplements to increase the nutritional value

9. **Colour retention agents**: In contrast to colourings, colour retention agents are used to preserve a food's existing colour.

10. **Emulsifiers :** Emulsifiers allow water and oils to remain mixed together in an emulsion, as in mayonnaise, ice cream, and homogenized milk.

11. **Flavours** : Flavours are additives that give food a particular taste or smell, and may be derived from natural ingredients or created artificially.

12. **Flavour enhancers**: Flavour enhancers enhance a food's existing flavors. A popular example is monosodium glutamate. Some flavor enhancers have their own flavors that are independent of the food.

13. **Flour treatment agents :** Flour treatment agents are added to flour to improve its color or its use in baking.

14. **Glazing agents:** Glazing agents provide a shiny appearance or protective coating to foods.

15. **Humectants**: Humectants prevent foods from drying out.

16. **Tracer gas :** Tracer gas allow for package integrity testing to prevent foods from being exposed to atmosphere, thus guaranteeing shelf life.

17. **Preservatives :** Preservatives prevent or inhibit spoilage of food due to fungi, bacteria and other microorganisms.

18. **Stabilizers**: Stabilizers, thickeners and gelling agents, like agar or pectin (used in jam for example) give food a firmer texture. While they are not true emulsifiers, they help to stabilize emulsions.

19. **Sweeteners**: Sweeteners are added to foods for flavouring. Sweeteners other than sugar are added to keep the food energy (calories) low, or because they have beneficial effects regarding diabetes mellitus, tooth decay, or diarrhoea.

20. **Thickeners** : Thickening agents are substances which, when added to the mixture, increase its viscosity without substantially modifying its other properties.

With the increasing use of processed foods since the 19th century, food additives are more widely used. Many countries regulate their use. For example, boric acid was widely used as a food preservative from the 1870s to the 1920s but was banned after World War I due to its toxicity, as demonstrated in animal and human studies. During World War II, the urgent need for cheap, available food preservatives led to it being used again, but it was finally banned in the 1950s. Such cases led to a general mistrust of food additives, and an application of the precautionary principle led to the conclusion that only additives that are known to be safe should be used in foods. In the United States, this led to the adoption of the Delaney clause, an amendment to the Federal Food, Drug, and Cosmetic Act of 1938, stating that no carcinogenic substances may be used as food additives. However, after the banning of cyclamates in the United States and Britain in 1969, saccharin, the only remaining legal artificial sweetener at the time, was found to cause cancer in rats. Widespread public outcry in the United States, partly communicated to Congress by postage-paid postcards supplied in the packaging of sweetened soft drinks, led to the retention of saccharin, despite its violation of the Delaney clause However, in 2000, saccharin was found to be carcinogenic in rats due only to their unique urine chemistry.

Probable Questions:

1. What is environmental toxicology?
2. Classify pesticides on the basis of target organisms.
3. Classify pesticides on the basis of mode of action.
4. What are the benefits of pesticides?
5. Write down the effects of pesticides on health, environment and economy.
6. How pesticide toxicity can be controlled?
7. What are the sources of heavy metal poisoning?
8. Write down Mechanism of heavy metal toxicity of any 3 metals.
9. What is the biochemical mechanism of heavy metal toxicity?
10. What are the effects of heavy metal on human health?
11. Write down the remediation method of heavy metal toxicity.
12. What is ionizing radiation? How it is measured?
13. What are the sources of radiation pollution?
14. What is ionizing radiation?
15. Describe different types of radiation toxicity.
16. what are the effects of radiation pollution?
17. What are the effects of ionizing radiation on DNA?
18. Describe the health hazards caused by food additives.
19. Describe different types of food additives with examples.
20. How radiation pollution can be controlled?

Suggested Readings:

1. Principles of Toxicology by Stephen Roberts.
2. Toxicology Handbook by Lindsay Murray
3. Principles of Ecotoxicology by C.H. Walker
4. Casarett & Doull's Toxicology: The Basic Science by Curtis D. Klaassen

Unit-IV

Toxicokinetics: Absorption, distribution, elimination

Objective: In this unit you will learn about absorption, distribution and elimination of toxin.

Introduction:

The sequence between exposure to a chemical and the generation of an adverse effect can be divided into two aspects (Figure 3.1); toxicokinetics or the delivery of the compound to its site of action and toxicodynamics or the response at the site of action. This subdivision is particularly useful in risk assessment.

Toxicokinetics is the study of the movement of chemicals around the body. It includes absorption (transfer from the site of administration into the general circulation), distribution (*via* the general circulation into and out of the tissues), and elimination (from the general circulation by metabolism or excretion). The term toxicokinetics has useful connotations with respect to the high doses used in toxicity studies, but it may be misleading if interpreted as the 'movement of toxicants around the body' since, as all toxicologists agree, 'all things are toxic and it is only the dose which renders a compound toxic'. Toxicodynamics relates to the processes and changes that occur in the target tissue, such as metabolic bioactivation and covalent binding, and result in an adverse effect.

Useful toxicokinetic data may be derived using a radiolabelled dose of the chemical, *i.e.* in which a proton in the molecule is replaced by a tritium atom or a carbon or sulfur atom is replaced by the radioactive equivalent (^{14}C or ^{35}S). Such studies are invaluable in following the fate of the chemical skeleton as it is transferred from the site of administration into the blood, is distributed to the tissues, and is eliminated as carbon dioxide or more likely as metabolites in air, urine, or bile. The advantage of using the radiolabelled chemical is that measured radioactivity reflects both the chemical and its metabolites, and this allows quantitative balance studies to be performed, *e.g.* to determine how much of the dose is absorbed, which organs accumulate the compound, and the pathways of metabolism. However, such simple radioactive absorption, distribution, metabolism, and excretion (ADME) studies provide only a part of the total picture, because the lack of chemical specificity in the methods does not allow an assessment of how much of the chemical is absorbed intact and how much is distributed around the body as the parent chemical.Afurther advantage of radiolabelling studies is that radiochromatographic methods can be invaluable in the separation and identification of metabolites, which is an important aspect of the fate of the chemical in the body. Thus, initial ADME studies define the overall fate of the chemical in the body and recognize the main chemical species (parent compound and/or metabolites) that are present in the circulation and in the urine and faeces following metabolism and excretion.

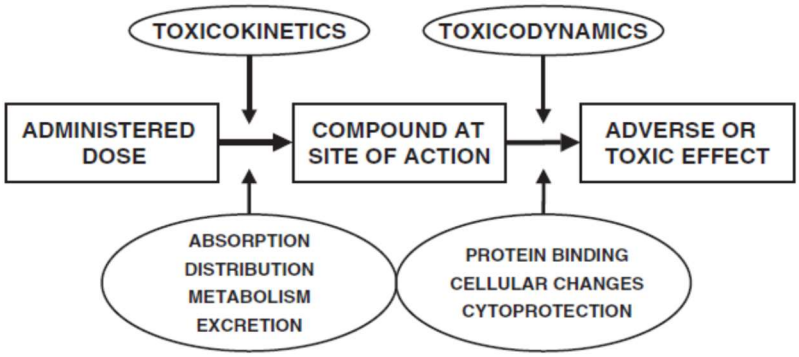


Figure 3.1 *The relationship between delivery of the administered dose to the target site and the generation of the adverse or toxic response*

In recent years, it has been recognized that measurement of the circulating concentrationsofthechemicaland/oritsmetabolitescanprovideusefulinformation on both the magnitude and the duration of exposure of targets for toxicity. The term toxicokinetics is sometimes restricted to studies based on measurements of blood or plasma concentrations, since these provide a vital link between the dosing of experimental animals and the amounts of the chemical in the general circulation (Figure 3.2). Such information can be of great value in the interpretation of species differences in toxic response, and in estimating the possible risk to humans of hazards identified in animal experiments. Toxicokinetic data are also useful in extrapolating across different routes of exposure or administration, as well as from single doses to chronic administration. Chemical-specific toxicokinetic measurements are essential if the results of *in vitro* toxicity tests are to be interpretedlogically.

The ever increasing sensitivity of modern analytical techniques should allow the measurements of 'toxicokinetics' in humans receiving the compound at safe expo- sure levels. Thus, toxicokinetic differences between test animals and humans are open to direct measurement, and such data should increase confidence in the extrapolation process. In contrast, it is unethical intentionally to generate potentially adverse effects in humans and therefore data on inter-species differences in toxicodynamics are limited to observations following accidental poisonings, mild and reversible biomarkers of the potential adverse effect, and *in vitro* studies related to the mode of action of the chemical in animals. The toxicokinetics of a chemical are determined by measuring the concentrations ofthechemicalinplasma(usually)orbloodatvarioustimesfollowingasingledose. The fundamental parameters that define the rates and extents of distribution and elimination are derived from data following an intravenous dose (Figure 3.2). The parameters relating to absorption from an extravascular site of administration, such as gut, lungs, *etc.*, are derived from comparisons of data following an extravascular dose with an intravenous dose. Additional useful information can be obtained from measurements of the concentrations in plasma (or blood) over a period of 24 h in animals treated chronically with the chemical since the area under the plasma concentration–time curve often referred to as 'area under the curve' (AUC) is the best indication of exposure.

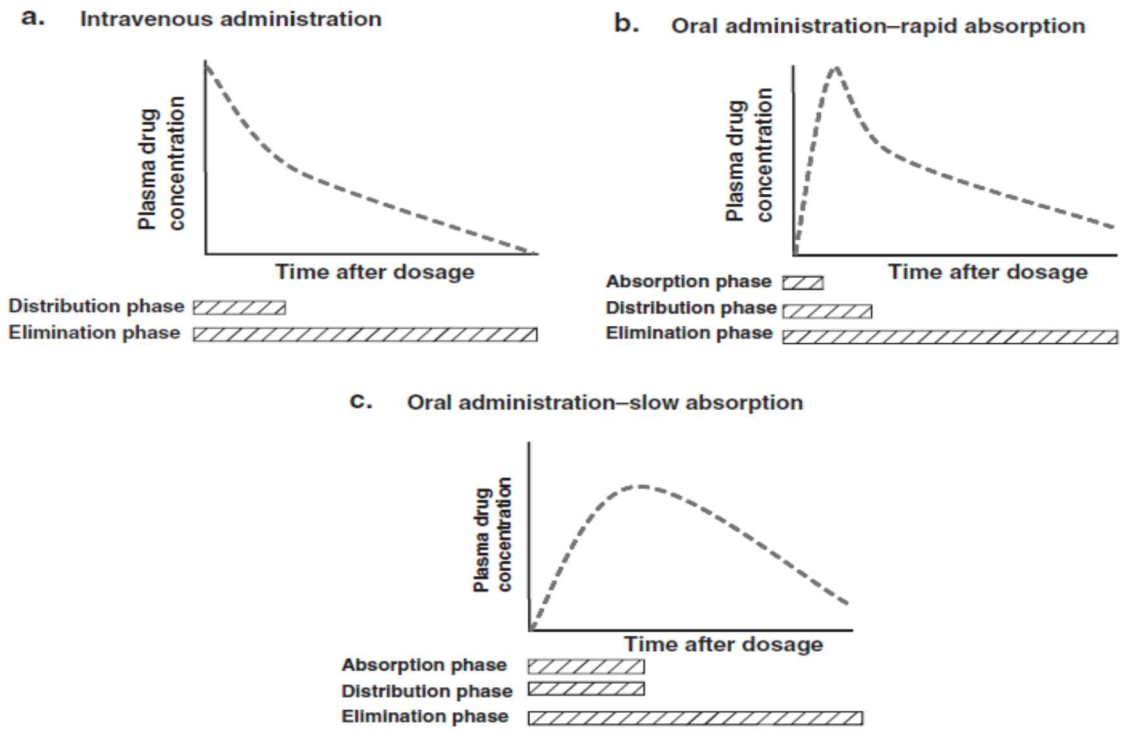


Figure 3.2 *The plasma concentration–time profiles of a chemical following intravenous and oral dosage*

The interpretation of toxicokinetic data requires an understanding of both the biological basis of the processes of absorption, distribution, and elimination and the way that simple measurements of plasma or blood concentrations can be converted intousefulquantitativekineticparametersthatdescribetheseprocesses.Themathematicsusedtodefineandd escribethemovementofachemicaloundthebodycan display various levels of sophistication and complexity. Compartmental analysis (Figure3.3) allows the derivation of a mathematical equation which fits the data and allows the prediction of plasma concentrations at time points that were not measured directly and also outside the confines of the period of experimental observations. Physiologically based pharmacokinetic (PBPK) modelling (Figure 3.4) allows a greater interpretation of the data in biologically relevant terms but requires a sophisticated database to produce valid results. PBPK models (see below) can be used to bridge the gap between species, based on physiological differences and *in vitro* metabolic data, and extended to a biologically based dose–response model by the incorporation of *in vitro* response data.

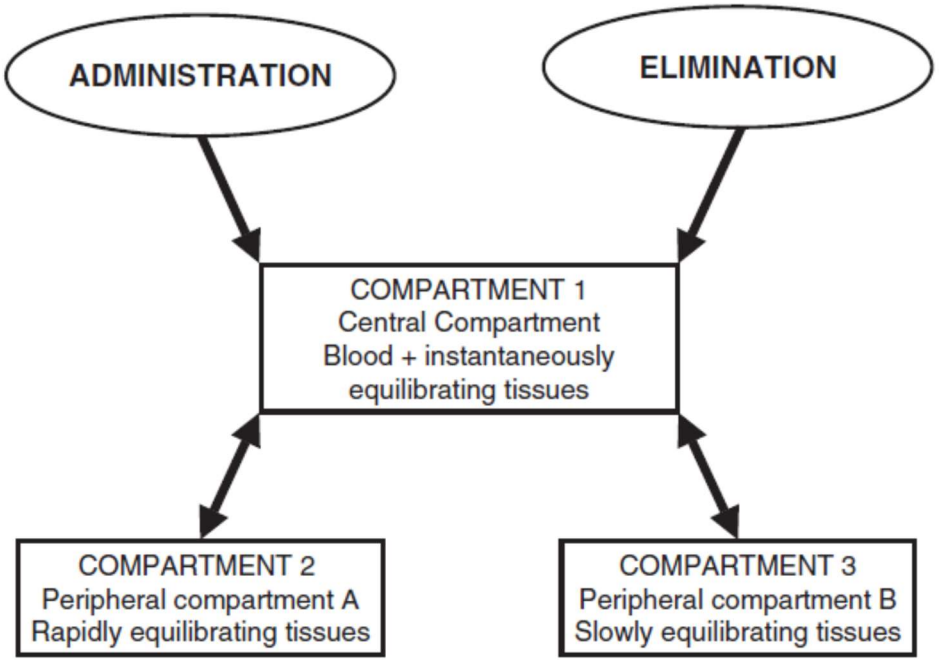


Figure 3.3 *Compartmental analysis. In the example shown, the body is considered to consist of two peripheral compartments that equilibrate with the central compartment. Strictly speaking the only property that links tissues that are part of the same "compartment" is the rate of transfer into and out of the tissue. The central compartment usually comprises blood and well-perfused tissues and equilibrates instantaneously. In the example shown, the compound is eliminated from the central compartment, for example by extraction by the liver or kidneys. The number of compartments necessary in the mathematical model fitted to the data depends on the number of exponential terms necessary to describe the plasma concentration–time curve. The mathematical model can be used to estimate the concentration in plasma or blood at any time after dosage*

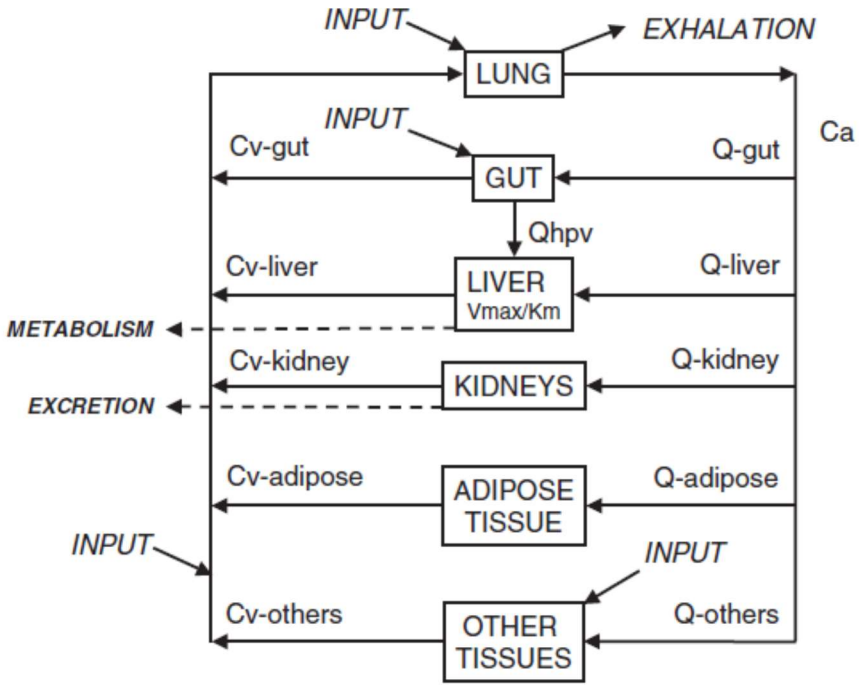


Figure 3.4 *Physiologically based pharmacokinetic model (PBPK). The PBPK model is derived from known rates of organ blood flow, the partition coefficient of the chemical between blood and the tissue, and the rates of the process of elimination, such as V_{max} and K_m for enzymes. PBPK modelling represents a powerful technique for estimating the dose delivered to specific tissues and can facilitate inter-species extrapolation by replacing animal blood flows and enzyme kinetic constants with human data. Removal across an organ equals (C_a–C_v) times the organ blood flow (Q)*

I. ABSORPTION:

The term absorption describes the process of the transfer of the parent chemical from the site of administration into the general circulation, and applies whenever the chemical is administered *via* an extravascular route (*i.e.* not by direct intravascular injection). The term 'absorption' is also used to describe the extent to which the radioactivity from a radiolabelled chemical is transferred from the site of administration into the excreta and/or expired air. However, many chemicals will be metabolized or transformed during their passage from the site of administration into the general circulation, so that little parent chemical may reach the general circulation, despite the fact that all of the radiolabel may leave the site of administration and be eliminated in the urine. This raises the possibility of confusion in discussing the 'extent of absorption' depending on whether the data refer to the parent chemical *per se*, or to radiolabel (which will include the chemical plus metabolites). This confusion is resolved by the proper use of the term bioavailability given below to describe the extent of absorption.

Rate of Absorption :

The rate of absorption may be of toxicological importance because it is a major determinant of the peak plasma concentration and, therefore, the likelihood of acute toxic effects. Transfer of chemicals from the gut lumen, lungs, or skin into the general circulation involves movement across cell membranes, and simple passive diffusion of the unionized molecule down a concentration gradient is the most important mechanism. Lipid-soluble molecules tend to cross cell membranes easily and are absorbed more rapidly than water-soluble ones. The gut wall and lungs provide a large and permeable surface area and allow rapid absorption; in contrast the skin is relatively impermeable and

even highly lipid-soluble chemicals can enter only slowly. The lipid solubility and rate of absorption depend on the extent of ionization of the chemical. Compounds are most absorbed from regions of the gastrointestinal tractatwhichtheyareleastionized.Weakbasesarenotabsorbedfromthestomach, but are absorbed from the duodenum which has a higher luminal pH, whereas weak acids are absorbed from the stomach. The rate of absorption can be affected by the vehicle in which the compound is given, because rapid absorption requires the establishment of a molecular solution of the chemical in the gut lumen. Extremely lipid- soluble compounds, such as dioxins, may be only partially absorbed, because they do not form a molecular solution in the aqueous phase of the intestinal contents. There are few membrane barriers to absorption following subcutaneous or intra-muscular dosage, and the absorption rate may be limited by the water solubility of the injected materials; slow absorption occurs with lipid-soluble compounds injected in an oily vehicle (which contrasts with the rapid absorption possible if such a dose is given *via* the gastrointestinal tract).Irrespective of the route of administration, the rate of absorption is determined from the early time points after dosing (Figure3.5), and is usually described by an absorption rate constant or absorption half-life.

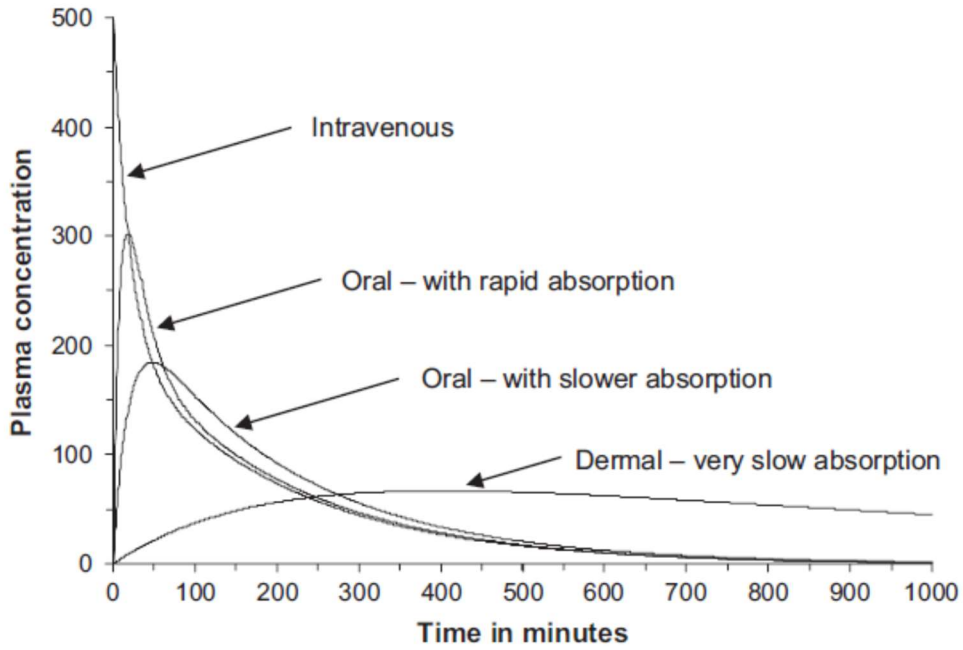


Figure 3.5 *The influence of the rate of absorption of a chemical on the plasma concentration–time curve. A relatively flat low profile is obtained when the rate of absorption is less than the rate of elimination, and this pattern is normally seen with transdermal absorption*

Extent of Absorption

The extent of absorption is important in determining the total body exposure or internal dose, and therefore is an important variable during chronic toxicity studies and/or chronic human exposure. The extent of absorption depends on the extent to which the chemical is transferred from the site of administration, such as the gut lumen, into the local tissue, and the extent to which it is metabolized or broken down by local tissues prior to reaching the general circulation. An additional variable affecting the extent of absorption is the rate of removal from the site of administration by other processes compared with the rate of absorption.

Chemicals given *via* the gastrointestinal tract may be subject to a wide range of pH values and metabolizing enzymes in the gut lumen, gut wall, and liver before they reach the general circulation. The initial loss of chemical prior to it ever entering the blood is termed first-pass metabolism or presystemic metabolism; it may in some cases remove up to 100% of the administered dose so that none of the parent chemical reaches the general circulation. The intestinal lumen contains a range of

hydrolytic enzymes involved in the digestion of nutrients. The gut wall can perform similar hydrolytic reactions and contains enzymes involved in oxidation, such as cytochrome P450 3A4, and conjugation of foreign chemicals. Enterocytes contain P-glycoprotein (PGP) which transports a range of absorbed complex foreign chemicals from the cytosol back into the gut lumen, which can increase the likelihood of first-pass metabolism in the gut lumen or gut wall, or incomplete absorption from the gut lumen. The portal circulation drains into the hepatic portal vein which carries compounds absorbed across the gut wall to the liver, which is the main site of foreign compound metabolism, and is responsible for most first-pass metabolism. The other main reason for incomplete absorption of the parent chemical occurs when the rate of absorption is so slow that the chemical is lost from the body before absorption is complete. Examples of this include incomplete absorption of very water-soluble chemical from the gut and their loss in the faeces, or incomplete dermal absorption, before the chemical is removed from the skin by washing.

Irrespective of the reason that is responsible for the incomplete absorption of the chemical as the parent compound, it is essential that there is a parameter which defines the extent of transfer of the intact chemical from the site of administration into the general circulation. This parameter is the bioavailability, which is simply the fraction of the dose administered that reaches the general circulation as the parent compound. (The term bioavailability is perhaps the most misused of all kinetic parametersandissometimesusedincorrectlyinageneralsenseastheamountavailable specifically to the site of toxicity).The fraction absorbed or bioavailability (F) is determined by comparison with intravenous (i.v.) dosing (where $F = 1$ by definition). The bio-availability can be determined from the area under the plasma concentration–time curve (AUC) of theparentcompound(seeFigure3.6),orthepercentagedoseexcretedinurineasthe parent compound, *i.e.* for an oraldose:

$$F = \frac{\text{AUC oral}}{\text{AUC}} \times \frac{\text{dose i.v.}}{\text{dose oral}}$$

$$F = \frac{\%\text{ in urine as parent compound after oral dosing}}{\%\text{ in urine as parent compound after intravenous dosing}}$$

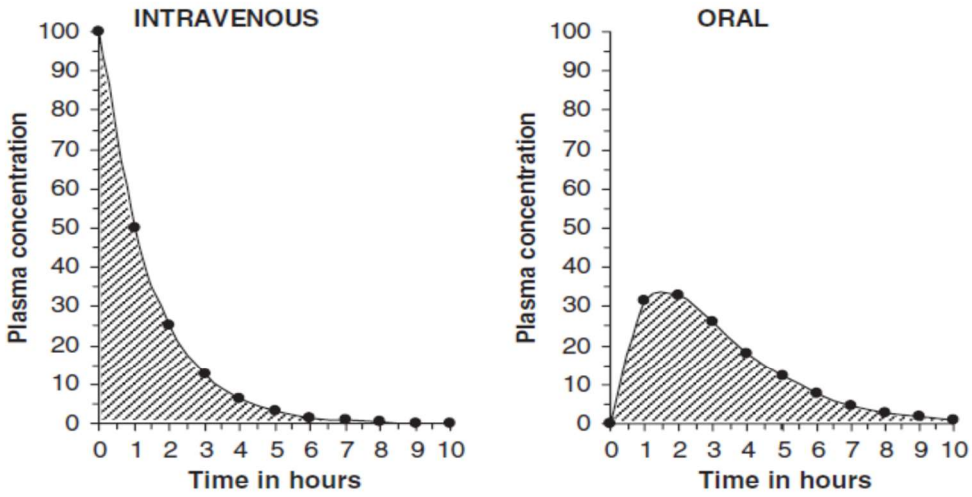


Figure 3.6 *The relationship between the area under the plasma concentration–time curve (AUC) and bioavailability. By definition, the bioavailability (fraction absorbed as the parent compound) is 1 for an intravenous dose. For other routes the bioavailability is given by the AUC for that route divided by the AUC after an intravenous dose (normalized to the same dose in mg kg^{-1})*

II. DISTRIBUTION:

Distributionisthereversibletransferofthechemicalbetweenthegeneralcirculation and the tissues. Irreversible processes such as excretion, metabolism, or covalent binding are part of elimination and do not contribute to distribution parameters. The important distribution parameters relate to the rate and extent ofdistribution.

Rate of Distribution

The rate at which a chemical may enter or leave a tissue may be limited by two factors:

(i) the ability of the compound to cross cell membranesand
(ii) the blood flow to the tissues in which the chemicalaccumulates.

The rate of distribution of highly water-soluble compounds may be slow due to their slow transfer from plasma into body tissues such as liver and muscle; water-soluble compounds do not accumulate in adipose tissue. In contrast, very lipid-soluble chemicals may rapidly cross cell membranes but the rate of distribution may be slow because they accumulate in adipose tissue, and their overall distribution rate may be limited by blood flow to adiposetissue.
Highly lipid-soluble chemicals may show two distribution phases: a rapid initial equilibration between blood and well perfused tissues, and a slower equilibration between blood and poorly perfused tissues (Figure 3.7). The rate of distribution is indicated by the distribution rate constant(s), which is(are) determined from the decrease in plasma concentrations in early time points after an intra venous dose.The rate constants refer to a mean rate of removal from the circulation and may not correlate with uptake into a specific tissue (for which the PBPK approach is more appropriate; see Figure 3.4). Once an equilibrium has been reached between the general circulation and a tissue, any process which lowers the blood (plasma) concentration will cause a parallel decrease in the tissue concentration (Figure 3.8). Thus the elimination half-life measured from plasma or blood samples is also the elimination half-life fromtissues.

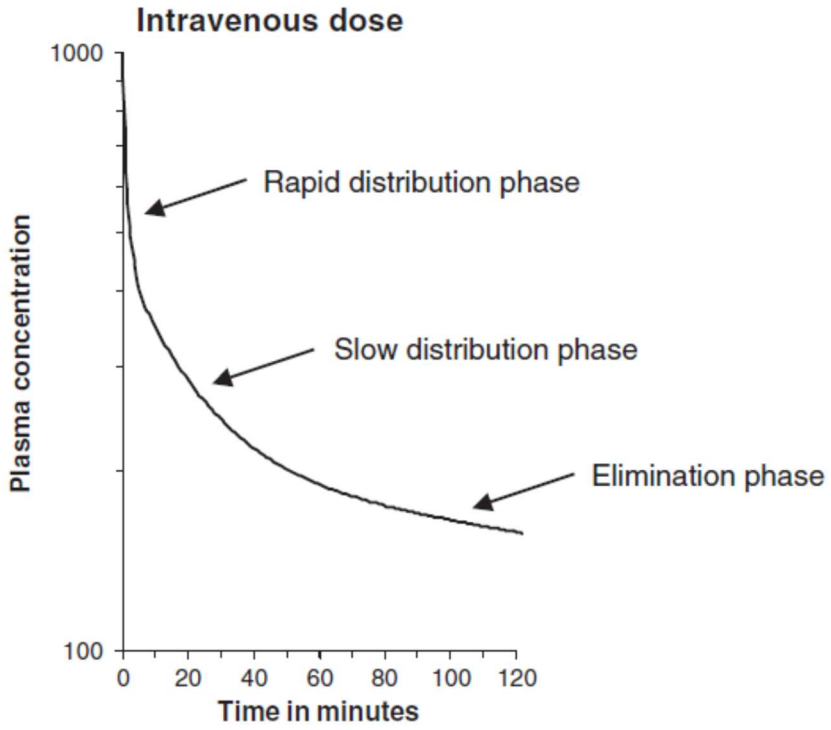


Figure 3.7 *The plasma concentration–time curve for a chemical that requires a three-compartment model (see Figure 3.3)*

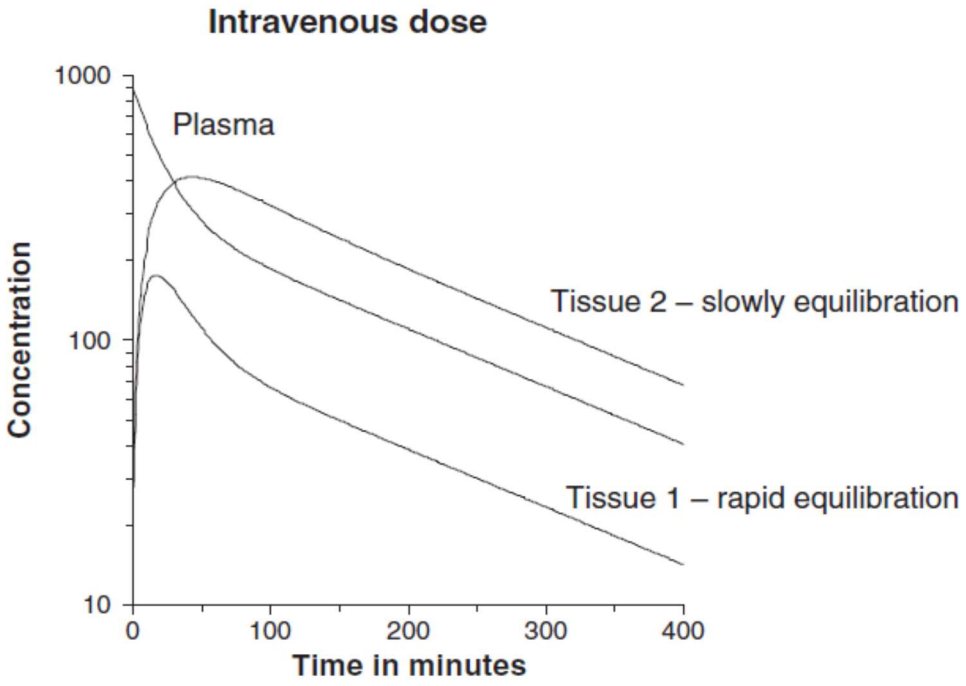


Figure 3.8 *Tissue distribution of a chemical after an intravenous bolus dose. Tissue 1 shows a greater rate of uptake and reaches equilibrium before tissue 2. Tissue 1 shows a lower affinity than tissue 2, so that the concentrations are lower. The concentrations measured in toxicokinetic studies are usually the total concentration (free + bound to proteins or present in cellular lipids) and tissue 2 may show greater tissue binding than tissue 1. The concentrations in all tissues decrease in parallel once all tissues have reached equilibrium with plasma*

Extent of Distribution :

The extent of tissue distribution of a chemical depends on the relative affinity of the blood or plasma compared with the tissues. Highly water-soluble compounds that are unable to cross cell membranes readily (*e.g.* tubocurarine) are largely restricted to extracellular fluid (about 13 L per 70 kg body weight). Water-soluble compounds capable of crossing cell membranes (*e.g.* caffeine, ethanol) are largely present in total body water(about 41Lper 70kg body weight).When one or more body tissues has an affinity for the chemical, such as reversible tissue binding, then the blood (plasma) concentration will be lower than if the compound was evenly distributed through body water. Lipid-soluble compounds frequently show extensive uptake into tissues and may be present in the lipids of cell membranes, adipocytes, central nervous system (CNS), *etc.*; the partitioning between circulating lipoproteins and tissue constituents is complex and may result in extremely low plasma concentrations. A factor which may further complicate the plasma/tissue partitioning is that some chemicals bind reversibly to circulating proteins such as albumin (for acid molecules) and a_1-acid glycoprotein (for basic molecules).

The internal environment of the brain is controlled by the endothelial cells of the blood capillaries to the brain which have tight junctions between adjacent cells, fewer and smaller pores, little endocytosis, and the presence of transporters such as PGP which can extrude chemicals that diffuse across the blood brain barrier. In consequence, water-soluble molecules cannot 'leak' into the brain between endothelial cells (as could happen, for example, in muscle capillaries) and are excluded from the brain. The endothelial membranes have specific transporters for the uptake of essential water- soluble nutrients and some ions and also for the exclusion of organic acids. This so- called blood–brain barrier serves to exclude most water-soluble compounds, so that CNS toxicity may be limited. In contrast, lipid-soluble chemicals readily cross the blood–brain barrier and the CNS is a common site for toxicity (*e.g.* organic

solvents). Similar permeability barriers are present in the choroid plexus, retina, and testes. The extent and pattern of tissue distribution can be investigated by direct measurement of tissue concentrations in animals. Tissue concentrations cannot be measured in human studies and, therefore, the extent of distribution in humans has to be determined based solely on the concentrations remaining in plasma or blood after distribution is complete. The parameter used to reflect the extent of distribu- tion is the apparent volume of distribution (*V*), which relates the total amount of the chemical in the body (*Ab*) to the circulating concentration (*C*) at any time after dis- tribution is complete:

$$V = \frac{Ab}{C}$$

V may be regarded as the volume of plasma in which the body load appears to have been dissolved and simply represents a dilution factor. The volumes of distribution of tubocurarine and caffeine are about 13 and 41 L per 70 kg because of their restricted distribution (see above). However, when a chemical shows a more extensive reversible uptake into one or more tissues the plasma concentration will be lowered and the value of *V* will increase. For highly lipid-soluble chemicals, such as organochlorine pesticides, which accumulate in adipose tissue, the plasma concentration may be so low that the value of *V* may be many litres for each kilogram of body weight.This is not are alvolume of plasma and therefore is called the apparent volume of distribution. It is an important parameter because extensive reversible distribution into tissues, which will give a high value of *V*, is associated with a low elimination rate and a long half-life (see below). It must be emphasized that the apparent volume of distribution simply reflects the extent to which the chemical has moved out of the site of measurement (the general circulation) into tissues, and it does not reflect uptake into any specific tissue(s).

Information on the uptake into specific tissues requires sampling of hat specific tissue, although PBPK modelling can provide useful estimates of tissue concentrates based on *in vitro* partition coefficients and organ blood flows. Once equilibrium has been reached for a tissue, the tissue/plasma ratio will remain constant, so that as the chemical is eliminated from the plasma, the chemical will leave the tissue,maintaining the same ratio (Figure3.8).

III. ELIMINATION

The parameter most commonly used to describe the rate of elimination of a chemical is the half-life (Figure3.9). Most toxicokinetic processes are first-order reactions, *i.e.* the rate at which the process occurs is proportional to the amount of chemical present. High rates (expressed as mass/time) occur at high concentrations and the rate decreases as the concentration decreases; in consequence the decrease is an exponential curve. The usual way to analyze exponential changes is to use logarithmically transformed data which converts an exponential into a straight line. The slope of the line is the rate constant (*k*)for the process and the half-life for the process is calculated as 0.693/*k*. Rate constants and half-lives can be determined for absorption, distribution, and elimination processes.

There are two important biological variables that determine the rate at which a chemical can be eliminated from the body: (i) the functional capacity/ability of the organs of elimination to remove the chemical from the body (the clearance) and (ii) the extent of distribution of the chemical from the general circulation into tissues. The clearance of a chemical is determined by the ability of the organs of elimination (*e.g.*the liver ,kidney, or lungs) to extract the chemical from the plasma or blood and permanently remove it by metabolism or excretion. (Note that this is different from distribution in which the chemical is free to leave the tissue and re-enter the blood when the concentration in the general circulation decreases.)

The mechanisms of elimination depend on the chemical characteristics of the compound:

- volatile chemicals areexhaled,
- water-soluble chemicals are eliminated in the urine and/or bileand
- lipid-soluble chemicals are eliminated by metabolism to more water-soluble molecules, which are then eliminated in the urine and/orbile.

Foreign compound metabolism is an enormous subject and involves a wide range of enzyme systems. Foreign chemicals (xenobiotics) may be metabolized by the enzymes of normal intermediary metabolism, *e.g.* esterases will hydrolyse ester groups. Alternatively, chemicals may be metabolized by enzymes such a scytochrome P450, a primary function of which is xenobiotic metabolism. Species differences in metabolism can be a major source of differences in toxic response.The usual consequence of metabolism is the formation of an inactive excretory products of that species with low metabolizing ability will be likely to show greater toxicity. However, for many com- pounds, metabolism is a critical step in the generation of a toxic or reactive chemical entity(bioactivation) ,and for such compounds high rates of metabolism will be linked with greater toxicity. If a chemical undergoes metabolic activation then toxicokinetic studies should measure both the parent chemical and the active metabolite. If the metabolite is so reactive that it does not leave the tissue in which it is produced (*e.g.* alkylating metabolites of chemical carcinogens), then toxicokinetic studies should define the delivery of the parent chemical to the tissues, and the process of local activation should be regarded as part of tissue sensitivity (toxicodynamics) because it is not strictly speaking part of toxicokinetics, *i.e.* the movement of the chemical and/or metabolites around the body.

The best measure of the ability of the organs of elimination to remove the compound from the body is the clearance (*CL*):

$$CL = \frac{\text{rate of elimination}}{\text{plasma concentration}}$$

Because the rate of elimination is proportional to the concentration (see Figure 3.9), clearance is a constant for first-order processes and is independent of dose. It can be regarded as the volume of plasma (or blood) cleared of compound within a unit of time (*e.g.* mL min^{-1}).

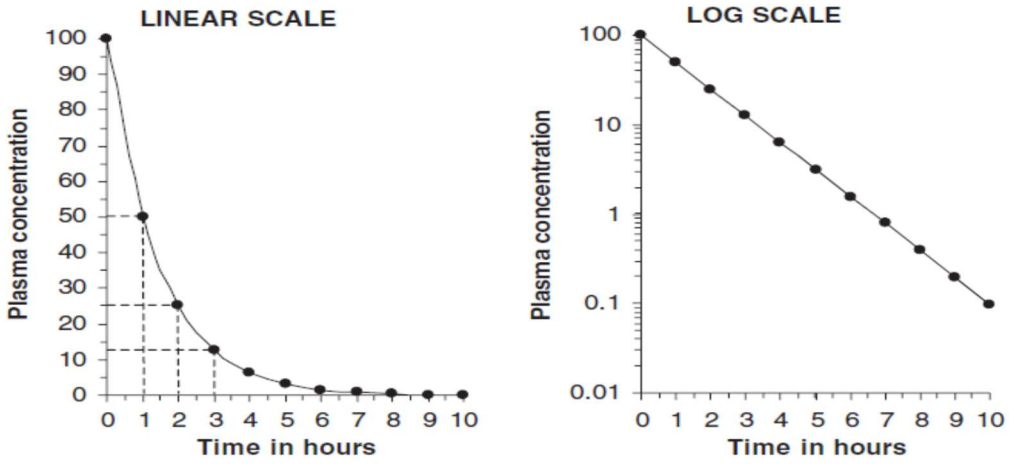


Figure 3.9 *The half-life of a chemical and its determination from plasma data. In the example in this figure the half-life is 1 h. Logarithmic conversion allows the concentration data to be fitted by linear regression analysis; the half-life is calculated as 0.693/slope. Plasma kinetic data are usually fitted by a non-linear least-squares method and there are various programmes available, such as Win-Nonlin*

Renal clearance depends on the extent of protein binding, tubular secretion and passive reabsorption in the renal tubule; it can be measured directly from the concentrations present in plasma and urine:

The total clearance or plasma clearance (which is the sum of all elimination processes, *i.e.* renal + metabolic, *etc.*) is possibly the most important toxicokinetic parameter. It is measured from the total amount of compound available for removal (*i.e.* an intravenous dose) and the total area under the plasma concentration–time curve (AUC) extrapolated to infinity.

$$CL = \frac{\text{Dose i.v.}}{\text{AUC i.v.}}$$

Plasma clearance reflects the overall ability of the body to remove permanently the chemical from the plasma. Plasma clearance is the parameter that is altered by factors such as enzyme induction, liver disease, kidney disease, inter-individual or inter-species differences in hepatic enzymes or in some cases organ blood flow. Once the chemical is in the general circulation, the same volume of plasma will be cleared of chemical per minute (*i.e.* the clearance value) applies irrespective of the route of delivery of chemical into the circulation. However, the bioavailability (*F*) will determine the proportion of the dose reaching the general circulation.Therefore, bioavailability has to be taken into account if clearance is calculated from data from a non-intravenous route (*e.g.*oral).

$$CL = \frac{\text{dose oral} \times F}{\text{AUC oral}}$$

Measurement of dose/AUC for an oral dose determines *CL/F*, which contains two potentially independent variables – the amount of chemical delivered to the blood from the site of administration and the clearance of chemical present in theblood.

The overall rate of elimination, as indicated by the terminal half-life (t^{-1}), is dependent on two physiologically related and independent variables:

$$t_{\frac{1}{2}} = \frac{0.693V}{CL}$$

where *CL* is the ability to extract and remove irreversibly the compound from the general circulation, and *V* the extent to which the compound has left the general circulation in a reversible equilibrium with tissues.Therefore, a chemical may have along half-life because the organs of elimination have a low ability to remove it from plasma and/or because it is extensively distributed to body tissues and only a small proportion of the total body burden remains in the plasma and is available for elimination. Chemicals that are extremely lipid-soluble and are sequestered in adipose tissue are eliminated slowly. Lipid- soluble organochlorine compounds,which are not substrates for P450 oxidation, due to the blocking of possible sites of oxidation by chloro-substituents, are eliminated extremely slowly: for example, the half-life of 2,3,7,8-tetrachlorodibenzodioxin (TCDD) is about 8 years inhumans.

Probable Questions:

1. Define toxicokinetics. Which parameters affect it?
2. How a toxic chemical is absorbed. Give suitable examples.
3. How a toxic chemical is eliminated. Give suitable examples.
4. How a toxic chemical is distributed. Give suitable examples.

Suggested Readings:

1. Principles of Toxicology by Stephen Roberts.
2. Toxicology Handbook by Lindsay Murray
3. Principles of Ecotoxicology by C.H. Walker
4. Casarett&Doull's Toxicology: The Basic Science by Curtis D. Klaasse

Unit-V

Organ toxicity: Hepato, Nephro, Respiratory, Reproductive system

Objective: In this unit you will learn different aspects of toxicity of various vital organs like liver, kidney, lungs and gonads.

Organ toxicity: Organ toxicity means the capacity of substances (xenobiotics) to damage various organs such as kidneys, liver, heart, lungs, nerves etc.

Hepatotoxicity

Hepatotoxicity: It is a toxicants-induced damage of liver, bile duct and gall bladder. The liver is exposed to high very high amount of xenobiotics or its metabolites because of extensive blood supply for the metabolic process of xenobiotics and other substances. The liver is plays a central role in transforming and clearing xenobiotics from the body. The over use of some medicinal drugs or other substances (e.g., natural, agricultural, industrial, herbal, chemical etc.) can induce hepatotoxicity.

Forms of hepatotoxicity: The various forms of hepatotoxicity are-

1. **Steatosis (Fatty liver):** Lipid accumulation in the adipocytes.
2. **Necrosis:** Death of hepatocytes.
3. **Cirrhosis:** Chronic fibrosis due to alcohol intake.
4. **Cholestasis:** Backup of bile salts into the liver.
5. **Hypersensitivity:** Hepatic necrosis due to immune response.
6. **Cancer:** Liver cancer.

Hepatotoxins:

The substances that cause liver injury is called hepatotoxins. There are more than 900 hepatotoxins causing liver damage. Some most familiar hepatotoxins are-

1. **Antipyretic-analgesics (Paracetamol or Acetaminophen):** The overdose of paracetamol causes acute liver failure worldwide.Actually damage to the liver is not due to paracetamol but to its metabolites (NAPQI = N-acetyl benzoquinone imine) of paracetamol produced by the action of cytochrome p450 enzyme in the liver. Normally NAPQI is detoxified in conjugation with glutathione, but its overuse produces a large amount of NAPQI that inhibits the detoxification process and causes liver damage.

2. **NSAIDs (Non steroidal anti-inflammatory drugs):** The NSAIDs (e.g., Aspirin, Ibuprofen, diclofenac, Aceclofenac, nimesulide, piroxicam etc.) are nonnarcotic, nonopoidanalgesic, antipyretic and anti-inflammatory drugs. The individual analgesic rarely induces liver damage. But, worldwide over use of these drugs is showing hepatotoxicity. It is actually dose-dependent hepatotoxic drugs.

3. **Glucocorticoids (corticosterone, cortisone, cortisol):** They are so named due to their effect on carbohydrate metabolism. They cause enlargement of liver due to storage of glycogen that may causes side effect in children. But the prolong use of these drugs causes fattyliver.

4. **Ioniazid (Antituberculosis drugs):** It is associated with upto 20% elevation of liver enzymes (SGOT & SGPT) and severe hepatotoxicity to 1-2% of patients.

5. **Natural products:** The natural hepatotoxins are amanita mushrooms, aflatoxins, alkaloids & green tea extract.

6. **Industrial toxins (Arsenic, CCl_4, vinyl chloride):** They may cause liverdamage.

Nephrotoxicity

The kidneys are the highly susceptible to the toxicants because a high volume of blood with toxicants flows through these organs for the filtration of all kind of toxins from the blood. The toxicants (especially lipid soluble) are concentrated within the cell during this process that can cause nephrotoxicity.

Forms of nephrotoxicity:-

1. Decrease rate of excretion of bodywaste.
2. Inability to maintain body fluid &electrolytes.
3. Decrease synthesis of essential hormone like erythropoietin (Function: It promotes the production of bloodcells).

Nephrotoxic agents & their function:

i) **Cardiovascular drugs as nephrotoxins:** Diuretics (thiazides, furosemide), vasodilators, beta blockers, ACE inhibitors etc.

ii)

iii) **NSAIDs :** Aspirin, ibuprofen, diclofenacetc.

iv) **Antibiotics:** Gentamicin, amphotericin B, cisplatin, ciprofloxacin, rifampicinetc.

v) **Antacids (PPI & H_2 antagonists):** Ranitidine, cemetidineetc.

vi) **Heavy metals:** Lead, mercury &cadmium

vii) **Others:** Lithium salt, gold salt, Heroin, fluorideetc.

Respiratory toxicity

Respiratory toxicity: The toxicants-induced damage of respiratory system (Upper respiratory system e.g., nose, pharynx, larynx & trachea & lower respiratory system e.g., bronchi, bronchioles & lungs &alveoli).

Forms of respiratory toxicity:

1. Asthma
2. Bronchitis

3. Emphysema
4. Laryngitis /pharyngitis
5. Allergic reactions
6. Pneumoconiosis
7. Lung cancer

Respiratory toxicants: There are numerous respiratory toxicants in environment-

i. SO_x, CO_x, NO_x , Ammonia, Chlorine, Fluorine, bromine.

ii. Arsenic & arsenic compounds, cadmium, lead, mercury, nickel, pyrethrum

iii. Aldrin, Dieldrin, Endrin, formaldehyde, kerosene, methane, ethanol, methanol, phenol, xylene, benzene, caffeine, colchicines, DDT

iv. Hydrogen, hydrogen peroxide, HCN, ozone

Reproductive toxicity

The toxicants which are involved in the damage of male or female reproductive systems is called reproductive toxicity.

Forms of toxic effects:

1. Infertility
2. Impotence
3. Interruption of pregnancy: Abortion, fetal death, premature delivery etc.
4. Infant death
5. Altered sexratio
6. Chromosomal abnormalities & birth defects
7. Childhood cancer

Reproductive toxicants:

i. Steroids

ii. Colchicine

iii. DDT

iv. Etodolac

v. Gemfibrozil

vi. Lead & its compounds, cadmium,benzene

vii. Levonorgerserol

viii. Nifedipine, Rifampicin,streptozocin

ix. Cocaine

Reproductive toxicants:

i. Steroids
ii. Colchine
iii. DDT
iv. Etodolac
v. Gemfibrozil
vi. Lead & its compounds, cadmium,benzene
vii. Levonorgerserol
viii. Nifedipine, Rifampicin,streptozocin
ix. Cocaine

Probable Questions:

1. What is organ toxicity?
2. Define hepatotoxicity? Describe different forms of hepatotoxicity.
3. Describe different kinds of hepatotoxins.
4. What is nephrotoxicity?
5. Discuss about Nephrotoxic agents & their functions.

6. Define Respiratory toxicity? Describe different forms of respiratory toxicity.
7. Describe different kinds of respiratory toxicants.
8. What is reproductive toxicity? What are toxic effects of toxins on reproductive system?
9. Name some chemicals which act as toxins to reproductive system.

Suggested Readings:

1. Principles of Toxicology by Stephen Roberts.
2. Toxicology Handbook by Lindsay Murray
3. Principles of Ecotoxicology by C.H. Walker
4. Casarett & Doull's Toxicology: The Basic Science by Curtis D. Klaassen.

Unit-VI

Plant Allelochemicals : types and its role in insect-plant interaction

Objective: In this unit you will learn about plant allelochemicals. Its types and its role in insect -plant interaction.

Introduction:

Allelopathy is a biological phenomenon by which an organism produces one or more biochemicals that influence the germination, growth, survival, and reproduction of other organisms. These biochemicals are known as allelochemicals and can have beneficial (positive allelopathy) or detrimental (negative allelopathy) effects on the target organisms and the community. Allelochemicals are a subset of secondary metabolites, which are not required for metabolism (i.e. growth, development and reproduction) of the allelopathic organism. Allelochemicals with negative allelopathic effects are an important part of plant defence against herbivory. The production of allelochemicals are affected by biotic factors such as nutrients available, and abiotic factors such as temperature and pH.

Allelopathy is characteristic of certain plants, algae, bacteria, coral, and fungi. Allelopathic interactions are an important factor in determining species distribution and abundance within plant communities, and are also thought to be important in the success of many invasive plants. For specific examples, see black crowberry (*Empetrum hermaphroditum*), spotted knapweed (*Centaurea maculosa*), garlic mustard (*Alliaria petiolata*), *Casuarina/Allocasuarina* spp, and nutsedge. The process by which a plant acquires more of the available resources (such as nutrients, water or light) from the environment without any chemical action on the surrounding plants is called resource competition. This process is not negative allelopathy, although both processes can act together to enhance the survival rate of the plant species.

Allelopathy describes those situations and events where chemicals produced by higher plants, algae, fungi, or microorganisms cause some effect, either inhibitory or stimulatory, on other members of the plant or microbial community . Unlike competition for a resource, the central principle in allelopathy arises from the fact that plants and microorganisms collectively produce thousands of chemicals, and many of these chemicals are released from the producing organism by leaching, exudation , volatilization , or decomposition processes. Subsequently, some of these compounds (known as allelochemicals) alter the growth or physiological functions of organisms that encounter them during growth. For example, almost pure droplets of sorgoleone (a quinone) are exuded from the roots of *Sorghum* species, and sorgoleone inhibits growth in plants that contact it by blocking photosynthesis and respiration. While the word "allelopathy" was first used in the 1930s, the phenomenon that it describes was suggested by natural philosophers more than two thousand years ago as they observed that some plants did not grow well near other kinds of plants.

Research conducted in the last half of the twentieth century demonstrated cases of growth inhibition by allelochemicals that influenced vegetational patterns, rate and sequences in plant succession, weed abundance, crop productivity, and problems in replanting fruit and other crops. Investigators have focused on identifying the producing plants and the chemicals they give off, the physiological effects on receiving species, and how climatic and soil conditions change the action of allelochemicals. Cinnamic and benzoic acids, flavonoids , and various terpenes are the most commonly found allelochemicals, but several hundred chemicals have been identified, including many other classes of secondary plant compounds. A few allelochemicals have been developed as herbicides and pesticides, and it may be possible to genetically engineer a crop to produce its own herbicides.

Allelochemicals:

A substance (semiochemical) produced by members of one species that influences the behaviour or growth of members of another species. Allelochemicals can be divided into several categories. *Kairomones* benefit the receiving organism but cause disadvantage to the producer. For example, many plants (e.g. cabbages) release aromatic chemicals that attract insect predators, while parasites often exploit the pheromones released by their hosts to locate a suitable host; certain insect predators detect their prey in a similar way. *Allomones* benefit the producer but have no effect on the receiver. For example, many members of the beetle family Lycidae emit pungent chemicals that warn potential predators of their distasteful nature. Hence they are protected from predation, while the impact on the potential predator is neutral. The flowers of certain orchids emit allomones that mimic the sex pheromones of their bee or wasp pollinator. Males of the respective insect species attempt to copulate with the orchid flower, and pollinate it in the process, thus benefiting the orchid, while the cost to the deceived male insect is minimal. *Synomones* are beneficial to both producer and recipient. For example, pine trees damaged by beetles often emit terpenes that attract parasitoid insects that parasitize the pest beetles. Hence the parasitoid finds a suitable host, and the tree's pests are controlled.

History:

The term allelopathy from the Greek-derived compounds *allelo-* and *-pathy* (meaning "mutual harm" or "suffering"), was first used in 1937 by the Austrian professor Hans Molisch in the book *Der Einfluss einer Pflanze auf die andere - Allelopathie* (The Effect of Plants on Each Other - Allelopathy) published in German. He used the term to describe biochemical interactions that inhibit the growth of neighbouring plants, by another plant. In 1971, Whittaker and Feeny published a study in the journal *Science*, which defined allelochemicals as all chemical interactions among organisms. In 1984, Elroy Leon Rice in his monograph on allelopathy enlarged the definition to include all direct positive or negative effects of a plant on another plant or on micro-organisms by the liberation of biochemicals into the natural environment. Over the next ten years, the term was used by other researchers to describe broader chemical interactions between organisms, and by 1996 the International Allelopathy Society (IAS) defined allelopathy as "Any process involving secondary metabolites produced by plants, algae, bacteria and fungi that influences the growth and development of agriculture and biological systems." In more recent times, plant researchers have begun to switch back to the original definition of substances that are produced by one plant that inhibit another plant. Confusing the issue more, zoologists have borrowed the term to describe chemical interactions between invertebrates like corals and sponges.

Long before the term allelopathy was used, people observed the negative effects that one plant could have on another. Theophrastus, who lived around 300 BC noticed the inhibitory effects of pigweed on alfalfa. In China around the first century AD, the author of *Shennong Ben Cao Jing* described 267 plants that had pesticidal abilities, including those with allelopathic effects. In 1832, the Swiss botanist De Candolle suggested that crop plant exudates were responsible for an agriculture problem called soil sickness.

Allelopathy is not universally accepted among ecologists and many have argued that its effects cannot be distinguished from the competition which results when two (or more) organisms attempt to use the same limited resource, to the detriment of one or both. Allelopathy is a direct negative effect on one organism resulting from the input of substances into the environment by another. In the 1970s, great effort went into distinguishing competitive and allelopathic effects by some researchers, while in the 1990s others argued that the effects were often interdependent and could not readily be distinguished.

However, by 1994 D. L. Liu and J. V. Lowett at the Department of Agronomy and Soil Science, University of New England in Armidale, NSW, Australia wrote two papers in the *Journal of Chemical Ecology* that developed methods to separate the allelochemical effects from other competitive effects, using barley plants and inventing a process to examine the allelochemicals directly. In 1994, M-C Nilsson at the Swedish University of Agricultural Sciences in Umeå, showed

in a field study that allelopathy exerted by *Empetrum hermaphroditum* reduced growth of Scots pine seedlings by c. 40%, and that below-ground resource competition by *E. hermaphroditum* accounted for the remaining growth reduction. For this work she inserted PVC-tubes into the ground to reduce below-ground competition or added charcoal to soil surface to reduce the impact of allelopathy, as well as a treatment combining the two methods.

Application :

The possible application of allelopathy in agriculture is the subject of much research. Current research is focused on the effects of weeds on crops, crops on weeds, and crops on crops. This research furthers the possibility of using allelochemicals as growth regulators and natural herbicides, to promote sustainable agriculture. A number of such allelochemicals are commercially available or in the process of large-scale manufacture. For example, Leptospermone is a purported thermochemical in lemon bottlebrush (*Callistemon citrinus*). Although it was found to be too weak as a commercial herbicide, a chemical analog of it, mesotrione (tradename Callisto), was found to be effective. It is sold to control broadleaf weeds in corn but also seems to be an effective control for crabgrass in lawns. Sheeja (1993) reported the allelopathic interaction of the weeds *Chromolaena odorata* (*Eupatorium odoratum*) and *Lantana camara* on selected major crops.

Many crop cultivars show strong allelopathic properties, of which rice (*Oryza sativa*) has been most studied. Rice allelopathy depends on variety and origin: Japonica rice is more allelopathic than Indica and Japonica-Indica hybrid. More recently, critical review on rice allelopathy and the possibility for weed management reported that allelopathic characteristics in rice are quantitatively inherited and several allelopathy-involved traits have been identified.

Many invasive plant species interfere with native plants through allelopathy. A famous case of purported allelopathy is in desert shrubs. One of the most widely known early examples was *Salvia leucophylla*, because it was on the cover of the journal *Science* in 1964. Bare zones around the shrubs were hypothesized to be caused by volatile terpenes emitted by the shrubs. However, like many allelopathy studies, it was based on artificial lab experiments and unwarranted extrapolations to natural ecosystems. In 1970, *Science* published a study where caging the shrubs to exclude rodents and birds allowed grass to grow in the bare zones. A detailed history of this story can be found in Halsey 2004.

Allelopathy has been shown to play a crucial role in forests, influencing the composition of the vegetation growth, and also provides an explanation for the patterns of forest regeneration. The black walnut *(Juglans nigra)* produces the allelochemical juglone, which affects some species greatly while others not at all. The leaf litter and root exudates of some *Eucalyptus* species are allelopathic for certain soil microbes and plant species. The tree of heaven, *Ailanthus altissima*, produces allelochemicals in its roots that inhibit the growth of many plants. The pace of evaluating allelochemicals released by higher plants in nature has greatly accelerated, with promising results in field screening.Garlic mustard is an invasive plant species in North American temperate forests. Its success may be partly due to its excretion of an unidentified allelochemical that interferes with mutualisms between native tree roots and their mycorrhizal fungi.

A study of *Kochia scoparia* in northern Montana by two high school students showed that when *Kochia* precedes spring wheat (*Triticum aestivum*), it reduces the spring wheat's growth. Effects included delayed emergence, decreased rate of growth, decreased final height and decreased average vegetative dry weight of spring wheat plants. A larger study later showed that *Kochia* seems to exhibit allelopathy on various crops in northern Montana

Effect of some allelochemicals on insects:

Phenolics (allomones): non-nitrogen compounds, hydroxyl group attached to the benzene rings; they affect nutritional quality of plants; the major groups are phenylpropanoids, flavonoids,

quinones. They have deleterious effects on larval growth of the insects. Compounds harmful to one insect may have little effect on another. For example, proanthocyanins or condensed tanninsare feeding inhibitors, however anthocyanins promote pollinator attraction, rotenone, an isoflavanoid has insecticidal properties. Protein inhibitors in plants are found in seeds, tubers and foliage and inhibitory activity of protein inhibitors is specific to digestive proteinases.Terpenoids: monoterpenes act as attractants/repellents, diterpenes exhibit considerable biological activity in relation to the action of toxins and hormones produced by plants. These chemicals are non toxic to the plant itself but on being consumed by insects are activated into lethal cytotoxins. Terpene induce cytochrome P-450 in insect to higher activity. Such activity may influence the hormone balance or pheromone products in the insect so that regulation of reproductive processes by these allelochemicals is implicated. Oligosaccharides are also reported to regulate not only activation of defense mechanism but also regulate the various aspects of plant product and morphogenesis.If the plant allelochemicals stimulate mating, dependence of the female on the plant is greater so that the adjustment of fecundity to the carrying capacity of the environment is better. For example, some phytophagous insects do not mate without eating the pollen of particular hosts, thus flower stimulates vitellogenesis and induces the female to a state for oviposition.

Probable Questions:

1. What is allelochemicals?
2. Define allelopathy.
3. Discuss about effect of allelochemicals on insects.
4. What are the applications of allelopathy?

Suggested Readings:

1. Principles of Toxicology by Stephen Roberts.
2. Toxicology Handbook by Lindsay Murray
3. Principles of Ecotoxicology by C.H. Walker
4. Casarett & Doull's Toxicology: The Basic Science by Curtis D. Klaassen.

Unit-VII

Plant signalling chemicals, insect response

Objective: In this unit you will learn about Plant signalling chemicals and insect response

Introduction:

Plants and insects have been living together for more than 350 million years. In co- evolution, both have evolved strategies to avoid each other's defense systems. This evolutionary arms race between plants and insects has resulted in the development of an elegant defense system in plants that has the ability to recognize the nonself molecules or signals from damaged cells, much like the animals, and activates the plant immune response against the herbivores. To counter the herbivore attack, plants produce specialized morphological structures or secondary metabolites and proteins that have toxic, repellent, and/or antinutitional effects on the herbivores. Plants confront the herbivores both directly by affecting host plant preference or survival and reproductive success (direct defense), and indirectly through other species such as natural enemies of the insect pests (indirect defense). Direct defenses are mediated by plant characteristics that affect the herbivore's biology such as mechanical protection on the surface of the plants (e.g., hairs,trichomes,thorns,spines,andthickerleaves)orproductionoftoxicchemicalssuchasterpenoids, alkaloids, anthocyanins, phenols, and quinones) that either kill or retard the development of the herbivores. Indirect defenses against insects are mediated by the release of a blend of volatiles that specifically attract natural enemies of the herbivores and/or by providing food (e.g., extra floral nectar) and housing to enhance the effectiveness of the natural enemies. Understanding the nature of gene expression of the plant defensive traits will have a tremendous application in designing crop plants with better protection against the herbivores. This in turn will reduce the need for use of harmful pesticides for insect control.

Host plant defences against insects:

Plants respond to herbivore attack through an intricate and dynamic defense system that includes structural barriers, toxic chemicals, and attraction of natural enemies of the target pests. Both defense mechanisms (direct and indirect) may be present constitutively or induced after damage by the herbivores. Induced response in plants is one of the important components of pest control in agriculture, and has been exploited for regulation of insect herbivore population.Over the past few decades, considerable progress has been made in studying induced responses in plants against different stresses, and has become an important topic in evolutionary biology and ecology. Although induced responses have some metabolic costs, they are very important when aimed at alleviating the stress of immediate concern, as most of these chemicals are produced in response to herbivore attack. Induced defenses make the plants phenotypically plastic, and thereby, decrease the chances of the attacking insects to adapt to the induced chemicals.

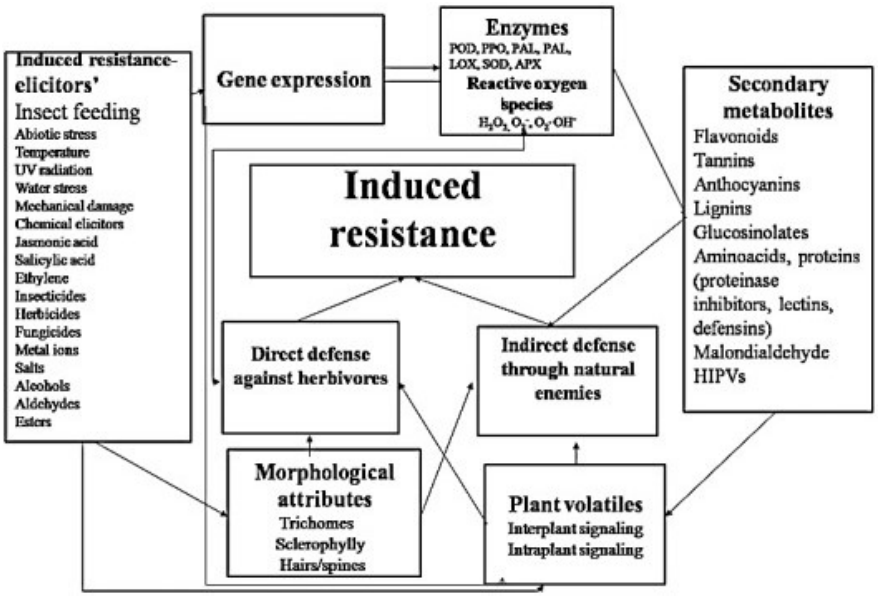


Figure 1. Mechanism of induced resistance in plants POD, peroxidase; PPO, polyphenol oxidase; PAL, phenylalanine ammonia lyase; TAL, tyrosine alanine ammonia lyase; LOX, lipoxygenase; SOD, superoxide dismutase; APX, ascorbate peroxidase; HIPVs, herbivore induced plant volatiles.

Changes in defensive constituents of a plant on account of insect attack develop unpredictability in the plant environment for insect herbivores, which in turn, affects the fitness and behavior of the herbivores. If induced response occurs very early, it is of great benefit to the plant, and reduces the subsequent herbivore and pathogen attack, besides improving overall fitness of the plant. Plants with high variability in defensive chemicals exhibit a better defense compared with those with moderate variability. Progress in insect-plant interactions has improved our understanding of the evolution of defensive approaches deployed by plants against herbivory; however, the underlying mechanisms of defense are less clearlyunderstood.

Direct defenses:

Plant structural traits such as leaf surface wax, thorns or trichomes, and cell wall thickness and lignification form the first physical barrier to feeding by the herbivores, and the secondary metabolites such act as toxins and also affect growth, development, and digestibility reducers form the next barriers that defend the plant from subsequent attack. Moreover, synergistic effect among different defensive components enhances the defensive system of plants against the herbivores invaders. In tomato, alkaloids, phenolics, proteinase inhibitors (PIs), and the oxidative enzymes, when ingested separately result in a reduced affect, but act together in a synergistic manner, affecting the insect during ingestion, digestion and metabolism. In *Nicotiana attenuate* (Torr. ex Watson), trypsin proteinase inhibitors and nicotine expression, contributed synergistically to the defensive response against *Spodopteraexigua*(Hub.). The role of morphological and biochemical constituents in host plant resistance (HPR), and induced responses to insect damage will be discussed below.

Morphological structures:

Plant structures are the first line of defense against herbivory, and play an important role in HPR to insects. The first line of plant defense against insect pests is the erection of a physical barrier either through the formation of a waxy cuticle, and/or the development of spines, setae, and trichomes. Structural defenses includes morphological andanatomical traits that confer a fitness advantage to the plant by directly deterring the herbivores from feeding, and range from

prominent protrubances on a plant to microscopic changes in cell wall thickness as a result of lignification and suberization. Structural traits such as spines and thorns (spinescence), trichomes (pubescence), toughened or hardened leaves (sclerophylly), incorporation of granular minerals into plant tissues, and divaricated branching (shoots with wiry stems produced at wide axillary angles) play a leading role in plant protection against herbivory. Sclerophylly refers to the hardened leaves, and plays an active role in plant defense against herbivores by reducing the palatability and digestibility of the tissues, thereby, reducing the herbivore damage.Spinescence includes plant structures such as spines, thorns and prickles. It has been reported to defend the plants against many insects. Pubescence consists of the layer of hairs trichomes) extending from the epidermis of the above ground plant parts including stem, leaves, and even fruits, and occur in several forms such as straight, spiral, stellate, hooked, and glandular. Chamarthi et al. reported that leaf glossiness, plumule and leaf sheath pigmentation were responsible for shoot fly *Atherigonasoccata*(Rondani) resistance in sorghum *Sorghum bicolor* (L.)(Moench).

Trichomes:

Trichomes play an imperative role in plant defense against many insect pests and involve both toxic and deterrent effects. Trichome density negatively affects the ovipositional behavior, feeding and larval nutrition of insect pests. In addition, dense trichomes affect the herbivory mechanically, and interfere with the movement of insects and other arthropods on the plant surface, thereby, reducing their access to leaf epidermis. These can be, straight, spiral, hooked, branched, or un-branched and can be glandular or nonglandular. Glandular trichomes secrete secondary metabolites including flavonoids, terpenoids, and alkaloids that can be poisonous, repellent, or trap insects and other organisms, thus forming a combination of structural and chemicaldefense.

Secondary metabolites and plant defense:

Secondary metabolites are the compounds that do not affect the normal growth and development of a plant, but reduce the palatability of the plant tissues in which they are produced. The defensive (secondary) metabolites can be either constitutive stored as inactive forms or induced in response to the insect or microbe attack. The former are known as phytoanticipins and the latter as phytoalexins. The phytoanticipins are mainly activated by β-glucosidase during herbivory, which in turn mediate the release of various biocidal aglycone metabolites. The classic examples of phytoanticipins are glucosinolates that are hydrolyzed by myrosinases (endogenous β-thioglucosideglucohydrolases) during tissue disruption. Other phytoanticipins include Benzoxazinoids (BXs), which are widely distributed among Poaceae. Hydrolyzation of BX-glucosides by plastid-targeted β-glucosidases during tissue damage leads to the production of biocidal aglycone BXs, which play an important role in plant defense against insects. Phytoalexins include isoflavonoids, terpenoids, alkaloids, etc., that influence the performance and survival of the herbivores. The secondary metabolites not only defend the plants from different stresses, but also increase the fitness of the plants. It has been reported that maize HPR to corn earworm, *Helicoverpazea*(Boddie) is mainly due to the presence of the secondary metabolites C-glycosyl flavone maysin [2"-*O* - a-L-rhamnosyl-6-*C*- (6-deoxy-*xylo*-hexos-4-ulosyl) luteolin] and the phenylpropanoid product, chlorogenic acid. Compound, 4, 4-dimethyl cyclooctene has been found to be responsible for shoot fly *A. soccata*resistance in sorghum *S. bicolor*.

Secondary metabolites have been primarily studied as the mediators of direct defense, however much is to be done to reveal the unidentified or emerging signaling pathways. Mass spectrometry used for the secondary metabolite profiling and gene expression analysis by high-throughput sequencing has made this field more exciting and cost-effective. Study on secondary metabolites could lead to the identification of new signaling molecules involved in plant resistance against herbivores and other stresses. Ultimately genes and enzymes involved in the biosynthesis of these metabolites could be identified. Role of some of the secondary metabolites in plant defense will be discussed below.

Plant phenolics:

Among the secondary metabolites, plant phenols constitute one of the most common and widespread group of defensive compounds, which play a major role in HPR against herbivores, including insects. Phenols act as a defensive mechanism not only against herbivores, but also against microorganisms and competing plants. Qualitative and quantitative alterations in phenols and elevation in activities of oxidative enzyme in response to insect attack is a general phenomenon.

Lignin, a phenolic heteropolymer plays a central role in plant defense against insects and pathogens. It limits the entry of pathogens by blocking physically or increasing the leaf toughness that reduces the feeding by herbivores, and also decreases the nutritional content of the leaf. Lignin synthesis has been found to be induced by herbivory or pathogen attack and its rapid deposition reduce further growth of the pathogen or herbivore fecundity. Increase in expression of lignin associated genes (*CAD/ CAD*-like genes) in plants infected with pests and pathogens have been documented.

Oxidation of phenols catalyzed by polyphenol oxidase (PPO) and peroxidase (POD) is a potential defense mechanism in plants against herbivorous insects. Quinones formed by oxidation of phenols bind covalently to leaf proteins, and inhibit the protein digestion in herbivores. In addition, quinones also exhibit direct toxicity to insects. Alkylation of amino acids reduces the nutritional value of plant proteins for insects, which in turn negatively affects the insect growth and development. Phenols also play an important role in cyclic reduction of reactive oxygen species (ROS) such as superoxide anion and hydroxide radicals, H_2O_2, and singlet oxygen, which in turn activate a cascade of reactions leading to the activation of defensive enzymes. Simple phenolics (salicylates) act as antifeedant to insect herbivores suchas*Operophterabrumata*(L.) in *Salix* leaves, and there is a negative correlation between the salicylate levels and the larval growth, however, salicylic acid (SA) is much more important as phytohormone than as deterrent.

Flavonoids:

Flavonoids play a central role in various facets of plant life especially in plant- environment interactions. These defend plants against various biotic and abiotic stresses including UV radiations, pathogens and insect pests. Flavonoids are cytotoxic and interact with different enzymes through complexation. Both flavonoids and isoflavonoids protect the plant against insect pests by influencing the behavior, and growth and development of insects. In addition, flavonoids scavenge the free radicals including ROS, and reduce their formation by chelating the metals. Flavonoids are divided into various classes that include anthocyanins, flavones, flavonols, flavanones, dihydroflavonols, chalcones, aurones, flavan, and proanthocyanidins. More than 5,000 flavonids have been reported in plants. A number of flavones such as flavonols, flavones, proanthocyanidins, flavan 3-ols, flavonones, flavans, and isoflavonoids have been investigated as feeding deterrents against many insect pests. Flavonoids such as flavones 5-hydroxyisoderricin, 7-methoxy-8- (3-methylbutadienyl)-flavanone and 5- methoxyisoronchocarpin isolated from *Tephrosiavillosa*(L.), *T. purpurea*(L.), and *T. vogelii*Hook, respectively have been found as feeding deterrents against *Spodopteraexempta*(Walk.), and *Spodopteralittoralis*Bios. Over-expressing a transcription factor controlling flavonoid production in Arabidopsis has been reported to confer resistance against *Spodopterafrugi-perda*(J.E. Smith). Angustone A, licoisoflavone B, angustone B, and angustone C. Isoflavones, licoisoflavone A, luteone, licoisoflavone B, and wighteone have been found to be not only feed- ing deterrents to insects, but also have antifungal activity against the fungi, *Colletotrichum gloeosporiode*(Penz.) and *Cladosporium cladosporioides*(Fres.). Isoflavonoids (judaicin, judaicin-7-*O*-glu-coside, 2-methoxyjudaicin, and maackiain) isolated from the wild relatives of chickpea act as antifeedant against *Helicoverpaarmig-era* (Hubner) at 100 ppm. Judaicin and maackiain were also found to be deterrent to *S. littoralis and S. frugiperda,* respectively. Cyanopropenyl glycoside and alliarinoside strongly inhibit feeding by the native American butterfly, *Pieris napi oleracea* L., while flavone glycoside, isovitexin-6"-D-β-glucopyranoside acts as a direct feeding deterrent to

the lateinstars.

Tannins:

Tannins have a strong deleterious effect on phytophagous insects and affect the insect growth and development by binding to the proteins, reduce nutrient absorption efficiency, and cause midgut lesions. Tannins are astringent (mouth puckering) bitter polyphenols and act as feeding deterrents to many insect pests. They precipitate proteins nonspecifically (including the digestive enzymes of herbivores), by hydrogen bonding or covalent bonding of protein-NH_2 groups. In addition, tannins also chelate the metal ions, thereby reducing their bioavailability to herbivores. When ingested, tannins reduce the digestibility of the proteins thereby decrease the nutritive value of plants and plant parts to herbivores. Role of tannins in plant defense against various stresses and their induction in response to insect damage has been studied in many plants. For example, e.g., in *Populus*species and in *Pinus sylvestris*L. However, no effect of herbivore damage on tannin content was observed in *Quercus serrata* (Thunb.) and *Betula pendula* Roth. Like proteinase inhibitors and oxidative enzymes, tannins have been reported to be systemically induced in neighboring leaves of the damagedplant.

Condensed tannins are oligomeric or polymeric flavonoids, also known as proanthocyanidins. They have diverse structures and functions. They act as feeding deterrents against some insects such as, *Lymantria dispar*(L.), *Euproctischrysorrhoea*(L.) and *O. brumata*. Condensed tannins such as (+) -catechin, (+) - gallocatechin, and vanillin in leaves of *Quercus robur*L. inhibited winter moth larvae, *O. brumata*. Procyanindin polymers have been found as feeding deterrent to *Aphis craccivora*(Koch) in groundnut. Condensed tannins from Alaska paper birch (coated on birch leaves at 3% dry wt.) reduced the pupal mass and survival of *Rheumapterahastata*(L.) larvae. It has been reported that induction of tannins in *Populustremuloides*Michx. leaves in response to wound- and herbivore occurs by transcriptional activation of the flavonoid pathway. Genes responsible for the production of tannins in response to wounding have been identified and are activated by the expression of a condensed tannins regulatory gene, *PtMYB134*, which is itself induced by damage. Furthermore, induction of tannin is also stimulated by light stress, and exposure to UV light in hybrid poplar. However, some polyphagous insect species have the ability to tolerate gallotannins, e.g., *Shistocercagregaria*(Forsk.) tolerates tanninsby hydrolyzing them rapidly to avoid any damaging effects by restricting the passage of tannins by adsorbing them on the thick peritrophic membrane, and by inhibiting the tannin protein complex formation by surfactants in themidgut.

Plant defensive proteins:

Ecologically, in insect-plant interaction, interrelationship between two is important for the survival of the both. Insects always look for a true and healthy host plant that can provide them proper food and could be suitable for mating, oviposition and also provides food for the offspring. The nutritional requirements of insects are similar to other animals, and any imbalance in digestion and utilization of plant proteins by the insects' results in drastic effects on insect physiology. Alteration of gene expression under stress including insect attack leads to qualitative and quantitative changes in proteins, which in turn play an important role in signal transduction, and oxidative defense. Many plant proteins ingested by insects are stable, and remain intact in the midgut, and also move across the gut wall into the hemolymph. An alteration in the protein's amino acid content or sequence influences the function of that protein. Likewise, anti-insect activity of a proteolysis-susceptible toxic protein can be improved by administration of protease inhibitors (PIs), which prevent degradation of the toxic proteins, and allows them to exert their defensive function. Better understanding of protein structure and post-translational modifications contributing to stability in the herbivore gut would assist in predicting toxicity and mechanism of plant resistance proteins (PRPs). Recent advances in microarray and proteomic approaches have revealed that a wide spectrum of PRPs is involved in plant defense against herbivores. Due to diverse feeding habits of arthropods, multiple signaling pathways including jasmonic acid (JA), SA and/or ethylene (ET) regulate arthropod-inducible proteins.

Plant lectins:

Lectins are carbohydrate-binding (glyco) proteins, ubiquitous in nature, and have protective function against a range of pests. The insecticidal activities of different plant lectins have been utilized as naturally occurring insecticides against insect pests. One of the most important properties of lectins is their survival in the digestive system of herbivores that gives them a strong insecticidal potential. They act as antinutritive and/or toxic substances by binding to membrane glycosyl groups lining the digestive tract, leading to an array of harmful systemic reac-tions. Lectins are stable over a large range of pH and damage the luminal epithelial membranes, thereby interfere with the nutrient digestion and absorption. Disruption of lipid, carbohydrate, and protein metabolism causes enlargement and/or atrophy of key tis-sues, which in turn alters the hormonal and immunological status, threatening the growth and development of insects.Lectins have been found to be promising against homopteran, lepidopteran, and coleopteran insects. Insecticidal properties of *Galanthus nivalis*L. agglutinin (GNA) were the first plant lectin shown to be active against hemipteran insect.Efficacies of carbohydrate binding plant lectins such as GNA, *Phaseolus* haemagglutinin, and wheat germ agglutinin, have been studied in detail against many insect pests. Mannose - binding lectins have been reported to be effective against sucking insects, because of their interaction with a specific carbohydrate residue of the cell membrane. Expression of lectin coding genes in trans-genic plants and their defense against insects has been worked out in many plants, e.g., GNA, PSA (*Pisum sativum* L.; pea), WGA (*Triticum vulgare* Kunth; wheatgerm), ConA (*Canavalia ensiformis*(L.); jack bean), AIA (*Artocarpusintegrifolia*Forst.; jack fruit), OSA (*Oryza sativa* L.; rice), ASAL (*Allium sativum* L.), and UDA (*Urticadioica*L.; stinging nettle). The *Arum maculatum*L. lectin has been found effective against the aphids *Lipaphiserysimi*(Kalt.) and *A. craccivora*when incorporated in an artificialdiet.

Studies on the mechanism of action of the mannose-specific lectin, GNA against brown planthopper (*Nilaparvatalugens*(Stal.) in rice has shown that GNA binds to the luminal surface of the midgut epithelial cells within the planthopper by recognizing the cell surface carbohydrate moieties of glycoproteins and/ or other glycoconjugates in the gut. Immuno- labeling GNA assay has shown its presence in the fat bodies, ovarioles, and hemolymph, indicating the ability of GNA to cross the midgut epithelial barrier and pass into the insect's circulatory system leading to systemic toxic effect. Partial resistance to homopteran insect pests has been reported in transgenic plants expressing snowdrop lectin in tobacco, rice, andwheat.

Plant lectins are induced by elicitors as an induced response to various stresses. JA induced the expression of *NICTABA* lectin in tobacco leaves. Induction of NICTABA by herbivores infestation including *S. littoralis*, *Manduca sexta*L. and *Tetranychusurticae*Koch has been reported in tobacco plants. Expression of a mannose-binding jacalin-like lectin called Hessian fly, *Mayetiola destructor* (Say) responsive protein 1 (HFR1), and two chimerolectin like proteins called HFR2 and HFR3 have been reported to be induced by the larvae of Hessian fly, *M. destructor* in wheat. Differences in feeding behavior of insects results in expression of different lectins, e.g., larvae of the fall armyworm, *S. frugiperda*induced HFR2, but not HFR3 expression while the phloem-feeding bird cherry-oat aphid, *Rhopalosiphumpadi*Koch, induced HFR3 andHFR2, but latter was expressed much later (12 d) than the former (24 h). Several jasmonate-inducible lectins are expressed in leaf tissues of monocots such as rice, bar-ley, wheat, rye, and maize. Advancement of our understanding in induction of plant lectins in response to various stresses, especially herbivory, and their role in plant defense has the potential for utilization of these entomotoxic lectins in crop protection through genetic engineering. Although, transformation of lectin genes into plants seems to be very attractive and effective, care is needed, because of possible toxicity of some lectins to non-target organisms, including mammals.

Proteinase inhibitors:

Proteinase inhibitors (PIs) cover one of the most abundant defensive classes of proteins in plants.

Higher concentration of PIs occurs in storage organs such as seeds and tubers, and 1 to 10% of their total proteins comprise of PIs, which inhibit different types of enzymes and play an important role in plant defense against insect herbivory. PIs bind to the digestive enzymes in insect gut and inhibit their activity, thereby reduce protein digestion, resulting in the short-age of amino acids, and slow development and/or starvation of the insects. The defensive function of many PIs against insect pests, directly or by expression in transgenic plants to improve plant resistance against insects has been studied against many lepidopteran, and hemipteran insects. The success of transgenic crops in expressing PIs against insect pests has accentuated the need to understand the mechanisms, and interactions of multiple PIs with other defenses, and the adaptive responses of the herbivores.

Many classes of PIs are induced in plants in response to stresses. Kunitz proteinase inhibitors (KPIs) are the serine PIs (SPIs), which are among the most strongly upregulated defense genes in response to wounding or herbivore feeding in plants. The SPIs from *Solanum nigrum* L. have been found to adversely affect a number of insect pests. Progress in genome sequencing has resulted in identification of a large number of proteinase inhibitors and other defense components induced in plants on account of herbivore damage. Although most of the KPIs in plants are upregulated in response to insect herbivory, their degree of induction varies as per the insect plant interaction. Various KPIs allow plants to deal with multiple generations of insects by providing a genetic storehouse of varied PIs. However, some insects respond to PIs by constitutive or induced production of PI-insensitive proteases or by inactivation of ingested PIs, thereby, preventing them from binding to sensitive proteases. Such a feeding response by insects negatively affects the PI activity, and may result in even greater damage to the plants. This counter defense by the insects is a major hindrance to manipulation and utilization of PIs for a longer-lasting plant defense, and there is a need to understand the mechanisms by which insects counteract the PI-based plant defense.

Enzymes:

One of the important aspects of HPR against insects is the disruption of insect's nutrition. The enzymes that impair the nutrient uptake by insects through the formation of electrophiles includes peroxidases (PODs), polyphenol oxidases (PPOs), ascorbate peroxidases, and other peroxidases by oxidizing mono- or dihydroxyphenols, that lead to the formation of reactive o-quinones, which in turn polymerize or form covalent adducts with the nucleophilic groups of proteins due to their electrophilic nature (e.g., -SH or e-NH_2 of Lys). Other important antioxidative enzymes include lipoxygenases, phenylalanine ammonia lyase, superoxide dismutase, etc. Induction of antioxidative enzymes in plants following herbivory has received considerable attention in recent years.

Peroxidases (POD):

Oxidative state of the host plants has been associated with HPR to insects, which results in production of ROS, that are subsequently eliminated by antioxidative enzymes. POD constitutes one such group of enzymes, which scavenges the ROS besides having other defensive roles. PODs are an important component of the immediate response of plants to insect damage. PODs are monomeric hemoproteins distributed as soluble, membrane-bound, and cell wall-bound within the cells, and are widely spread in plants and include several isozymes, whose expression depends on tissue, developmental stage, and environmental stimuli. A number of process are regulated by PODs that have direct or indirect role in plant defense, including lignification, suberization, somatic embryo-genesis, auxin metabolism, and wound healing. Role of PODs in plant resistance to insect pests has been studied in various plant systems. Production of phenoxy and other oxidative radicals by the PODs in association with phenols directly deter the feeding by insects and/or produces toxins that reduce the plant digestibility, which in turn leads to nutrient deficiency in insects with drastic effects on their growth and development. In addition, PODs

have been reported to have direct toxicity in guts of herbivores. PODs have been purified and characterized from many plants where they were induced in response to insect attack.

Polyphenol oxidases (PPO):

The PPOs are important enzymes in plants that regulate feeding, growth, and development of insect pests, and play a leading role in plant defense against the biotic and abiotic stresses. PPOs can function in following ways: a) PPO-generated quinones could alkylate essential amino acids, decreasing plant nutritional quality, (b) quinones may produce oxidative stress in the gut lumen through redox cycling, and (c) quinones and ROS produced by phenolic oxidation, could be absorbed and have toxic effects on herbivores. The PPOs are metalloenzymes that catalyze the oxidation of monophenols and *o*-diphenols to quinones, which are highly reactive intermediate compounds that readily polymerize, and react with nucleophilic side chain of amino acids and crosslink proteins, thereby reducing the availability of such proteins, and affect the nutritional quality of the food. Under acidic conditions, quinones form semiquinone radicals that in turn give rise to ROS, while under basic conditions; quinines react with cellular nucleophiles. Quinines are more toxic to plant herbivores than the original phenols. In addition to their role in digestibility and palatability of plant tissues, melanin formation by PPOs increases the cell wall resistance to insects and pathogens. Induction of PPO activity under abiotic and biotic stresses and by treatment with compounds related to the octadecanoid pathway makes it an important tool in plant resistance against different stresses. The PPO genes are differentially induced by signaling molecules and injury due to wounding, and pathogen, or insect infestation. Correlation between induction of PPO activity and insect fitness has been reported in many plants including tomato and lettuce. Although PPOs accumulate in leaves, roots, stems and flowers of the plants, young tissues with greater vulnerability to insect attack exhibit greater induction. The PPOs confer resistance to *Spodopteralitura*(Fab.), *H. armigera, Bemisiatabaci*(Gen.), *Tetranychuscinnabarinus*(Boisd.), *Myzuspersicae*(Sulzer), *Empoascafabae*(Harris), *Aphis medicaginis*(Koch), *S. exigua*, and *Agelasticaalni*(L.). However, induced PPO levels had no or limited impact on *L. dispar*, *Orgyialeucostigma*(JE Smith), and *Blissusocciduus*Barber.

Lipoxygenases:

Lipoxygenases (LOXs) are another group of anti-oxidative enzymes involved in plant defense against many stresses through octadecanoid pathway. They catalyze hydro- peroxidation of polyunsaturated fatty acids resulting in the formation of fatty acid hydroperoxides. The latter are enzymatically and/or chemically degraded to unstable and highly reactive aldehydes, γ-ketols, epoxides, and ROS such as hydroxyl radicals, singlet oxygen, superoxide ion and peroxyl, acyl and carboncentered radicals. The unstable reactive productsinteract with proteins resulting in protein-protein cross linking and amino acid damage that in turn affects the amino acid assimilation. In addition, lipid peroxidation end products also act as insect repellents or antixenosis and are toxic to insect pests (antibiosis). Major substrates of LOX in plants are linoleic and linolenic acids. One of the most important aspects of LOX in plant defense is the oxidation of linolenic acid in JA signaling pathway, which in turn plays a leading role in activation of plant defense, both directly by production of oxidative enzymes and protease inhibitors, and indirectly through the production of volatile organic compounds (VOC) that attract the natural enemies of insect pests. Oxygenation of polyunsaturated fatty acids has been found to be catalyzed by LOX, which results in the production of hydroperoxides that are metabolized to compounds such as JA and traumatin.

Induction of LOX activity in response to herbivory has been studied in many plants such as soybean in response to two-spot-ted spider mite, *T. urticeae*, in tomato in response to aphids, *Macrosiphium euphorbiae* Thom., and *M. persicae*, in *N. attenuate* following infestation by

*Myzusnicotianae*Black. and in wheat following *Sitobionavenae*(F.) infestation. The *N. attenuata*plants deficient in LOX are more vulnerable to attack by *M. sexta,* which also attract the new herbivores such as *Empoasca*spp, as compared with the plants where LOX3-mediated defense reduced larval growth, food consumption, and frass production. Maize plants transformed with the wheat oxalate oxidase gene had upregulation of LOX transcripts and elevation of free phenolics (14-fold), which were positively associated with resistance to the European corn borer, *O.nubilalis*.

Indirect defenses:

The defensive response in plants to attract natural enemies of herbivores plays a pivotal role in protecting the plants against herbivore attack. Indirect defenses can be constitutive or induced as a result of combined action of mechanical damage and elicitors from the attacking herbivore. Production of volatiles and the secretion of extra floral nectar (EFN) mediate interactions of plants with natural enemies of the insect pests (i.e., parasitoids or predators), which actively reduce the numbers of feeding herbivores. Induced indirect defenses have received increasing attention recently and have been studied on the genetic, biochemical, physiological, and ecological levels.

Herbivore induced plant volatiles (HIPVs):

Plants indirectly defend themselves from herbivore feeding by emitting a blend of volatiles and non-volatile compounds. Herbivore-induced plant volatiles (HIPVs) play an important role in plant defence by either attracting the natural enemies of the herbivores or by acting as feeding and/or oviposition deterrent. HIPVs are the lipophilic compounds with higher vapor pressure which are released from the leaves, flowers, and fruits into the atmosphere, and into the soil from the roots by plants in response herbivore attack. The HIPV's produced vary according to the plant and herbivore species, the developmental stage and condition of the plants and the herbivores. An optimum quantity of volatile compounds is normally released by the plants into the atmosphere, whereas a different blend of volatiles is produced in response to herbivory. The volatile blend released by plants in response to insect attack is specific for a particular insect-plant system, including natural enemies and the neighboring plants. The HIPVs mediate the interactions between plants and arthropods, microorganisms, undamaged neighbouring plants, or intraplant signalling that warns undamaged sites within the plant. Depending upon the modes of feeding of insect pests, different defense signaling pathways are activated, which induce the production of specific volatile compounds.

The HIPVs include terpenes, green leafy volatiles (GLVs), ethylene, methyl salicylate and other VOCs. The well-studied metabolites of hydroperoxide lyase (HPL) branch of oxylipin-pathway producing stress-inducible compounds are the GLVs. GLVs are reactive electrophile species involved in stress and defense signals. GLVs consist of C6-aldehydes [(Z)-3-hexenal, n-hexanal] and their respective derivatives such as (Z)-3-hexenol, -3-hexen-1-yl acetate, and the corresponding E-isomers. To understand the role of C6-aldehydes and their respective derivatives in plant defense, the GLVs levels have been altered either by application of elicitors, or by manipulating genetically the HPL expression in plants. GLVs play an important role in plant defense by attracting natural enemies. Plant volatiles such as methyl salicylates and the C_{16}-homoterpene 4, 8, 12-trimethyl-1, 3(*E*), 7(*E*), 11-tridecatetraene [(*E, E*)-TMTT] have been found to attract the predatory mites. The most frequent component of the HIPVs is methyl salicylate (MeSA), and has been reported in the headspace of many insect-infested plants including lima bean, and Arabidopsis. MeSA is a ubiquitous component of many leaf and floral blends and MeSA baited sticky cards attract many insect predators including the big- eyed bug, *Geocoris pal-lens* Stal., ladybird beetle, *Stethorus punctum picipes*(Casey), green lacewing *Chrysopanigricornis* Burmeister, and other natural enemies. Ulland et al. reported the inhibition of oviposition of cabbage moths *Mamestrabrassicae* L. by MeSA released during infestation,suggesting that MeSA can also be detected by the attacking herbivores. Methyl benzoate (MeBA), which structurally resembles MeSA, has also been detected from insect-

infested plants. *S. frugiperda*infestation in rice induces emission of about 30 volatiles, including MeSA and MeBA, which are highly attractant to the natural enemies of *S. frugiperda*, such as, *Cotesiamarginiventris*(Cresson). However, there is an ecological cost of using HIPVs to engineer natural enemies; because HIPVs have the potential of attracting crop pests. For example, Colorado potato beetles, *Leptinotarsadecemlineata*(Say) is attracted to a blend of volatiles consisting of cis-3-hexenyl acetate, linalool, and MeSA.

Compounds such as ester methyl salicylate (MeSA), mono-terpenes myrcene and β-ocimene, homoterpene (*E*, *E*)-4, 8, 12-trimethyltrideca-1, 3, 7, 11-tetraene (TMTT), and sesquiterpene (*E*, *E*)-α-farnesene are emitted hours after infestation. Systemic release of VOCs is one of the best studied responses specific to herbivores. The HIPVs defend the plants either directly by repelling, deterring and toxicity to the herbivore or indirectly by attracting the natural enemies of the attackers, and thus, protect the plants from further damage. Lipoxygenase and Shikimic acid pathway metabolites and terpenoid pathway products (terpenoids) play an important role in plant defense, both directly and indirectly. Period specific volatile emission has been observed in many plants e.g., lima bean leaves attacked by *S. littoralis*, and hybrid poplar (*Populustrichocarpa*Torr. and A. Grey X *deltoides*) leaves infested by forest tent caterpillar, *L. dispar*emitted blend of volatiles containing (*E*)-β-ocimene and other mono-, sesqui- and homoterpenes. Maize plants when exposed to -3-hexanol induced the volatile blend emission that is usu-ally released after caterpillar infestation, and attracts the natural enemies.Priming of the volatile emission signals has been reported in many plants. Engelberth et al. reported that application of GLV compounds such as (Z)-3-hexanal, (Z)-3-hexen-1-ol, and -3-hexenyl acetate individually and blend of volatiles to the maize seedlings enabled the seedlings to respond to wounding and beet armyworm, *S. exigua*caterpillar regurgitate, and resulted in accumulation of JA and sesquiterpenes as compared with the control plants. Similar observations were recordedby Kessler et al. in *N. attenuata*in response to *M. sexta* infestation, where low damage was shown by plants primed with clipped sagebrush-released volatiles. Thus, priming plays an important role in plant defense by incomplete turning on of defense related processes to reduce the biochemical investments until the onset of actual attack. However, there are a few reports where some non-target insect pests were also attracted on account of volatile emission in infested plants, thereby, increasing the insect attack on the plant.

Transgenic Arabidopsis with overexpression of strawberry nerolidol synthase, a terpene synthase (TPS) responsible for the production of sesquiterpene alcohol (3S)-(*E*)-nerolidol has been reported to attract the predatory mite, *P. persimilis*. The parasitic wasp, *Cotesiamarginiventris*(Cresson) was attracted to the lepidopteran larvae infesting transgenic maize plants with over- expression of the corn *TPS10* gene responsible for the formation of (*E*)-β-farnesene, (*E*)-α- bergamotene, and other herbivore induced sesquiterpene hydrocarbons.

In addition to the plant volatiles released from aerial parts of the plant, roots have also been found to release diverse volatiles that defend the plants from belowground insect pests by acting as antimicrobial and antiherbivore, and also by attracting the natural enemies of the root feeding insect pests. Root feeding insect, *Diuraphisnoxia*(Mord.) triggers the emission of 1,8-cineole,a monoterpene volatile, which is toxic and repellent to some insects. Sesquiterpene (*E*)-β-caryophyllene produced by maize roots in response to feeding by the larvae of *Diabroticavirgiferavirgifera*LeConte attracts the nematode *Heterorhabditismegidis*Poinar. However, root emitted volatiles such as 1,8-Cineole inhibits the growth of *Brassica campestris* seedlings due to the inhibition cell proliferation more severely than cell elongation because root growth requires both elongation and proliferation of the constituent cells, and also due to the interference with nuclear as well as organelle DNA synthesis in root apical meristem and alteration in root phospholipids and sterol composition.

Defense elicitors (insect oral secretion):

Plants undergo a dynamic change in transcriptomes, proteomes, and metabolomes in response to herbivore-induced physical and chemical cues such as insect oral secretions (OS) and compounds in the oviposition fluids. It is generally believed that insect-induced plant responses are mediated by oral secretions and regurgitates of the herbivore. The defenses generated by various elicitors differ based on the type of the elicitor and the biological processes involved. A potential elicitor of herbivore-induced plant volatiles from the regurgitate of *Pieris brassicae*L. larvae has been identified as β-glucosidase which results in emission of a volatile blend from mechanically wounded cabbage leaves that attract the parasitic wasp, *Cotesiaglomerata*(L.).Fatty acid-amino acid conjugates (FACs) are the major components in the oral secretions of insects. The first FAC elicitor identified was volicition, N-(17-hydroxylinolenoyl)-L-glutamine (volicitin), detected in the OS of beet armyworm larvae, *S. exigua*. Volicitin when applied on *Zea mays* L. induced the emission of elicitor that attracts the natural enemies of the feeding larvae. N-linolenoyl-glu isolated from regurgitate of tobacco hornworm, *M. sexta*has been found to be a potential elicitor of volatile emissions in tobacco plants. The FACs in OS of insects have been found to activate mitogen-activated pro-tein kinase (MAPK) pathway, that regulate plant growth and development, and play an important role in signaling transduction in responses to various stresses including cold, heat, ROS, UV, drought, pathogen and insect attack. FACs in oral secretions of *M. sexta*, when applied to the wounded leaves have been found to activate signaling processes that lead to the activation of MAPKs, salicylic acid-induced protein kinase (SIPK) and wound-induced protein kinase (WIPK), and bursts of jasmonic acid (JA), JA- isoleucine conjugate (JA-Ile), salicylic acid (SA), and ethylene. In wild rice, *Oryza minuta*Presl., expres-sion of putative MAPK, *OmMKKI*, is induced by brown plant hopper, *N. lugens*feeding. Several other FAC elicitors such as *N*-acyl Gln/Glu have been isolated from regurgitates of vari- ous lepidopteran species. The FACs has also been reported to induce accumulation of 7-*epi*- jasmonic acid, an octadecanoid-derived phytohormone, which is a potent elicitor of transcripts of herbivore-responsive genes in tobacco plants. The FACs in lepidopteran OS evokes specific responses such as transcriptomic and proteomic alteration, induction of nicotine, and proteinase inhibitors in *N. attenuate*. Besides FACs, other groups of elicitors identified in insect oral secretions include inceptins, and caeliferins. Inceptins are disulphide-bonded peptides formed by the proteolytic fragmentation of plastidic ATP synthase, γ-subunit, whereas caeliferins are sulfated fatty acids, in the oral secretion of *S. americana*(Stal.), and other grasshopper species. The lipase activity of grasshopper oral secretions evoked an immediate and quick accumulation of various oxylipins, such as, 13-hydroperoxy octadecatrienoic acid, 12-oxo-phytodienoic acid (OPDA), JA, and jasmonic acid-isoleucine in Arabidopsis. Furthermore, there was increase in cytosolic calcium, ethylene emission and activity of MAPKs on treatment with grasshopper oral secretions.

Role of phytohormones in induced resistance in plants:

Plant defense against herbivore attack involves many signal transduction pathways that are mediated by a network of phytohormones. Plant hormones play a critical role in regulating plant growth, development, and defensemechanisms. A number of plant hormones have been implicated in intra- and inter-plant communication in plants damaged by herbivores. Most of the plant defense responses against insects are activated by signal-transduction pathways mediated by JA, SA, and ethylene. Specific sets of defense related genes are activated by these path-ways upon wounding or by insect feeding. These hormones may act individually, synergistically or antagonistically, depending upon the attacker.

Jasmonic acid:

Although various phytohormones are involved in plant defense against herbivores, JA is the most important phytohormone linked to plant defense against herbivores and activates the expression of both direct and indirect defenses. JA is derived from linolenic acid through octadecanoid pathway and accumulates upon wounding and herbivory in plant tissues. Chewing of plant parts by insects causes the dioxygenation of linoleic acid (18:2) and linolenic acid (18:3) by specific

LOXs at C9 or C13 to form (9S)- or (13S)-hydroperoxy- octadecadi(tri)enoic acids, which are converted into 12-oxophytodienoic acid (12-OPDA) by allene oxide synthase and allene oxide cyclase. OPDA is transferred to the peroxisome, where it is reduced by OPDA reductase 3 (OPR3), forming JA. Oxidative burst produces ROS, which convert linolenic acid into phytoprostanes that signal trans-duction pathways. A broad spectrum of defensive responses are induced by jasmonates that include antioxidative enzymes, PIs, VOCs, alkaloid production, trichome formation, and secretion of EFN. A large numbers of genes involved in defense against herbivores are regulated by JA. Concentration of indole glucosinolate, an important defensive compound, is induced by jasmonates. In addition to its role in the production of JA, OPDA signals the defense pathways individually. For example, OPDA signaling regulates the CORONATIN-INSENSITIVE 1 (COI1) -dependent and -independent transcription, alters the intracellular calcium levels and cellular redox status. Jasmonates (most likely the JA-amino acid conjugate jasmonoyl-isoleucine) have been found to interact with the COI1 unit of an E3 ubiquitin ligase complex, termed SCFCOI1 (Skip/Cullin/F-box–COI1), which promotes binding of the COI1-unit to JAZ (jasmonate ZIM-domain) proteins, resulting in degradation of JAZ proteins, which otherwise suppress JA-inducible gene expression. JA has also been reported to affect calcium-dependent protein kinases (CDPK) transcript, and activity in potato plants. CDPKs comprise of a large family of serine/threonine kinases in plants (34 members in Arabidopsis) and play an important role in plant defense against a variety of biotic andabioticstressesthroughsignaltransduction.InadditiontotheroleplayedbyJAindirectresistance against insect pests through the induction of various defensive compounds, its role in indirect resistance has also been well established. For example, EFN produced by JA is used as an alternate food by natural enemies of insect pests. JA also induces the defense enzymes such as POD, and PPO.

Salicylic acid:

Salicylic acid (SA), a benzoic acid derivative, is an important phytohormone involved in regulation of plant defense. It is an important endogenous plant growth regulator that generates a wide range of metabolic and physiological responses in plants involved in defense in addition to their impact on plant growth and development. Responses to SA depend on a regulatory protein called Non-Expressor of Pathogenesis-Related Genes1 (*NPR1*). The *NPR1* gene is activated through redox pathways by SA accumulation and is translocated to the nucleus, however, it does not bind to DNA directly, but acts through transcription factors. SA induces greater defense against piercing and sucking type of insect pests than the chewing ones. SA signaling molecule is involved in local defense as well as induction of systemic resistance. Production of ROS by SA pathway has been proposed to induce resistance in plants against insect pests, e.g., in tomato plants against *H. armigera*. H_2O_2 induced by SA in plants defends them against various insect pests since H_2O_2 actively damages the digestive sys-tem of insects leading to reduced growth and development. Furthermore, SA signals the release of plant volatiles that attract the natural enemies of insect pests, e.g., Lima bean and tomato plants infested by spider mite attract the natural enemies of spider mite. However, it has been reported that SA and JA act antagonistically, where SA inhibits the activity of JA and vice versa. MeSA serves as a volatile signal to trigger induced defenses in plants, including HIPV emission, and a number of predaceous arthropods are attracted to MeSA under fieldconditions.

Ethylene:

Ethylene is an important phytohormone, which plays an active role in plant defense against many insects. Ethylene signaling pathway plays an important role in induced plant defense against herbivores and pathogens both directly and indirectly, however, there are limited reports on its role in indirect defense through the emission of HIPVs. ET signaling pathway works either synergistically or antagonistically, with JA in expression of plant defense responses against pathogens and herbivorous insects. It has been reported that ET and JA work together in tomato in PIs expression. Infestation by *A. alni*induced the emission of ethylene and release of various valatiles in *Alnusglutinosa*L. leaves in addition to mono-, sesqui and homoterpenes. ETprecursor, 1-amino-cyclopropane-1-carboxylic acid has been reported to enhance the volatile emission from

the JA treated detached leaves. Ethylene further induced the emission of volatiles induced by volicitin, JA or (Z)-3-hexen-ol in maize.

Role of Calcium ions (Ca2+) in plant defense:

Plant defense elicitors induced in plants upon herbivory undergo different signal transduction pathways. Ca signaling is one of the early events in insect-plant interaction, where Ca acts as a second messenger, which in turn mediates a number of plant signaling pathways. Herbivore induced signals rapidly spread over the leaf and leads to a strong Ca-dependent trans-membrane potential (Vm) depolarization in the damage zone, and is followed by a transient Vm hyperpolarization in the surrounding area, and a constant depolarization at distances greater than 6–7 mm. Organelle and apoplastic fluid Ca concentration is generally higher (about 10 to 10 times) as compared with that in the cytosol (100 and 200 nM). However, upon insect attack, the cytosolic Ca increases, which in turn activates the calcium-sensing proteins such as calmodulin, calmodulin-bind-ing proteins, and calcium- dependent protein kinases (CDPKs) that promote the signaling events such as, phosphorylation and transcriptional change. However, CDPKs are the important proteins against biotic and abiotic stresses, which form Ca sensors that contain a protein kinase domain and a calmodulin like domain (including an EF-hand calcium-binding site) in a single polypeptide. *NtCDPK2* regulates the activation of stress-induced MAP kinases in tobacco. Involvement of two Arabidopsis CPKs (CPK3 and CPK13) in herbivory-induced signaling network through *HsfB2a*- mediated regulation of the defense-related transcriptional machinery has been observed in tobacco. Damage by *S. littoralis*larvae on *Phaseolus lunatus*L. induced Ca not only in cells adjacent to the feeding site, but throughout the leaf. Expression of calmodulin binding proteins involved in plant defense signaling increased considerably in wheat damaged by *D. noxia*and Arabidopsis by *M.persicae*.

Role of reactive oxygen species (ROS) in plant defense:

Oxidative state of plants is an important tactic that enables plants to defend against various stresses. Rapid and transient genera-tion of ROS is a common phenomenon in plants on account of oxidative stress due to biotic and abiotic factors. ROS play versatile signaling functions that mediate multiple responses, and can also act directly as toxins. However, production of ROS on account of biotic stress isstill debatable. ROS include partially reduced forms of oxygen such as superoxide (O), hydrogen peroxide (H_2O_2), and hydroxyl radicals (HO). Distinct signaling pathways are activated by different types of ROS especially the ones involving MAPKs. Rapid increase in ROS content under stress conditions is referred as “oxidative burst.” Following insect attack, ROS accumulate in apoplastic as well as in symplastic regions, besides their main concentration in exo-cellular matrix, peroxisomes/mitochondria, and plasma mem-brane. Apoplastic burst of ROS acts as a first barrier against subsequent attack by the pathogens and herbivores. Being highly reactive, ROS can potentially react with and/or cause damage to proteins, lipids, and nucleic acids. However, to prevent the self-toxicity of ROS, plant cells have developed ROS scavenging systems for removing the excess ROS to maintain a relatively low and constant ROS concentration.Among all the ROS, high stability and freely diffusible H_2O_2 is a central component of induced defense response in plants against different stresses. Although H_2O_2 is produced in various ways, the oxidative burst is supposed to occur through the activation of membrane bound NADPH complex. NADPH oxidase generates superoxide anion at the plasma membrane or in the apoplast extracellularly, which is then converted to H_2O_2 by superoxide dismutase (SOD). Besides having direct effect on the pathogens and herbivores, H_2O_2 stimulates a cascade of reactions that lead to the expression of defense genes, which pre-vent the plants from subsequent attack by pathogens and herbivores. H_2O_2 application in Arabidopsis results in up- and downregulation of many genes (113 and 62 genes, respectively), suggesting that ROS act as secondary messengers to control gene expression. ROS also play an important role in mediating cross-linking of cell wall components by peroxidase, and also for the activation of many defenses related genes. Oxidative changes in plants after insect attack cause oxidative damage to insect mid-gut, mainly due to accumulation of H_2O_2. Many physiological and

molecular responses in plants against insect attack are triggered by H_2O_2, and its levels remain elevated as long as the herbivore attack persists. Induction of H_2O_2 has been studied in oat, wheat, barley and groundnut against *D. noxia, R. padi*, *Schizaphisgraminum*Rond., *H. armigera*and *S. litura*. Argandona et al. observed induction of H_2O_2 in barley infested with *S. graminum*after 20 min of infestation, indicating that H_2O_2 could be the beginning of a cascade of physiological and molecular events leading to production of further defensive components, and protection of plants from subsequent damage.ROSmediatethedefensivegeneactivationandestablishadditionaldefensesbyregulating the transcription and/or by interacting with other signal components like phosphorylation in plant systems in response to a variety of stresses.

Gene expression:

The basic process of plant defense:

Extensive rearrangements in gene expression occur in plants in response to herbivory with hundreds, and even up to several thousands of genes getting up- or downregulated. Advances in genomics and transcriptomics including availability of whole-genome sequence data, expressed sequence tags (ESTs), and microarrays, has led to better understanding of the changes in gene-expression profiles in response to insect attackDNA microarrays provide a closer and complete view of gene- expression patterns and signaling responses mediated by insect elicitors and plant signals, and has proven to be exceptional tools to monitor the expression of thousands of genes simultaneously. However, with the advent of next-generation sequencing (NGS) technologies, it is anticipated that microarrays will be soon replaced by some new and innovative technologies like RNA-sequencing, RAD-sequencing, and reduced represented sequencing etc., for measuring gene expression directly. Expression quantitative trait loci (eQTL) mapping has revolutionized the area of gene expression. The eQTL mapping is having the advantage of dealing with thousands of traits at a time and has been used in many plants including Arabidopsis and rice. Investigation of inducible defenses in Arabidopsis against *P. rapae*and *Brassica oleracea* var *capitata*L. and *Brassica nigra*L., or the aphid *Brevicorynebrassicae*L. by microarrays has been studied extensively. Responses against feeding of *D. noxia*(Mord.), *S. graminum, M.nicotianae,persicae*and *S. avenae*on foliage of Arabidopsis, celery, sorghum, *Apiumgraveolens*L. cereal, tobacco or wheat plants have been well established.

Change in gene expression profiles after herbivory has shown a substantial reallocation of plant resources to defense. Gene expression levels have also been used to analyze the differences in transcriptional profiles of different genotypes within a plant species. A large numbers of genes (2182) are expressed by the aphid, *M. persicae*as compared with caterpillar, *P. rapae*(186) attack. Lepidopterans usually elicit changes in the expression of genes involved in glucosinolate metabolism in Brassicaceae, detoxification, cell survival, and signal transduction, while the aphids regulate the expression of genes involved in cell wall modifications, oxidative stress, calcium-dependent signaling, and glucosinolate synthesis. Different attackers face differentresponses in plants based on the feeding behavior and the plant attacked; e.g., transcriptional changes in *Arabidopsis thaliana* (L.) in response to feeding by aphid, *M. persicae*and whitefy, *Bemisiatabaci*(Gen.). Different plants respond differently to the same herbivore, e.g., two white cabbage cultivars differ considerably in gene expression in response to feeding by *P. rapae*. Combination of various technologies such as genetic, genomic tools including microarrays, deep sequencing, and transcriptional profiling tools and proteomics through mass spectrometry will advance our understanding of molecular mechanisms of plant defense against insect herbivores to a greater extent.

Transgenerational induced resistance to herbivores:

Biotic and abiotic stresses in plants have been found to induce resistance not only in the maternal plants, but also in the offspring. This maternally induced resistance (transgenerational immunity)

has been found to protect the progeny of plants exposed to herbivory from insect pests, besides producing vigorous seeds and seed-lings. However, there are only few reports on transgenerational immunity of plants against insect pests. Wild radish plants, *R. raphanistrum*damaged by *P. rapae*or treated with JA produce offspring's with high levels of induced resistance to this insect. Arabidopsis plants exposed to stresses such as, cold, heat and flood, resulted in a higher homologous recombination frequency and increased genome methylation, which in turn induced the resistance to stress in the progeny. Maternal plants with low to intermediate levels of herbivore damage could produce the seeds that are more vigorous and seedlings that are resistant to insect pests. However, further studies are required to understand the genetic and molecular mechanisms of such signaling interactions. Furthermore, research on plant-insect interactions should be focused not only to genetic effects, but also toward the epigenetic regulation of plant defense pathways and insect responses, because a substantial body of evidence has been demonstrated for mobile siRNA signals and inheritance of DNA methylation based changes in gene expression. There is much need for in-depth studies on this subject to exploit it for pest management by manipulating the maternal ecology. An understanding of transgenerational induced resistance might answer some of the intricate questions regarding the ability of plants to withstand herbivore damage.

Probable Questions:

1. What are direct responses of plants to herbivores.
2. How secondary metabolites help plants in defence?
3. Describe the role of flavonoids in plant defence.
4. Describe the role tannins in plant defence
5. Describe the role enzymes in plant defence.
6. Describe the role polyphenol oxidases in plant defence.
7. Describe the role Lipoxygenases in plant defence.
8. Describe the role peroxidises in plant defence.
9. Describe the role Proteinase inhibitors in plant defence.
10. Describe the role plant lectins in plant defence.
11. Describe role of phytohormones in induced resistance in plants.
12. Describe the role plant jasmonic acid in plant defence.
13. Describe the role of ROS in plant defence.
14. Describe the role of ethylene in plant defence.
15. Describe the role of salicylic acid in plant defence.

Suggested Readings:

Mechanisms of Plant Defense against Insect Herbivores. War *et al.* 2012. Plant Signaling& Behavior 7:10, 1306-1320

HARD CORE THEORY PAPER (ZHT- 310)

Environmental Toxicology and Endocrinology

Group B: Endocrinology

Module	Unit	Content	Credit	Class	Time (h)	Page No.
ZHT - 310 (Environmental Toxicology and Endocrinology)	VIII	Classification of hormones; general principles and nature of hormone action, nature of hormone receptor.	2.0	1	1	
	IX	Biosynthesis, secretion and regulation of hormones: biosynthesis of protein and peptide hormones (Growth Hormone and Insulin), Post-Translational event and release; biosynthesis of steroid hormones and their regulations; biosynthesis of T3 and T4 and their regulation.		1	1	
	X	Physiological role of hormones: hormonal regulation of mineral metabolism and fluid volume.		1	1	
	XI	GI tract hormone source, composition and function.		1	1	
	XII	Neuroendocrine system and neurosecretion: neural control of glandular secretion; hypothalamic pituitary unit, neuroendocrine feedback.		1	1	

Unit-VIII

Classification of hormones; general principles and nature of hormone action, nature of hormone receptor

Objective: In this unit you will learn about classification of hormones, general principles and nature of hormone action. You will also know about various kind of hormone receptors.

Definition of Hormones:

Hormones are chemical messengers (may be of proteins, lipids or amines), secreted from special cells of endocrine glands and maintain the physiological activities very specifically on target cells through circulation and disintegrated after action.

Characteristics of Hormones:

The hormones possess the following specific properties:

1. They are chemical entities produced by special cells of endocrine glands.

2. They are transported to the target cells/ tissue/organ via circulation.

3. Their actions are species specific…

4. They are active in very minute quantities.

5. They are mostly water soluble.

6. They are low in molecular weight.

7. They are destroyed after their actions.

8. Chemically they are heterogeneous substances.

9. They cannot be stored for a long time; usually they are synthesized and secreted during the time of requirement.

10. They usually activate target cells by forming hormone receptor complex.

Mechanism of Hormone Action:

1. Enhancement of enzyme synthesis:

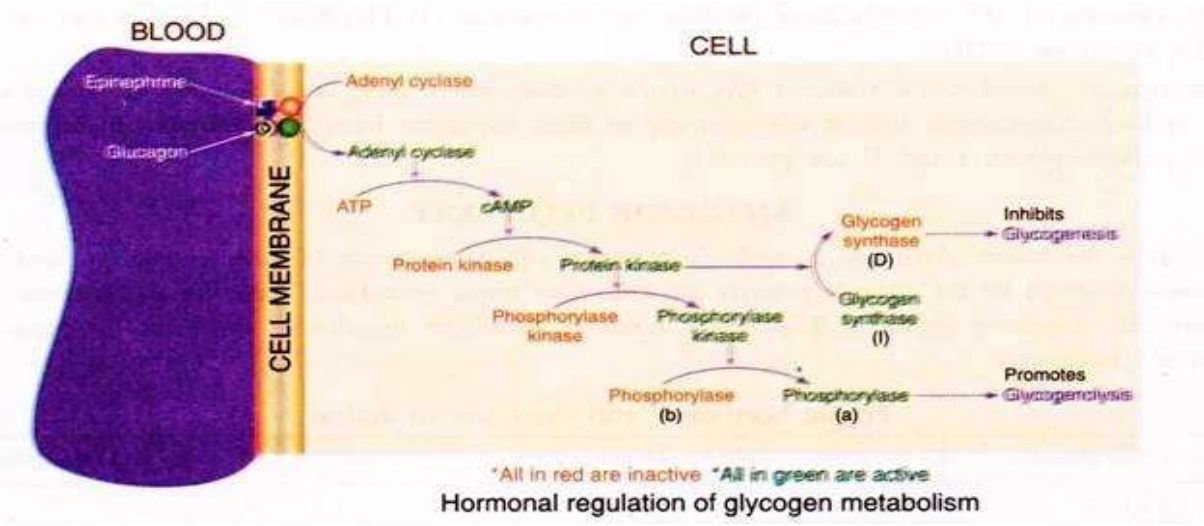
The steroid hormones and the thyroid hormones enter the cell and combine with the specific receptor protein to form 'receptor protein-hormone complex'. This complex will then bind to a specific site on DNA and initiate or enhance the synthesis of mRNA which in turn synthesizes the protein i.e. enzymes. Therefore the cell reactions speed up.

2. Change in cell permeability:

Hormones like insulin binds to a specific receptor on the cell membrane which results in alteration of the permeability of the cell to certain substances like glucose, amino acids and ions. The entry of these substances will bring a change in cell reactions.

3. Action through a second messenger (cAMP):

Hormones like epinephrine, glucagon bind to a regulatory site on the cell membrane. On the inner side of this regulatory site, an enzyme known as adenyl cyclase is present that converts ATP to cAMP which then activates certain protein kinases that in turn will phosphorylate certain enzymes. Some enzymes on phosphorylation become active whereas some other enzymes become inactive. Certain reactions are therefore stimulated while others are inhibited.



Hormonal regulation of glycogen metabolism

There are two mechanism by which hormones exerts its effect:

Mechanism - 1. Mode of Protein Hormone Action through Extracellular Receptors:

(i) Formation of Hormone Receptor Complex:
Every hormone has its own receptor. The number of receptors for each hormone varies. Insulin receptors for most cells is less than 100 but for some liver cells their number may be more than 1,00,000. The molecules of amino acid derivatives, peptides or polypeptide protein hormones bind to specific receptor molecules located on the plasma membrane to form the hormone receptor complex.

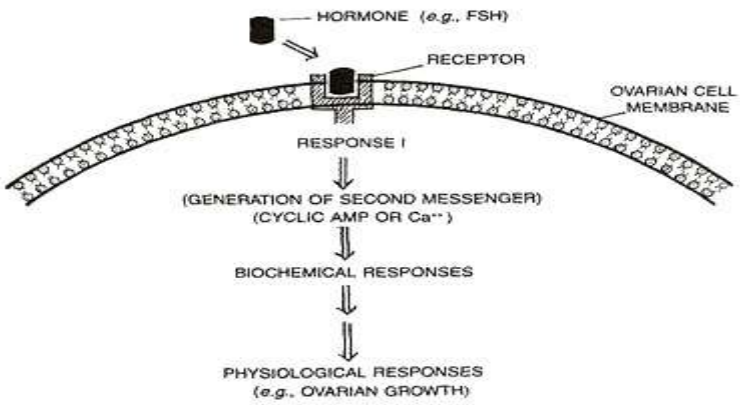


Fig. 22.18. Diagrammatic representation of mechanism of protein hormone action.

(ii) Formation of Secondary Messengers—the Mediators:

The hormone-receptor complex does not directly stimulates adenyl cyclase present in the cell membrane. It is done through a transducer G protein. Alfred Gilmans has shown that the G protein is a peripheral membrane protein consisting of ∝, β and γ subunits (Fig 22.19).It interconverts between a GDP form and GTP form. In muscle or liver cells, the hormones such as adrenaline bind receptor to form the hormone-receptor complex in the plasma membrane.

The hormone- receptor complex induces the release of GDP from the G protein. The α- subunit bearing GTP separates from the combined β and y subunits. The β and y subunits do not separate from each other. The activated β and γ subunits of G protein activate adenyl cyclase. The activated adenyl cyclase catalyses the formation of cyclic adenosine monophosphate (cAMP) from ATP.

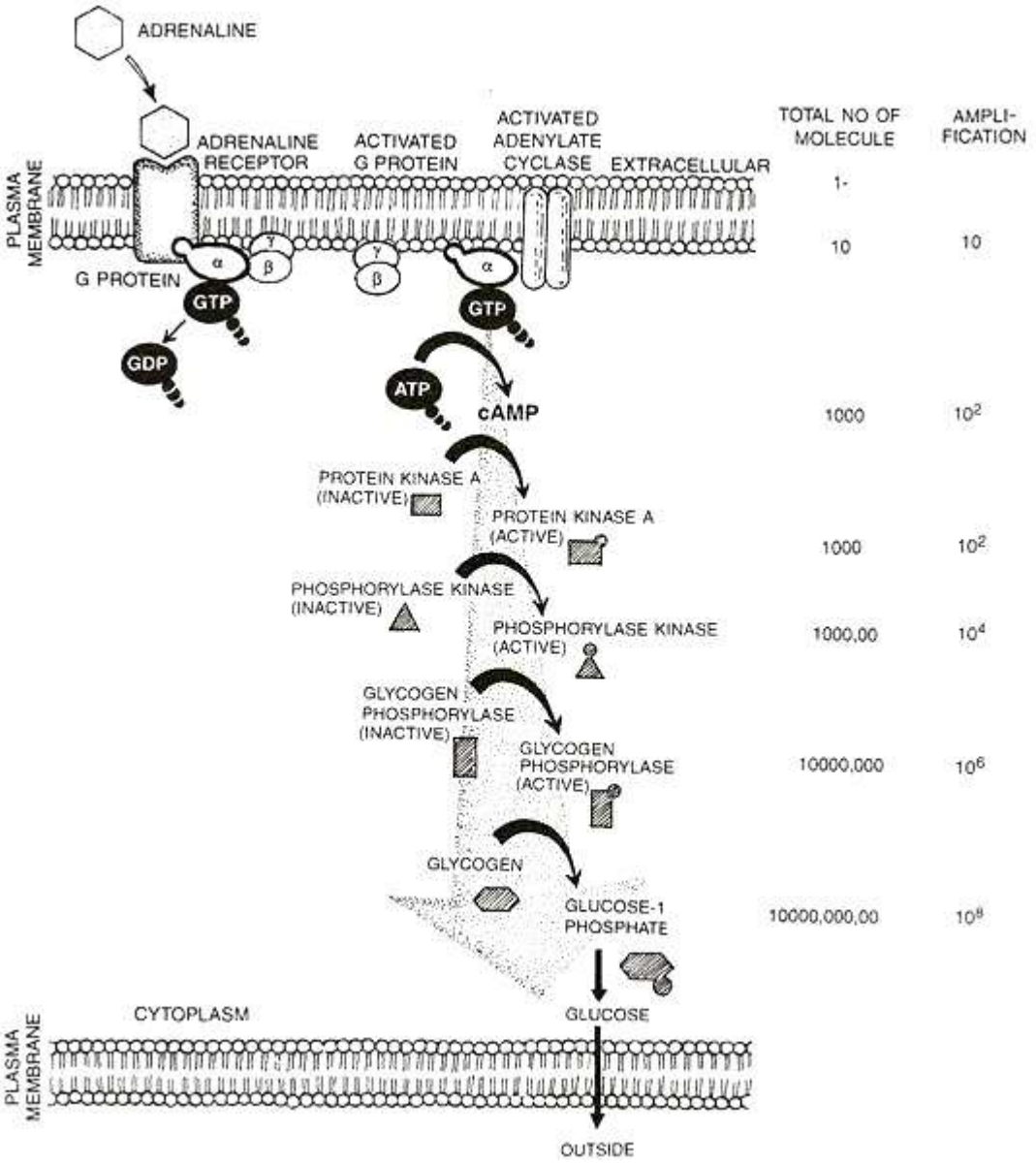
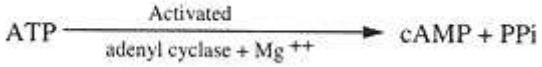


Fig. 22.19. Mode of hormone action through the extracellular receptor and amplification.

The hormone is called the first messenger and cAMP is termed the second messenger.



The hormones which interact with membrane-bound receptors normally do not enter the taget cell, but generate second messengers (e.g., cAMP).

Besides, cAMP, certain other intracellular second messengers are cyclic guanosine monophosphate (cGMP), diacyl-glycerol (DAG), inositol triphosphate (IP_3) and Ca^{++} responsible for amplification of signal. Earl W. Sutherland Jr (1915-1974) discovered cAMP in 1965. He got Nobel prize in physiology of medicine in 1971 for his discovery, "Role of cAMP in hormone action".

(iii) Amplification of Signal:

Single activated molecule of adenyl cyclase can generate about 100 cAMP molecules. Four molecules of cAMP now bind to inactive protein-kinase complex to activate protein-kinase A enzyme. Further steps as shown in involve cascade effect. In cascade effect, every activated molecule in turn activates many molecules of inactive enzyme of next category in the target cell. This process is repeated a number of times.

n the cytoplasm a molecule of protein kinase A activates several molecules of phosphorylase kinase. This enzyme changes inactive form of glycogen phosphorylase into active one.

Glycogen phosphorylase converts glycogen into glucose-1 phosphate. The latter changes to glucose. As a result single molecule of ademaline hormone may lead to the release of 100 million glucose molecules within 1 to 2 minutes. This increases the blood glucose level.

(iv) Antagonistic Effect:

The effect of hormones which act against each other are called antagonistic effects. Many body cells use more than one second messenger. In heart cells cAMP acts as a second messenger that increases muscle cell contraction in response to adrenaline, while cGMP acts as another second messenger which decreases muscle contraction in response to acetylcholine.

Thus the sympathetic and parasympathetic nervous systems achieve antagonize effect on heart beat. Another example of antagonistic effect is of insulin and glucagon. Insulin lowers blood sugar level and glucagon raises blood sugar level.

(v) Synergistic Effect:

When two or more hormones complement each other's actions and they are needed for full expression of the hormone effects are called synergistic effects. For example, the production and ejection of milk by mammary glands require the synergistic effects of oestrogens, progesterone, prolactin and oxytocin hormones.

Hormones that Bind to Cell Membrane Receptor mediate their actions through many second messengers, some of which are discussed below:

A. cAMP as the Second Messenger:

i. For the formation of cAMP from ATP needs: Receptor, GS protein, Adenylate cyclase.

ii. cAMP (Cyclic adenosine 3′-5′ monophosphate) is formed from ATP by the action of the enzyme adenylate cyclase and converted to physiologically inactivated 5′- AMP by the action of enzyme phosphodiesterase.

iii. Hormone receptor complex combines with G_s or G_i; (s = stimulatory, i = inhibitory) type of GTP dependent trimeric nucleotide regulatory complex of the cell membrane.

iv. Both G_s or G_1 are made up of 3 subunits. G_s contains $\alpha_s\beta\gamma$ and G_i contains $\alpha_i\beta\gamma$.

v. The α subunit (either G_s or G_i) is bound to GDP. When binding of hormone to R_s or R_i results in a receptor-mediated activation of G, then GDP is exchanged for GTP and the a subunit separates from the combined β and γ subunits.

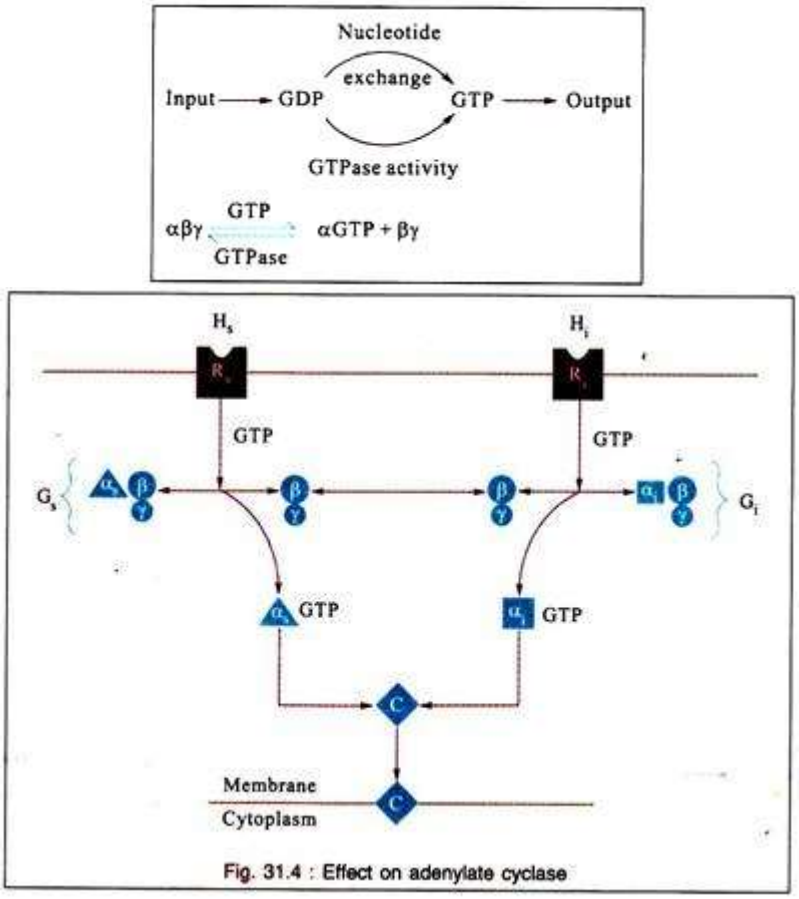


Fig. 31.4 : Effect on adenylate cyclase

vi. This GTP-α_s activates effectors (adenylate cyclase). The intrinsic GTPase activity of the α-subunit then converts GTP and GDP and leads to re-association of the a with βγ subunit.

vii. On the other hand, α_i-GTP inhibits adenylate cyclase by binding with it. This lowers the intracellular concentration of cAMP. Hormones that stimulate adenylate cyclase: ACTH, ADH, FSH, Glucagon. Hormones that inhibit adenylate cyclase: Acetylcholine, Angiotensin II.

viii. cAMP binds to a protein kinase that is a hetero tetrameric molecule consisting of 2 regulatory subunits (R) and 2 catalytic subunits (C). cAMP binding results in the following reaction.

$4cAMP + R_2C_2 \rightleftharpoons R_2\,(4cAMP) + 2C$

ix. The R_2C_2 complex has no enzymatic activity but the binding of cAMP by R dissociates R from C, thereby activating protein kinase. This activated protein kinase catalyzes the transfer of the y

phosphate of ATP (Mg^{++}) to a serine or threonine residue in a variety of proteins. Thus they regulate the conformational changes of phosphoprotein and physiologic effect occurs.

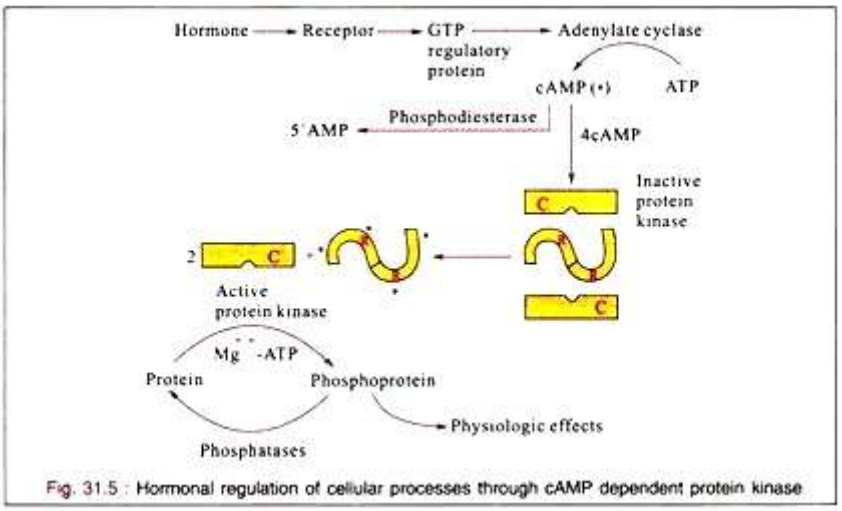


Fig. 31.5 : Hormonal regulation of cellular processes through cAMP dependent protein kinase

B. Role of cGMP in Hormone Action:

i. Hormones such as insulin and growth hormone, affect the guanylate cyclase cGMP system. This will increase the intracellular concentration of cGMP and activate cGMP dependent protein kinases.

ii. The active cGMP protein kinase would in turn bring about phosphorylation of specific cellular proteins to change their activities, leading to relaxation of smooth muscles, vasodilatation and other effects.

iii. The idea of cGMP as second messenger has not been accepted as yet. It is likely that Ca^{++} may act as second messenger to activate guanylate cyclase and thereby increasing the concentration of cGMP inside the cell.

iv. It appears that cGMP has its unique place in hormone action. The atriopeptins, a family of peptides, produced in cardiac atrial tissues cause natriuretic, diuresis, vasodilatation and inhibition of aldosterone secretion.

v. These peptides (e.g., atrial natriuretic factor) bind to and activate the membrane bound form of guanylate cyclase. This results in an increase of cGMP.

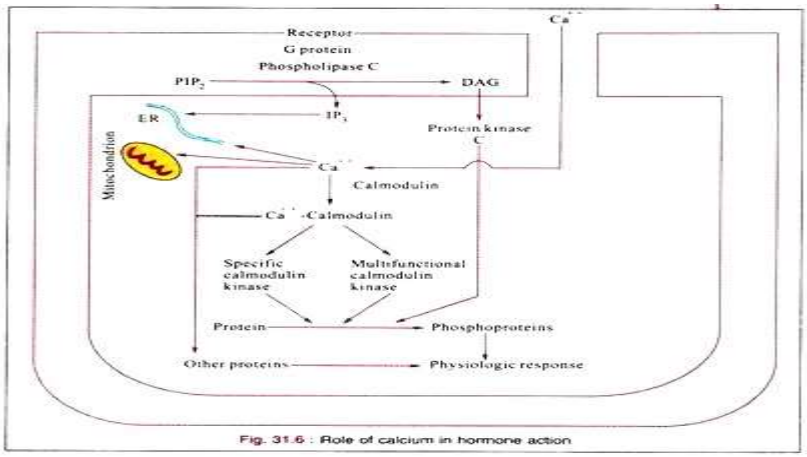


Fig. 31.6 : Role of calcium in hormone action

C. Role of Calcium in Hormone Action:

i. It is suggested that ionized calcium of the cytosol is the important signal for hormone action than cAMP.

ii. The extracellular calcium (Ca^{++}) concentration is about 5 mmol/L, the intracellular concentration of this free ion is much lower 0.1-10 µmol/L.

iii. The hormones that bind cell membrane receptor enhance membrane permeability to Ca^{++} and thereby increase Ca^{++} influx. This is probably accomplished by an Na^{+}/Ca^{++} exchange mechanism that has a high capacity but a low affinity for Ca^{++}. There is a $Ca^{2+}/2H^{+}$-ATPase dependent pump that extrudes Ca^{2+} in exchange for H^{+}. This has a high affinity for Ca^{2+} but a low capacity.

iv. Cell surface receptors such as those for acetylcholine, ADH, when occupied by their respective ligands, potent activators of phospholipase c.

v. Receptor binding and activation of phospholipase c are coupled by a unique G protein.

vi. Phospholipase c catalyses the hydrolysis of phosphatidyl inositol 4, 5-bisphosphate to inositol triphosphate and 1, 2 diacylglcerol.

vii. The diacylglycerol is itself capable of activating protein kinase c, the activity of which also depends upon free ionic calcium.

viii. Inositol triphosphate is an effective releaser of calcium from intracellular storage sites such as endoplasmic reticulum, and mitochondria.

ix. Thus, the hydrolysis of PIP_2 leads to activation of protein kinase c and promotes an increase of cytoplasmic calcium ion.

x. The calcium dependent regulatory protein is now referred to as calmodulin. Calmodulin has $4Ca^{++}$ binding sites. Ca^{++}– calmodulin complex can activate specific kinases. These then modify the conformational changes of phosphoprotein and alters physiologic responses.

xi. The activated protein kinase c can phosphorylate specific substrates and alter physiologic processes.

Mechanism - 2. Mode of Steroid Hormone Action through Intracellular Receptors:

Steroid hormones are lipid-soluble and easily pass through the cell membrane of a target cell into the cytoplasm. In the cytoplasm they bind to specific intracellular receptors (proteins) to form a hormone receptor complex that enters the nucleus.

In the nucleus, hormones which interact with intracellular receptors (e.g., steroid hormones, iodothyromines, etc.) mostly regulate gene expression or chromosome function by the interaction of hormone-receptor complex with the genome. Biochemical actions result in physiological and developmental effects (tissue growth and differentiation, etc.). In-fact the hormone receptor complex binds to a specific regulatory site on the chromosome and activates certain genes (DNA).

The activated gene transcribes mRNA which directs the synthesis of proteins and usually enzymes in the cytoplasm. The enzymes promote the metabolic reactions in the cell. The actions of lipid soluble hormones are slower and last longer than the action of water- soluble hormones.

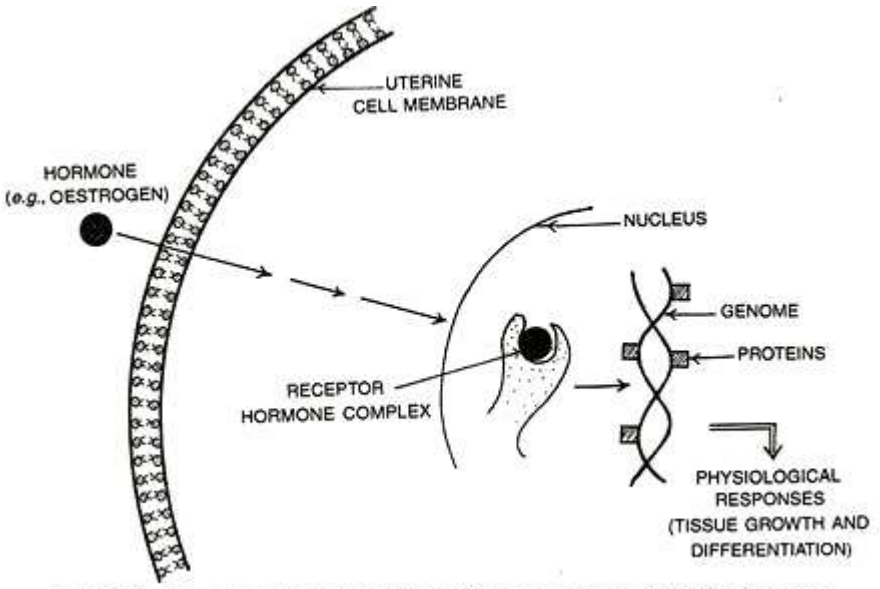


Fig. 22.20. Diagrammatic representation of the mechanism of Steroid hormone.

Role of Hormones as Messengers and Regulators (Role of Hormones in Homeostasis):

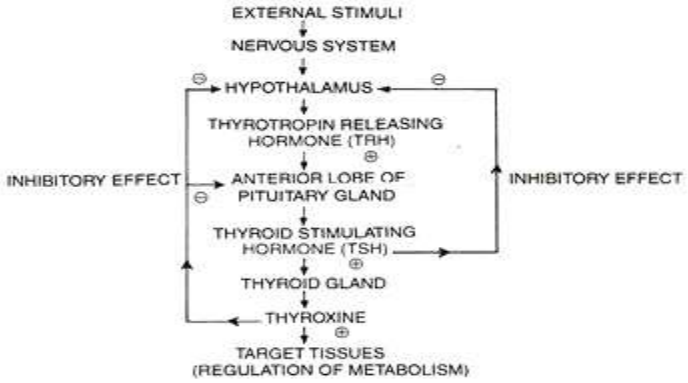


Fig. 22.21. Feed back control involving the hypothalamus, anterior lobe of pituitary gland, thyroid gland and target tissues.

Hormones as Messengers [Hypothalamus-hypophysial (pituitary) Axis]:

Hypothalamus is a part of the fore brain. Its hypothalamic nuclei— masses of grey matter containing neurons, are located in the white matter in the floor of the third ventricle of the brain. The neurons (neurosecretory cells) of hypothalamic nuclei secrete some hormones called neurohormones (releasing factors) into the blood.

The neurohormones are carried to the anterior lobe of the pituitary gland (= hypophysis) by a pair of hypophysial portal veins. In the pituitary gland (hypophysis) the neurohormones stimulate it to release various hormones. Hence the neurohormones are also called “releasing factors”.

Hormones as Regulators (Feed Back Control):

Homeostasis means keeping the internal environment of the body constant. Hormones help in maintaining internal environment of the body. When the secretion of hormones is under the control of factors or other hormones it is called feedback control. The regulation of secretion of thyroxine from the thyroid gland is an example of such feedback control mechanism.

Degradation and excretion of hormones:

All the hormones are degraded and excreted. Peptide hormones are degraded in the liver and/or kidney. The catecholamine's, steroids and the thyroid hor-mones are inactivated directly by enzymatic modification in the blood and/or in the liver.

Feed back control is of two types:

(i) Negative Feed Back Control:

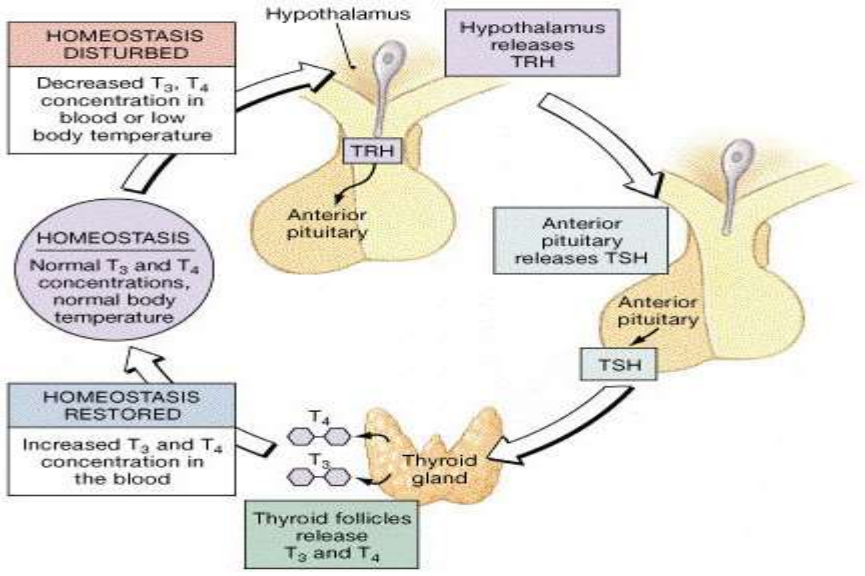
The receptors (sensory cells) present on the body of vertebrates constantly monitors the reference point of internal environment. Any changes in the internal environment can activates the receptor cells, which relay messages to the control centre (Brain or spinal cord). The control centre determines the deviation and activates the effectors. Effectors are generally muscles or glands. The effectors respond to the stimulus and corrects the reference point either by increasing or decreasing the activities. As soon as the system is corrected, the control centre and effectors are turned off by the mechanism called Negative feed-back.

In negative feed-back mechanism, changes occurring in the system automatically activates the corrective mechanism, which reverse the changes and bring back the system to the normal. The principle of thermostat is analog to the Negative feed-back mechanism. In thermostat, when the temperature exceeds the normal ranges, the receptor detects the changes and signals the control center of thermostat to turn off the heating plate, allowing the thermostat to cool down. When the thermostat cool down below the set point, it turn ON the heating plates, so the temperature starts rise again.

The mechanism of Negative feed-back in biological system can be illustrated with the example given below.

Negative feed-back mechanism of thyroid gland

Lower concentration of thyroxine hormone in blood alters the cellular activities ie. Decrease in basic metabolic rates or temperature. Decreases in BMR stimulates neurosecretory cells of hypothalamus to secrete thyrotropin releasing hormone (TRH). The releasing of TRH causes anterior pituitary gland to secrete thyroid stimulating hormone (TSH). This TSH then stimulates the thyroid gland to release thyroxine. Thyroxin causes an increase in the metabolic activity, generating ATP energy and heat and eventually restore homeostasis. Both the raised body temperature and higher thyroxine levels in the body feed-back to inhibit the releasing of TRH and TSH.



(ii) Positive Feed Back Control:

Positive feedback mechanism causes destabilizing effects in the body, so does not results in homeostasis. It is mainly responsible for amplification of the changes caused by the stimulus. Positive feedback is relatively less common than negative feedback, since it leads to unstable condition and extreme state. Most positive feedback mechanisms are harmful and in some cases resulting in death. For example, if a person breathes air that has very high carbon dioxide content. The amount of oxygen in blood decreases while the concentration of carbon-dioxide in blood increases. This is sensed by carbon dioxide receptors, which cause the breathing rate to increase. So the person breathes faster, taking in more carbon dioxide, which stimulates the receptors even more, so they breathe faster and faster which ultimately results in death.

In some cases, the positive feed-back is very useful, such as during blood clotting, fever, child birth, breast feeding etc. Positive feedback also plays a role in the contractions of the uterus during child birth. The contraction of uterine wall is caused by oxytocin hormone. In this case, stretching of the uterus by the fetus stimulates oxytocin release which results in contraction of uterus, and contraction causes further stretching and release of oxytocin; the cycle continues until the fetus is expelled from the uterus.

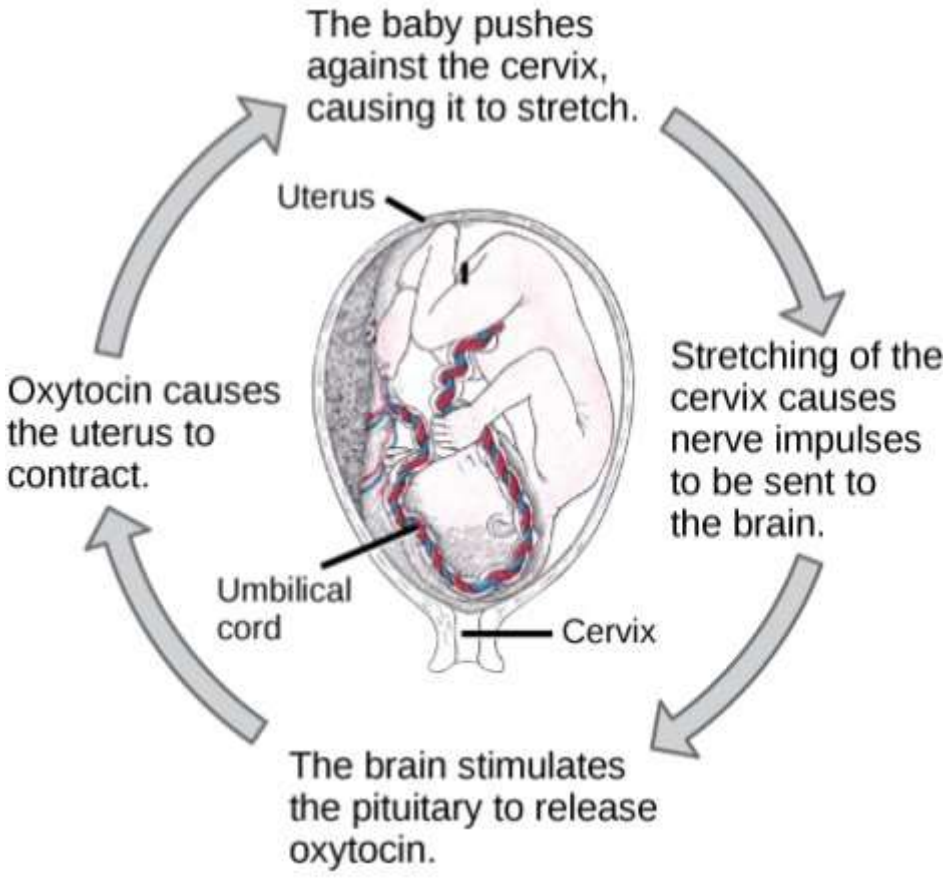


Figure: Regulation of oxytocin hormone; an example of positive feedback mechanism

Hormone Receptors:

Meaning of Hormone Receptors:

A hormone receptor is a receptor protein on the surface of a cell or in its interior that binds to a specific hormone. The hormone causes many changes that take place in the cell. Binding of hormones to hormone receptors often trigger the start of a biophysical signal that can lead to further signal transduction pathways, or trigger the activation or inhibition of genes.

Types of Hormone Receptors

Peptide Hormone Receptors:

Are often trans membrane proteins. They are also called G-protein- coupled receptors, sensory receptors or ionotropic receptors. These receptors generally function via intracellular second messengers, including cyclic AMP (cAMP), inositol 1, 4, 5-triphosphate (IP_3) and the calcium (Ca^{2+})—calmodulin system.

Steroid Hormone Receptors and Related Receptors:

Are generally soluble proteins that function through gene activation. Their response elements are DNA sequences (promoters) that are bound by the complex of the steroid bound to its receptor. The receptors themselves are zinc-finger proteins. These receptors include those for glucocorticoids, estrogens, androgens, thyroid hormone (T3), calcitriol (the active form of vitamin D), and the retinoids (vitamin A).

Receptors for Peptide Hormones:

With the exception of the thyroid hormone receptor, the receptors for amino acid derived and peptide hormones are located in the plasma membrane. Receptor structure is varied.Some receptors consist of a single polypeptide chain with a domain on either side of the membrane, connected by a membrane-spanning domain. Some receptors are comprised of a single polypeptide chain that is passed back and forth in serpentine fashion across the membrane, giving multiple intracellular, trans membrane, and extracellular domains. Other receptors are composed of multiple polypeptides. Ex. The insulin receptor is a disulfide linked tetramer with the β-subunits spanning the membrane and the α-subunits located on the exterior surface.

Subsequent to hormone binding, a signal is transduced to the interior of the cell, where second messengers and phosphorylated proteins generate appropriate metabolic responses. The main second messengers are cAMP, Ca^{2+}, inositol triphosphate (IP3), and diacylglycerol (DAG).
Proteins are phosphorylated on serine and threonine by cAMP-dependent protein kinase (PKA) and DAG-activated protein kinase C (PKC). Additionally a series of membrane-associated and intracellular tyrosine kinases phosphorylate specific tyrosine residues on target enzymes and other regulatory proteins.The hormone-binding signal of most, but not all, plasma membrane receptors is transduced to the interior of cells by the binding of receptor-ligand complexes to a series of membrane-localized GDP/GTP binding proteins known as G-proteins. The classic interactions between receptors, G-protein transducer, and membrane-localized adenylate cyclase are illustrated using the pancreatic hormone glucagon as an example.

When G-proteins bind to receptors, GTP exchanges with GDP bound to the α-subunit of the G-protein. The G_a-GTP complex binds adenylate cyclase, activating the enzyme. The activation of adenylate cyclase leads to cAMP production in the cytosol and to the activation of PKA, followed by regulatory phosphorylation of numerous enzymes. Stimulatory G-proteins are designated Gs, inhibitory G-proteins are designated Gi.

A second class of peptide hormones induces the transduction of 2 second messengers, DAG and IP3. Hormone binding is followed by interaction with a stimulatory G-protein which is followed in turn by G-protein activation of membrane-localized phospholipase C-y, (PLC-y). PLC-y hydrolyzes phosphatidylinositol bisphosphate to produce 2 messengers viz. IP_3, which is soluble in the cytosol, and DAG, which remains in the membrane phase.
Cytosolic IP_3 binds to sites on the endoplasmic reticulum, opening Ca^{2+} channels and allowing stored Ca^{2+} to flood the cytosol. There it activates numerous enzymes, many by activating their calmodulin or calmodulin-like subunits. DAG has 2 roles-it binds and activates PKC, and it opens Ca^{2+} channels in the plasma membrane, reinforcing the effect of IP_3. Like PKA, PKC phosphorylates serine and threonine residues of many proteins, thus modulating their catalytic activity.

Insulin Receptor:

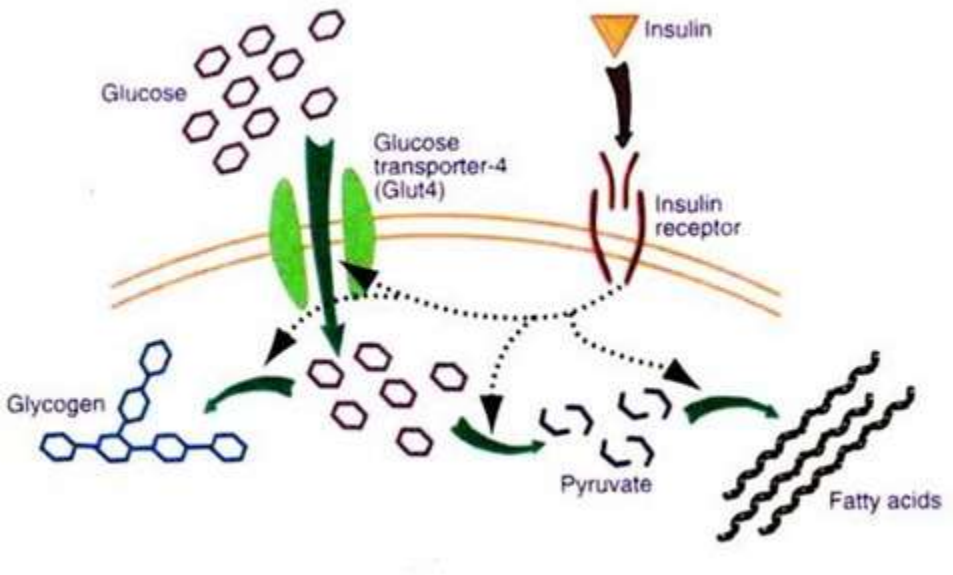
Is a trans membrane receptor that is activated by insulin. It belongs to the large class of tyrosine kinase receptors. Two alpha subunits and two beta subunits make up the insulin receptor. The beta subunits pass through the cellular membrane and are linked by disulfide bonds. The alpha and beta subunits are encoded by a single gene (INSR). The insulin receptor has been designated as CD_{220} (cluster of differentiation 220).

Function of insulin receptor-effect of insulin on glucose uptake and metabolism:
Insulin binds to its receptor which in turn starts many protein activation cascades.

These include—
i. Translocation of Glut-4 transporter to the plasma membrane and influx of glucose

ii. Glycogen synthesis

iii. Glycolysis and fatty acid synthesis



Insulin receptors (a family of tyrosine kinase receptors), mediate their activity by causing the addition of a phosphate group to particular tyrosine's on certain proteins within a cell. The 'substrate' proteins which are phosphorylated by the insulin receptor include a protein called 'IRS-1' for 'Insulin Receptor Substrate-1'.

IRS-1 binding and phosphorylation eventually leads to an increase in the high affinity glucose transporter (Glut4) molecules on the outer membrane of insulin-responsive tissues, including muscle cells and adipose tissue, and therefore to an increase in the uptake of glucose from blood into these tissues. Briefly, the glucose transporter ($Glut_4$) is transported from cellular vesicles to the cell surface, where it then can mediate the transport of glucose into the cell. Glycogen synthesis is also stimulated by the insulin receptor via IRS-1.

Pathology of insulin receptors:

The main activity of activation of the insulin receptor is inducing glucose uptake. For this reason 'insulin insensitivity', or a decrease in insulin receptor signalling, leads to diabetes mellitus type 2 – the cells are unable to take up glucose, and the result is hyperglycemia (an increase in circulating glucose), and all the sequelae which result from diabetes. Patients with insulin resistance may display acanthosis nigricans.A few patients with homozygous mutations in the INSR gene have been described, which causes Donohue syndrome or Leprechauns. This autosomal recessive disorder results in a totally non-functional insulin receptor. These patients have low set, often protuberant ears, flared nostrils, thickened lips, and severe growth retardation.

In most cases, the outlook for these patients is extremely poor with death occurring within the first year of life. Other mutations of the same gene cause the less severe Rabson-Mendenhall syndrome, in which patients have characteristically abnormal teeth, hypertrophic gingiva (gums) and enlargement of the pineal gland. Both diseases present with fluctuations of the glucose level—after a meal the glucose is initially very high, and then falls rapidly to abnormally low levels.

Degradation of insulin and its receptors:

Once an insulin molecule has docked onto the receptor and effected its action, it may be released back into the extracellular environment or it may be degraded by the cell. Degradation normally involves endocytosis of the insulin-receptor complex followed by the action of insulin degrading enzyme. Most insulin molecules are degraded by liver cells. It has been estimated that a typical insulin molecule is finally degraded about 71 minutes after its initial release into circulation.

It is a 62 kDa peptide that is activated by glucagon and is a member of the G- protein coupled family of receptors, coupled to Gs. Stimulation of the receptor results in activation of adenylate cyclase and increased levels of intracellular cAMP. Glucagon receptors are mainly expressed in liver and in kidney with lesser amounts found in heart, adipose tissue, spleen, thymus, adrenal glands, pancreas, cerebral cortex, and G.I. tract.

Steroid Hormone Receptors:

Are proteins that have a binding site for a particular steroid molecule. Their response elements are DNA sequences that are bound by the complex of the steroid bound to its receptor. The response element is part of the promoter of a gene. Binding by the receptor activates or represses, as the case may be, the gene controlled by that promoter. It is through this mechanism that steroid hormones turn genes on (or off).

The DNA sequence of the glucocorticoid (a protein homodimer) response element is:

5′-AGAACAnnnTGTTCT-3′

3′ TCTT GTnnnACAAGA-5′

where n represents any nucleotide (a palindromic sequence)

The glucocorticoid receptor, like all steroid hormone receptors, is a zinc-finger transcription factor; there are four zinc atoms each attached to four cysteine's.

For a steroid hormone to turn gene transcription on, its receptor must:

(i) Bind to the hormone

(ii) Bind to a second copy of itself to form a homodimer

(iii) Be in the nucleus, moving from the cytosol if necessary

(iv) Bind to its response element

(v) Activate other transcription factors to start transcription

Each of these functions depends upon a particular region of the protein (Ex. The zinc fingers for binding DNA). Mutations in any one region may upset the function of that region without necessarily interfering with other functions of the receptor.

Nuclear Receptor Superfamily:

The zinc-finger proteins that serve as receptors for glucocorticoids and progesterone are members of a large family of similar proteins that serve as receptors for a variety of small, hydrophobic molecules. These include other steroid hormones like the mineralocorticoid-aldoster- one, estrogens, the thyroid hormone (T_3), calcitriol (the active form of vitamin D), rednoids—vitamin A (retinol) and its relatives-retinal/retinoic acid, bile acids and fatty acids. These bind members of the superfamily called Peroxisome Proliferator Activated Receptors (PPARs). They got their name from their initial discovery as the receptors for drugs that increase the number and size of peroxisomes in cells.

In every case, the receptors consists of at least three functional modules or domains from N-terminal to C-terminal, these are:

i. A domain needed for the receptor to activate the promoters of the genes being controlled

ii. The zinc-finger domain needed for DNA binding (to the response element)

iii. The domain responsible for binding the particular hormone as well as the second unit of the dimer

Receptors for Thyroid Hormones:

Are members of a large family of nuclear receptors that include those of the steroid hormones. They function as hormone-activated transcription factors and thereby act by modulating gene expression.

Thyroid hormone receptors bind DNA in absence of hormone:

Usually leading to transcriptional repression. Hormone binding is associated with a conformational change in the receptor that causes it to function as a transcriptional activator.

Mammalian thyroid hormone receptors are encoded by two genes, designated alpha and beta. Further, the primary transcript for each gene can be alternatively spliced, generating different alpha and beta receptor isoforms. Currently, four different thyroid hormone receptors are recognized as-(i) α-1 (ii) α-2 (iii) β-1 and (iv) β-2.

Like other members of the nuclear receptor superfamily, thyroid hormone receptors encapsulate three functional domains:

i. A transactivation domain at the amino terminus that interacts with other transcription factors to form complexes that repress or activate transcription. There is considerable divergence in sequence of the transactivation domains of alpha and beta isoforms and between the two beta isoforms of the receptor.

ii. A DNA-binding domain that binds to sequences of promoter DNA known as hormone response elements.

iii. A ligand-binding and dimerization domain at the carboxy-terminus.

Disorders of thyroid hormone receptors:

A number of humans with a syndrome of thyroid hormone resistance have been identified, and found to have mutations in the receptor beta gene which abolish ligand binding. Clinically, such individuals show a type of hypothyroidism characterized by goiter, elevated serum concentrations of T_3 and thyroxine and normal or elevated serum concentrations of TSH.
More than half of affected children show attention-deficit disorder, which is intriguing considering the role of thyroid hormones in brain development. In most affected families, this disorder is transmitted as a dominant trait, which suggests that the mutant receptors act in a dominant negative manner.

Adrenergic Receptors (or Adrenoceptors):

Are a class of G-protein coupled receptors that are targets of the catecholamine's. Adrenergic receptors specifically bind their endogenous ligands, the catecholamine's adrenaline and noradrenalin (called epinephrine and norepinephrine), and are activated by these.

Many cells possess these receptors, and the binding of an agonist will generally cause a sympathetic response (i.e. the fight-or-flight response) viz. the heart rate will increase and the pupils will dilate, energy will be mobilized, and blood flow diverted from other, non-essential, organs to skeletal muscle. There are several types of adrenergic receptors, but there are two main groups viz. a-adrenergic and P-adrenergic.

α-Adrenergic receptors:

These receptors bind noradrenalin (norepinephrine) and adrenaline (epinephrine). Phenylephrine is a selective agonist of the a-receptor. They exist as α_1-adrenergic receptors and α_2-adrenergic receptors.

β-Adrenergic receptors:

These receptors are linked to Gs proteins, which in turn are linked to adenyl cyclase. Agonist binding thus causes a rise in the intracellular concentration of the second messenger cAMP. Downstream effectors of cAMP include cAMP-dependent protein kinase (PKA), which mediates some of the intracellular events following hormone binding.

Role in circulation:

Epinephrine reacts with both α and β-adrenoreceptors, causing vasoconstricdon and vasodilation, respectively. Although receptors are less sensitive to epinephrine, when activated, they override the vasodilation mediated by β-adrenoreceptors. The result is that high levels of circulating epinephrine

cause vasoconstriction. Lower levels of epinephrine dominates β-adrenoreceptor stimulation, producing an overall vasodilation.

The mechanism of adrenergic receptors:

Adrenaline or noradrenalin is receptor ligands to either α_1, α_2 or β-adrenergic receptors, a, couples to Gq, which results in increased intracellular Ca^{2+} which results in smooth muscle contraction. α_2 on the other hand, couples to Gi, which causes a decrease of cAMP activity, resulting in smooth muscle contraction. β receptors couple to Gs, and increase intracellular cAMP activity, resulting in heart muscle contraction, smooth muscle relaxation and glycogenolysis.

Functions of α-receptors:

α-Receptors have several functions in common. They are:
(i) Vasoconstriction of arteries to heart (coronary artery)

(ii) Vasoconstriction of veins

(iii) Decrease motility of smooth muscle in gastrointestinal tract

Alpha-1 adrenergic receptor:

Alpha-1 -adrenergic receptors are members of the G protein-coupled receptor superfamily. Upon activation, a heterotrimeric G-protein, Gq, activates phospholipase C (PLC), which causes an increase in IP_3 and calcium. This triggers all other effects. Specific actions of the β_1 receptor mainly involve smooth muscle contraction.
It causes vasoconstriction in many blood vessels including those of the skin & gastrointestinal system and to kidney (renal artery) and brain. Other areas of smooth muscle contraction are for instance – ureter, vas deferens, hairs (arrector pili muscles), uterus (when pregnant), urethral sphincter, bronchioles (although minor to the relaxing effect of β_2 receptor on bronchioles). Further effects include glycogenolysis and gluconeogenesis from adipose tissue and liver, as well as secretion from sweat glands and Na reabsorption from kidney.

Alpha-2 adrenergic receptor:

There are 3 highly homologous subtypes of α_2 receptors viz. α_2A, α_2B, and α_2C. Specific actions of the a_2-receptor include:

i. Inhibition of insulin release in pancreas

ii. Induction of glucagon release from pancreas

iii. Contraction of sphincters of the gastrointestinal tract

Beta-1 adrenergic receptor:

Specific actions of the β_1 receptor include:

i. Increase cardiac output, both by raising heart rate and increasing the volume expelled with each beat (increased ejection fraction)

ii. Renin release from juxtaglomerular cells

iii. Lipolysis in adipose tissue

Beta-2 adrenergic receptor:

Specific actions of the β_2 receptor include:

i. Smooth muscle relaxation, e.g. in bronchi

ii. Relaxes urinary sphincter and pregnant uterus

iii. Relaxes detrusor urinary muscle of bladder wall

iv. Dilates arteries to skeletal muscle

v. Glycogenolysis and gluconeogenesis

vi. Contract sphincters of GI tract

vii. Thickened secretions from salivary glands

viii. Inhibit histamine-release from mast cells

ix. Increase renin secretion from kidney

Comparison of different adrenergic receptors

Receptor type	Agonist potency order	Selected action of agonist	Mechanism	Agonists	Antagonists
α_1:A, B, D	Adrenaline ≥ Noradrenaline >> Isoprenaline	Smooth muscle contraction	Gq: Phospholipase C (PLC) activated, IP3 and Calcium up	Noradrenaline Phenylephrine Methoxamine Cirazoline	(Alpha blockers) Phenoxy-benzamine Phentolamine Prazosin Tamsulosin Terazosin
α_2: A, B, C	Adrenaline ≥ Noradrenaline >> Isoprenaline	Smooth muscle contraction and neurotransmitter inhibition	Gi: Adenylate cyclase inactivated, cAMP down	Clonidine lofexidine Xylazine Tizanine Guanfacine	(Alpha blockers) Metoprolol atenolol
β_1	Isoprenaline > Adrenaline = Noradrenaline	Heart muscle contraction	Gs: Adenylate cyclase activated, cAMP up	Noradrenaline Isoprenaline Dobutamine	(Beta blockers) Metoprolol atenolol
β_2	Isoprenaline > Adrenaline > > Noradrenaline	Smooth muscle relaxation	Gs: Adenylate cyclase activated, cAMP up	Salbutamol Bitolterol Mesylate Formoterol Isoprenaline Levalbuterol Metaproterenol Salmeterol Terbutaline Ritodrine	(Beta blockers) Butoxamine propranolol
β_3	Isoprenaline = Noradrenaline > Adrenaline	Enhance lipolysis	Gs: Adenylate cyclase activated, cAMP up	L-796568	

Probable Questions:

1. Define hormone. What are the main characteristics of a hormone?
2. How hormones exert their effect through extracellular receptors?
3. How steroid hormones exert their effect?
4. What is antagonistic effect and what is synergistic effect of hormone action?
5. State the role of cAMP as hormone second messenger.
6. State the role of cGMP as hormone second messenger.
7. State the role of Calcium ion as hormone second messenger.
8. Discuss about positive feed back of hormone action with suitable example.
9. Discuss about negative feed back of hormone action with suitable example.
10. What is hormone receptor? Describe its importance.
11. How insulin receptor exerts its effect in cell? What happen when there is defect in the receptor?
12. What are the characteristics of steroid receptors?
13. Discuss the role and types of adrenergic receptors.
14. Compare different types of adrenergic receptors.

Suggested Readings:

1. General Endocrinology. Turner and Bagnara. Sixth Edition.
2. Williams Textbook of Endocrinology. Tenth Edition.
3. Introduction to Endocrinology. Chandra S Negi. Second Edition
4. Endocrinology. Hadley and Levine. Sixth Edition

Unit-IX

Biosynthesis, secretion and regulation of hormones: biosynthesis of protein and peptide hormones (Growth Hormone and Insulin), Post-Translational event and release; biosynthesis of steroid hormones and their regulations; biosynthesis of T3 and T4 and their regulation

Objective: In this unit you will learn about biosynthesis release and regulations of different hormones such as growth hormone, insulin, thyroxine and steroid hormones.

Meaning of Growth Hormones:

Growth hormone (GH) also known as somatotropic hormone and is a peptide hormone secreted by acidophils of the anterior pituitary gland. GH is stored in large, dense granules present in acidophil cells. It is a single chain polypeptide with molecular weight of 22,000 having 191 amino acids and two disulphide bridges. As the name indicates, its action is on the growth of the body. It stimulates somatic growth and development and helps to maintain lean body mass and bone mass in adults.

Growth hormone (**GH**) or **somatotropin**, also known as **human growth hormone** (**hGH** or **HGH**) in its human form, is a peptide hormone that stimulates growth, cell reproduction, and cell regeneration in humans and other animals. It is thus important in human development. GH also stimulates production of IGF-1 and raises the concentration of glucose and free fatty acids. It is a type of mitogen which is specific only to the receptors on certain types of cells. GH is a 191-amino acid, single-chain polypeptide that is synthesized, stored and secreted by somatotropic cells within the lateral wings of the anterior pituitary gland.

A recombinant form of hGH called somatreopleopin (INN) is used as a prescription drug to treat children's growth disorders and adult growth hormone deficiency. In the United States, it is only available legally from pharmacies by prescription from a licensed health care provider. In recent years in the United States, some health care providers are prescribing growth hormone in the elderly to increase vitality. While legal, the efficacy and safety of this use for HGH has not been tested in a clinical trial. Many of the functions of hGH remain unknown.

In its role as an anabolic agent, HGH has been used by competitors in sports since at least 1982, and has been banned by the IOC and NCAA. Traditional urine analysis does not detect doping with HGH, so the ban was not enforced until the early 2000s, when blood tests that could distinguish between natural and artificial HGH were starting to be developed. Blood tests conducted by WADA at the 2004 Olympic Games in Athens, Greece targeted primarily HGH. Use of the drug for performance enhancement is not currently approved by the FDA.

GH has been studied for use in raising livestock more efficiently in industrial agriculture and several efforts have been made to obtain governmental approval to use GH in livestock production. These uses have been controversial. In the United States, the only FDA-approved use of GH for livestock is the use of a cow-specific form of GH called bovine somatotropin for increasing milk production in dairy cows. Retailers are permitted to label containers of milk as produced with or without bovine somatotropin.

The mammalian *GH* gene (also called *GH-normal* or *GH-N*) belongs to a gene cluster that includes the genes for prolactin and some placental lactogens, and is primarily expressed in the somatotroph

cells of the anterior pituitary gland. GH secretion occurs in a pulsatile fashion owing to the action of two hypothalamic factors, growth hormone releasing hormone (GHRH) which stimulates GH secretion, and somatostatin which inhibits GH secretion. GH secretion is also stimulated by ghrelin, an endogenous GH secretagogue that is primarily secreted by the gastrointestinal tract. In the circulation, GH is bound to the growth hormone binding protein (GHBP) which is a soluble truncated form of the growth hormone receptor (GHR). GHBP is generated either as an alternative splice form of the GHR transcript (in rodents) or by limited proteolysis of the GHR protein (in humans). Thus, GH in the circulation exists as bound and free forms, the predominance of each being dependent on the pulsatile pattern of its secretion.

GH secretion exhibits sexual dimorphism; it is secreted more frequently in females than in males. While this could reflect the differential effects of sex steroids on GH secretion and action, recent data suggest the existence of sex-specific differences in the GH/IGF-1 axis at birth. Moreover, inter-species differences in circulating GH profiles have been observed in mammals. In males, GH secretion occurs nocturnally in humans and in 3-4 hour intervals in rodents; while in females, rats have residual GH levels between periods of GH secretion which are absent in humans and mice.

Nomenclature

The names *somatotropin* (*STH*) or *somatotropic hormone* refers to the growth hormone produced naturally in animals and extracted from carcasses. Hormone extracted from human cadavers is abbreviated *hGH*. The main growth hormone produced by recombinant DNA technology has the approved generic name (INN) *somatropin* and the brand name *Humatrope*, and is properly abbreviated rhGH in the scientific literature. Since its introduction in 1992 Humatrope has been a banned sports doping agent, and in this context is referred to as HGH.

Structure

The major isoform of the human growth hormone is a protein of 191 amino acids and a molecular weight of 22,124 daltons. The structure includes four helices necessary for functional interaction with the GH receptor. It appears that, in structure, GH is evolutionarily homologous to prolactin and chorionic somatomammotropin. Despite marked structural similarities between growth hormone from different species, only human and Old World monkey growth hormones have significant effects on the human growth hormone receptor.

Several molecular isoforms of GH exist in the pituitary gland and are released to blood. In particular, a variant of approximately 20 kDa originated by an alternative splicing is present in a rather constant 1:9 ratio, while recently an additional variant of ~ 23-24 kDa has also been reported in post-exercise states at higher proportions. This variant has not been identified, but it has been suggested to coincide with a 22 kDa glycosylated variant of 23 kDa identified in the pituitary gland. Furthermore, these variants circulate partially bound to a protein (growth hormone-binding protein, GHBP), which is the truncated part of the growth hormone receptor, and an acid-labile subunit (ALS).

Regulation of Growth hormone secretion

Secretion of growth hormone (GH) in the pituitary is regulated by the neurosecretory nuclei of the hypothalamus. These cells release the peptides growth hormone-releasing hormone (GHRH or *somatocrinin*) and growth hormone-inhibiting hormone (GHIH or *somatostatin*) into the hypophyseal portal venous blood surrounding the pituitary. GH release in the pituitary is primarily determined by the balance of these two peptides, which in turn is affected by many physiological stimulators (e.g., exercise, nutrition, sleep) and inhibitors (e.g., free fatty acids) of GH secretion.

Somatotropic cells in the anterior pituitary gland then synthesize and secrete GH in a pulsatile manner, in response to these stimuli by the hypothalamus. The largest and most predictable of these GH peaks occurs about an hour after onset of sleep with plasma levels. Otherwise there is wide

variation between days and individuals. Nearly fifty percent of GH secretion occurs during the third and fourth NREM sleep stages. Surges of secretion during the day occur at 3- to 5-hour intervals.[3] The plasma concentration of GH during these peaks may range from 5 to even 45 ng/mL. Between the peaks, basal GH levels are low, usually less than 5 ng/mL for most of the day and night. Additional analysis of the pulsatile profile of GH described in all cases less than 1 ng/ml for basal levels while maximum peaks were situated around 10-20 ng/mL.

A number of factors are known to affect GH secretion, such as age, sex, diet, exercise, stress, and other hormones. Young adolescents secrete GH at the rate of about 700 μg/day, while healthy adults secrete GH at the rate of about 400 μg/day. Sleep deprivation generally suppresses GH release, particularly after early adulthood.

Mechanism of Action of Growth Hormones:

i. Receptors for growth hormone are present on the plasma membrane of cells.

ii. Belong to cytokine family of receptors.

iii. Presence of excess of GH down regulates the synthesis of its receptors.

iv. Many hours must elapse after administration of GH before anabolic and growth-promoting actions of the hormones to become evident.

v. Most of the actions of GH require the production of GH induced somatomedin C or insulin-like growth factor (IGF).

vi. The plasma half-life of IGF is much longer than that of GH.

Actions of the hormone can be broadly classified into two types:

a. Indirect growth promoting action

b. Direct anti-insulin action.

1. Indirect growth promoting action (Figs 6.9 and 6.10) is due to the action of growth hormone on liver. When the hormone acts on liver, liver secretes somatomedin C or insulin-like growth factor (IGF- I). This substance acts on skeletal and extraskeletal compartments.

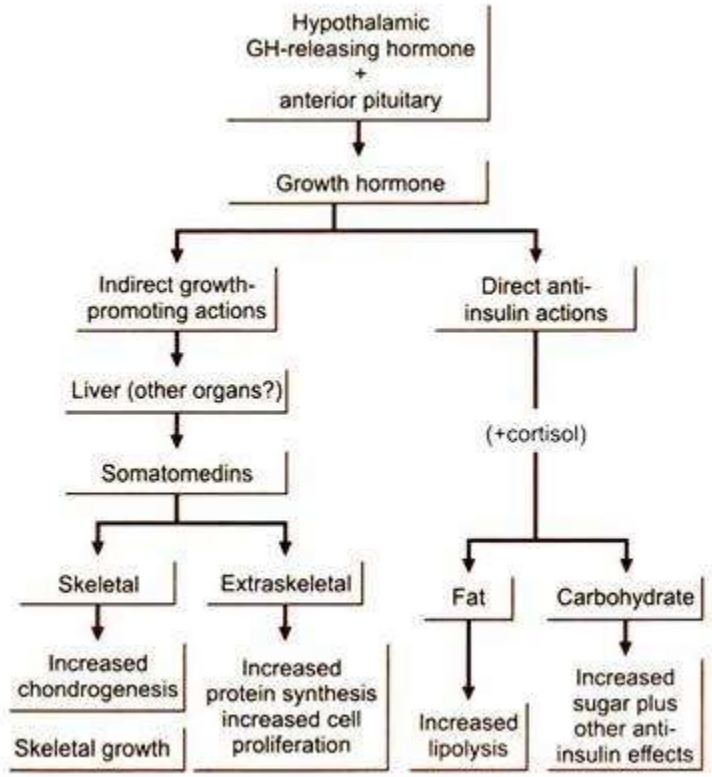


Fig. 6.9: Composite diagram showing actions of GH

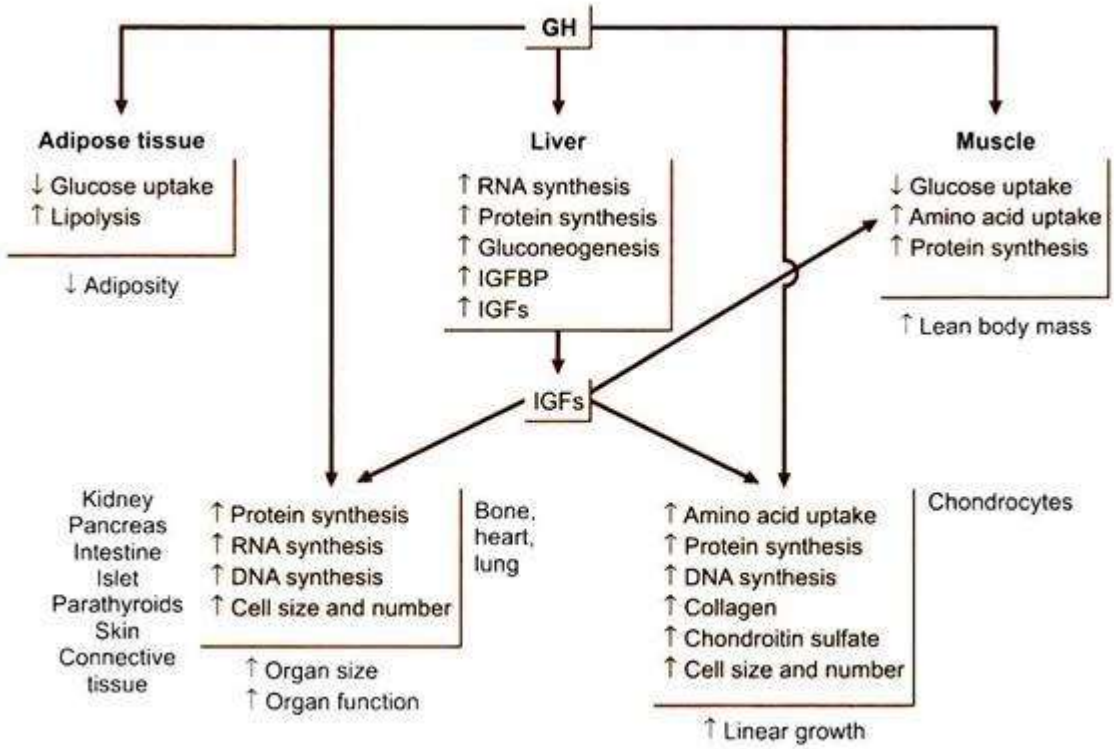


Fig. 6.10: Highlighting various intracellular actions of GH in the body

i. Skeletal compartment:

When somatomedin acts on epiphyseal plate present between the long bones, the epiphyseal plate gets widened. This gives space for the chondrogenesis of the long bones. The long bones grow linearly.

Hence, the height of the person increases. The long bones can grow only up to the age of about 18-20 years beyond which the epiphyseal plates get fused with long bones and there can be no more linear growth of body.

ii. Extra-skeletal compartment:

This in general refers to the growth of organ and tissues. The growth is brought about by hyperplasia (stimulating mitotic cell division and hence increase in cell number) and hypertrophy (increase cell size). The various tissues in the body grow. There will be increased protein synthesis because of which it brings about positive nitrogen balance. The proteins synthesized are incorporated for the growth of the organs.

The various parts of the body do not grow in equal proportion at the same time. The growth of the different parts of the body based on chronological age has been shown in Fig. 6.11.

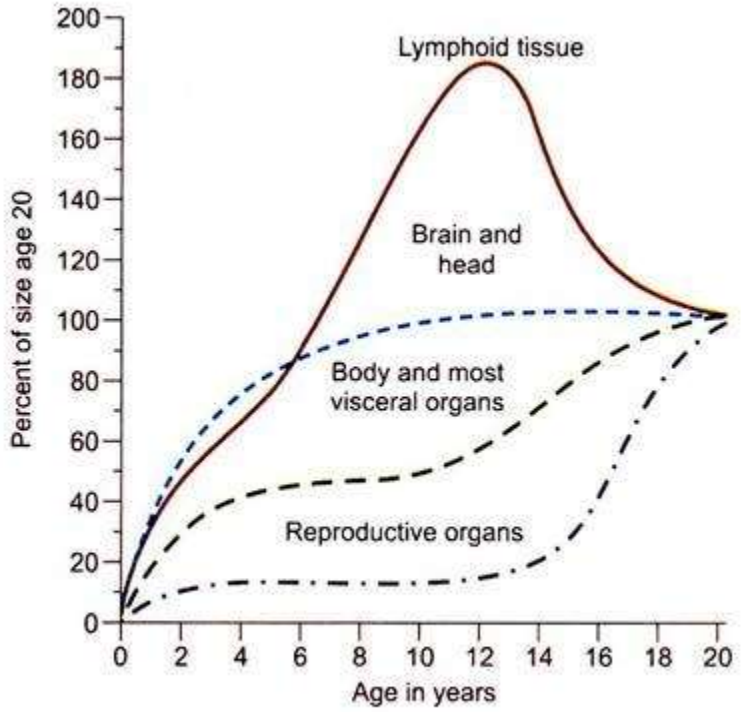


Fig. 6.11: Extent of growth of various tissues at different ages

2. Direct anti-insulin action:

This can be brought about in the target organs in presence of Cortisol (permissive action of Cortisol is required.

i. On carbohydrate metabolism:

It is a hyperglycemic agent. Increases the blood glucose level by:

a. Decreasing the peripheral utilization of glucose.

b. Increased gluconeogensis in liver.

Metahypophyseal diabetes:

Uncontrolled secretion of GH for a long time brings about increase in blood glucose level. This leads to increase stimulation of beta cells of islets of Langerhans to secrete insulin. After sometime, due to constant stimulation, the beta cells get exhausted and lead to development of diabetes mellitus.

ii. Fat metabolism:

Acts on the adipose tissue. Neutral fats and triglycerides are broken down to release the free fatty acids. They are utilized for energy supply to the tissues.

This can lead to increased production of keto acids. Growth hormone also promotes the retention of sodium, potassium, calcium and phosphate since these substances are required for the growth of the body.

Regulation of Secretion of Growth Hormones:

It is mainly by the negative feedback control by the free form of the hormone level in circulation.

Growth hormone releasing hormone (GRH) secreted from the hypothalamus acts on anterior pituitary gland and stimulates the secretion of growth hormone, which in turn increases insulin-like growth factor (IGF) I or somatomedin C secretion from liver. When IGF I level in circulation increases, it acts on hypothalamus to stimulate the secretion of somatostatin (SS). SS on reaching anterior pituitary decreases the secretion of growth hormone (Fig. 6.12).

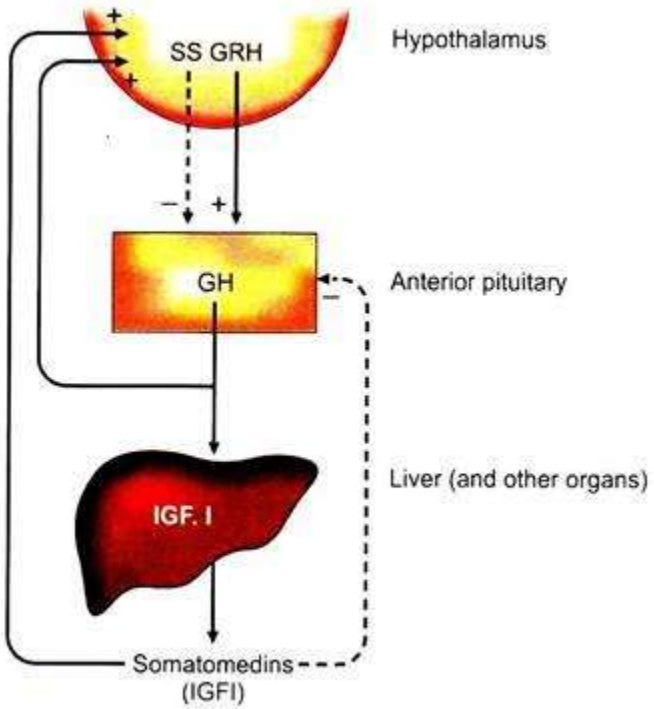


Fig. 6.12: Regulation of secretion of GH by feedback mechanism

IGF I also acts directly on anterior pituitary and exerts inhibitory influence on the secretion of growth hormone.GH secreted by the anterior pituitary gland is able to reach the hypothalamus through circulation and on reaching hypothalamus it stimulates the secretion of somatostatin. Somatostatin on reaching anterior pituitary inhibits further secretion of growth hormone.

Some of the other factors that increase the secretion of growth hormone are:

i. Increase in amino acids in circulation

ii. Hypoglycemia

iii. Free fatty acid decrease

iv. Exercise

v. At puberty

vi. Stage IV sleep.

The factors which inhibit the GH secretion are:

i. Dreaming or rapid eye movement (REM) sleep.

ii. Glucose increase.

iii. Cortisol.

iv. Obesity.

Applied Aspects of Growth Hormones:

Deficiency of GH in children:

i. Hypothalamic dysfunction

ii. Pituitary destruction

iii. Defective GHRH receptor

iv. Biologically incompetent GH or GH receptor

v. Failure to produce IGF

vi. GH receptor deficiency

vii. GH receptor unresponsiveness: Laron dwarfism

Dwarfism:

i. It's because of hyposecretion of GH from childhood.

ii. Person will have short stature. There will be a generalized stunted growth of the body.

iii. The person will have normal reproductive development.

iv. There will not be any mental abnormality and will have normal intelligent quotient (IQ).

v. Facial changes correspond with chronological age.

Achondroplasia is the most common form of dwarfism. The characteristic feature will be short limbs and normal trunk.

Laron dwarf:

i. It will be due to insensitivity of the tissues to GH.

ii. The receptors are non-responsive to GH.

iii. There can be normal or elevated level of GH in circulation.

Progeria:

Deficiency of growth hormone in adult. The person appears older at an younger age.

Dwarfism could also be due to:

i. Cretinism—thyroxine deficiency

ii. Gonadal dysgenesis

iii. Kaspar Hauser syndrome—psychosocial dwarfism

iv. Achondroplasia—child born to aged father

Frolich dwarf:

Destructive disease of part of anterior pituitary. At times may include post-pituitary and hypothalamus.

i. Stunted growth.

ii. Obesity

iii. Decreased sexual development

iv. Somnolence

v. Mentally subnormal

Deficiency of GH in adult:

i. Decreased muscle

ii. Decreased muscle strength and exercise performance

iii. Decreased lean body mass

iv. Decreased bone density

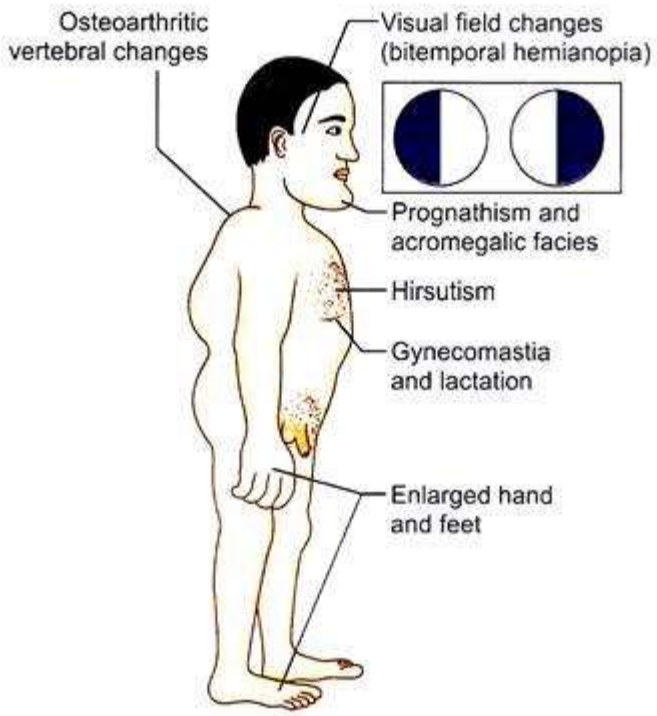


Fig. 6.13: Some of the important features of acromegaly

Acromegaly:

i. Hypersecretion of growth hormone after the puberty.

ii. Enlargement of hand and feet (acral parts of the body only can grow because of the ossification of the long bones).

iii. There will also be enlargement of mandible which results in prognatism. There will also be enlargement and protrusion of frontal bone. Because of this, the person may have gorilla-like appearance.

iv. Certain osteoarthritic changes are also observed leading to kyphosis.

v. There can be enlargement of viscera especially that of heart and may lead to cardiomegaly.

vi. There can be hirsutism (increased hair growth on anterior part of trunk) and gynecomastia (enlargement of breasts even in males) and lactation (secretion of milk).

vii. The person may suffer from bitemporal hemianopia (a type of visual field defect) due to the compressing on the medial part of optic chiasma by enlarged pituitary gland.

Gigantism:

i. Hypersecretion of hormone from childhood.

ii. Size of the person is pathologically big, but the person will be weak. Hence, the person is known as weak giant. There will not be proportionate growth of the contractile proteins in the muscles. Hence muscles are weak.

iii. The person is prone to develop early diabetes. This is because since growth hormone has hyperglycemic action, the sustained increase in blood glucose level may lead to exhaustion of beta cells of islets of Langerhans. So the person develops diabetes.

iv. The longevity of these people is restricted and die early.

Sheehan's syndrome:

i. Observed in female. Due to postpartum hemorrhage, there can be ischemic necrosis of pituitary gland.

ii. The pituitary gland secretion in general gets decreased.

iii. Symptoms include lethargy, sexually inactive, unable to withstand stress. Growth is inhibited and thyroid function is depressed.

iv. There can be atrophy of gonads. The menstrual cycle stops.

v. When there is general deficiency of all the hormones of anterior pituitary gland, this condition is known as panhypopituitarism.

Hyperprolactinemia:

It could be due to administration of dopamine antagonist/prolactin secreting adenomas.

Features:

a. Amenorrhea , b. Galactorrhea, c. Decreased libido, d. Impotence, e. Hypogonadism, f. Testosterone level low

Insulin:

Insulin is a type of protein hormone, which is synthesized in the β-cells of islets of Langerhans. The term insulin is derived from Latin word "Insula" means island. Banting and Best (1916) observed the role of insulin in glucose metabolism.

Structure of Insulin:

Insulin is a peptide hormone and its molecular weight is 5.7 Kdt. It is made up of two polypeptide chains α and β. Insulin is constituted by 51 amino acids, of which a-chain contains 21 amino acids and β-chain contains 30 amino acid residues. Besides the primary peptide bonds, the polypeptide chains are strengthened by disulphide bonds (-S-S-).

One intra -S-S- bond occurs in the a-chain in between 6 and 11 positions of cystine. Two inter -S-S- bonds are found in between a and p chain one in between 7^{th} position of both the chains and other in between 20^{th} position of α-chain and 19^{th} position of β-chain (Fig. 6.1).

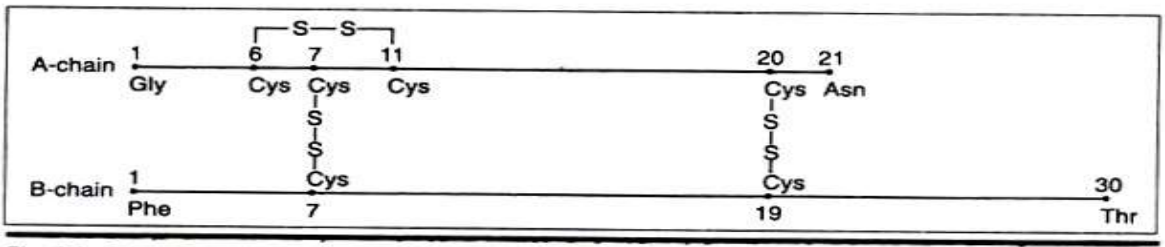


Fig. 6.1: Schematic representation of human insulin molecule

In different species of vertebrates, structure of insulin varies according to variation of amino acid residues. Variations occur at 8th, 9th and 10th position of a-chain and 30th position of β-chain.

Species	*α-chain*			*β-chain*
	8th	9th	10th	30th
Horse	Thr	Gly	Ile	Ala
Goat & Cattle	Ala	Ser	Val	Ala
Man	Thr	Ser	Ile	Thr
Rabbit	Thr	Ser	Ila	Ser

Biosynthesis of Insulin:
The synthesis of insulin takes place in p-cells of islets of Langerhans.

It is a complex phenomenon and it occurs in following ways:

1. Transcription of code:
Genes on chromosome 11 coding for insulin and are transcribed to mRNA in the nucleus.

2. Translation of the code:
After moving to the cytoplasm, mRNA is translated by the polysome attach to GER. Polypeptide synthesis is initiated with the formation of N-terminal signal peptide (leading sequence) which penetrates through the membrane of GER.

3. Synthesis of preproinsulin:
Further elongation directs the polypeptide chain into the lumen of GER, resulting in the formation of preproinsulin. It is constituted by 109 amino acid residues and mol. wt. is 11.5 kdt.

4. Separation of signal sequence:
In the lumen of GER, N-terminal signal peptide is hydrolysed away by signal peptidase. Thus signal peptide is cleaved and pro- insulin is formed in the cysternal space of GER. Pro-insulin consists of 86 amino acid residues and its mol. wt. is about 9 kd. Pro-insulin has disulphide bonds.

5. Transfer of pro-insulin:
Pro-insulin is transported from GER to the Golgi complex

6. Splitting of pro-insulin:

In Golgi cisternae pro-insulin is hydrolysed by trypsin like peptidase to yield a 53 amino acid insulin precursor and pro-c-peptide has 33 amino acids.

Under condition of excessive stimulation pro-insulin is secreted by vesicular exocytosis along with the insulin from p-cells (Fig. 6.2).

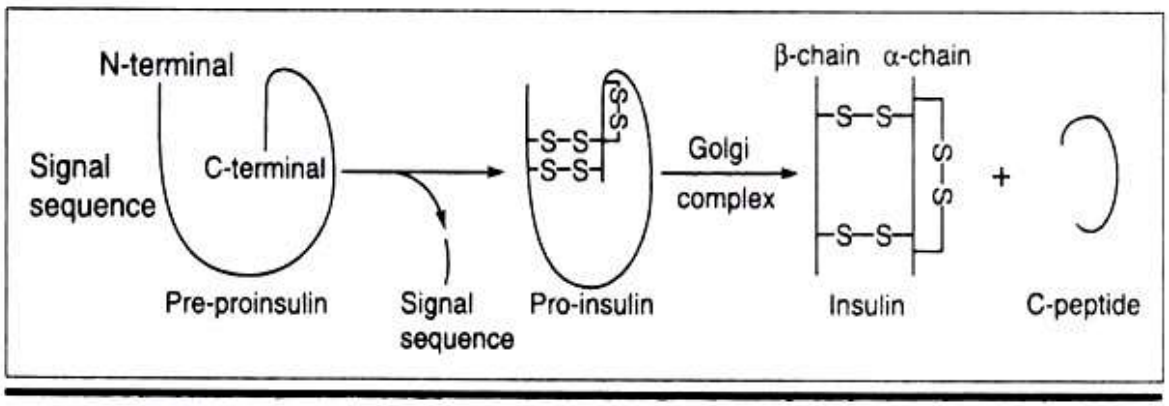


Fig. 6.2: Formation of insulin

7. Formation of insulin:

In the Golgi complex about 95% of the pro-insulin is converted to active insulin. Enzyme carboxylase peptidase hydropses c-terminal peptide bonds in the pro-c- peptide and the insulin precursor to release 2-c-terminal basic amino acids from each. Two molecules of Arg. are driven out from the insulin precursor and lead to the formation of active insulin (consists of 51 amino acids).From pro-c-peptide two amino acids Lys and Arg are separate out and leads to the formation of connective peptide or c-pep- tide (consists of 31 amino acids). Insulin and c-peptide are present in secretory granules of Golgi complex. In some species, insulin is combined with Zn within P-cells. After stimulation insulin is secreted by exocytosis (Fig. 6.3).

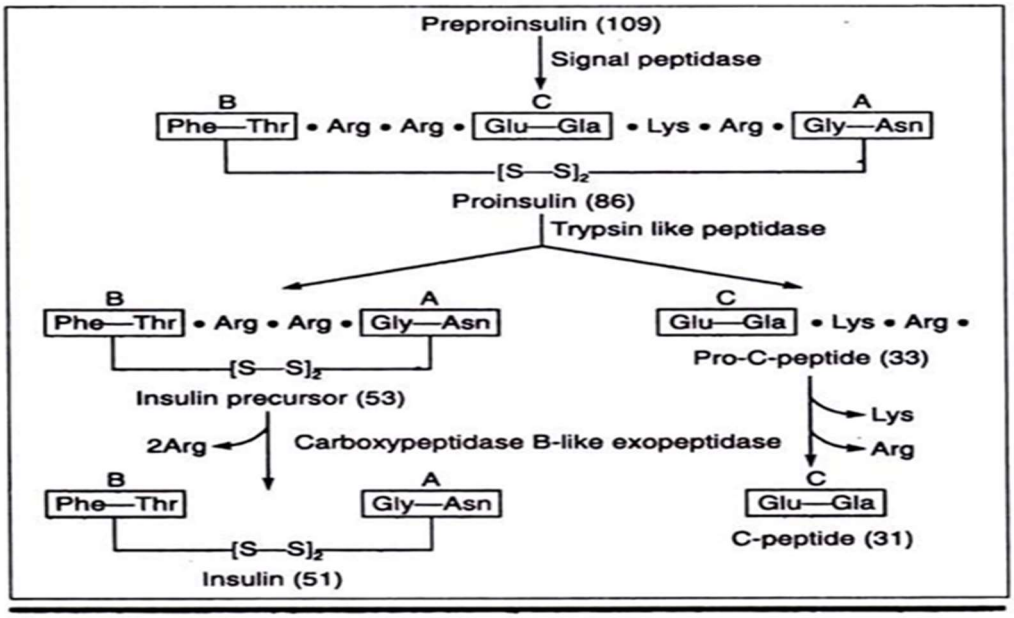


Fig. 6.3: Schematic representation of insulin synthesis

Transport of insulin:
Insulin is directly diffused within the blood sinusoids of the islets and is transported to the target organ.

Catabolism of insulin:
After biochemical reaction, insulin is degraded within the liver, kidney, skeletal muscles and placenta in presence of enzyme insulinase.

Control of secretion:

Insulin synthesis and secretion is controlled by following factors:

1. Carbohydrate meal:
Intake of carbohydrate rich food leads to raise the blood glucose which is signal for increased insulin secretion.

2. Amino acids:
Ingestion of protein causes an increase in plasma amino acids level. Elevated plasma arginine is particularly potent stimulus for insulin secretion.

3. Gastrointestinal hormone:
Intestinal hormones (GIP & VIP) secretion stimulates the insulin synthesis and secretion.

4. Epinephrine:
The synthesis and release of insulin are degraded by negative feedback mechanism of epinephrine in stress condition.

5. Glucagon:
Low blood sugar level stimulates the secretion of glucagon for glycogenesis, which in-turn inhibits the synthesis of insulin (Fig. 6.4).

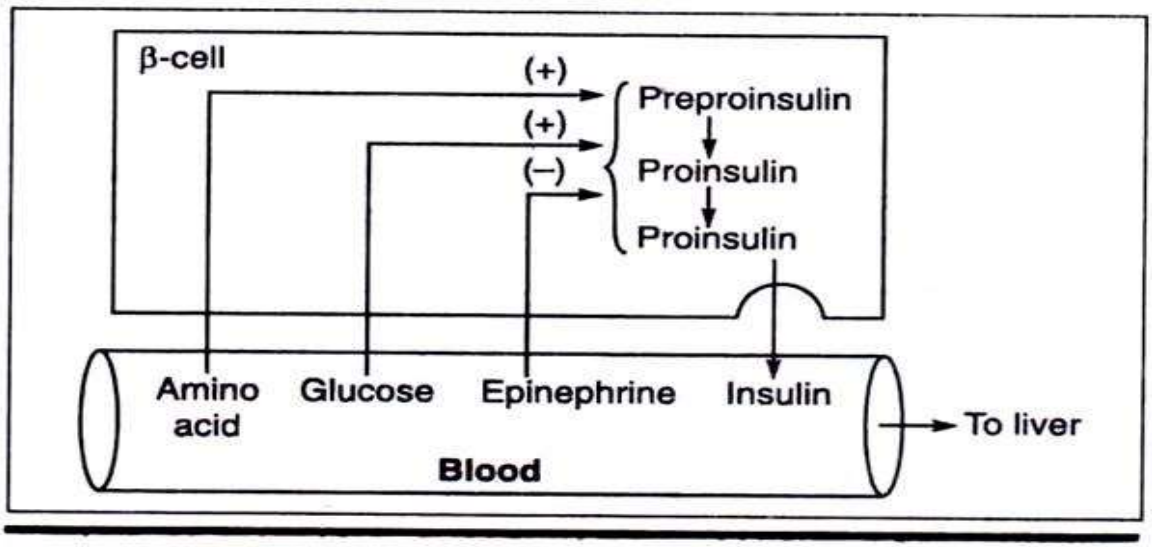


Fig. 6.4: Regulation of insulin release from β-cell

6. Somatostatin:

This hormone is secreted from D-cells of pancreatic islets and regulates the secretion α and β-cells.

Insulin Receptor:

a. Insulin acts on target tissues by binding to specific insulin receptors which are glycoproteins.

b. The human insulin receptor gene is found on chromosome 19. The insulin receptors are being constantly synthesized and degraded. Their half-life is 6 to 12 hours only.

c. It is synthesized as a single chain polypeptide, pro-receptor in the rough endoplasmic reticulum and is rapidly glycosylated in Golgi region.

d. The pro-receptor is cleaved to form mature α and β subunits ($\alpha_2\beta_2$) which is heterodimer, linked by S- S bonds.

e. Both subunits are extensively glycosylated and removal of sialic acid and galactose decreases insulin binding and insulin action.

f. Insulin receptors are found in target cell membrane.

g. Though insulin receptor is a heterodimer consisting of 2 subunits, designated α and β ($\alpha_2\beta_2$) linked by disulphide bonds.

h. The α subunit is entirely extracellular and it binds insulin, probably via a cystinerich domain.

i. The β subunit is a trans-membrane protein that performs the second major function of a receptor, i.e. signal transduction and insulin action.

j. Binding of insulin to the receptor stimulates its tyrosine kinase activity. Tyrosine kinase enzyme phosphorylates the phenolic -OH group of tyrosine residues in specific protein including that of a tyrosine in the chain of insulin receptor itself to modulate their activities, ATP + tyrosine protein → ADP + phosphotyrosine protein.

The cytoplasmic portion of p subunit has tyrosine kinase activity and an auto-phosphorylation site.

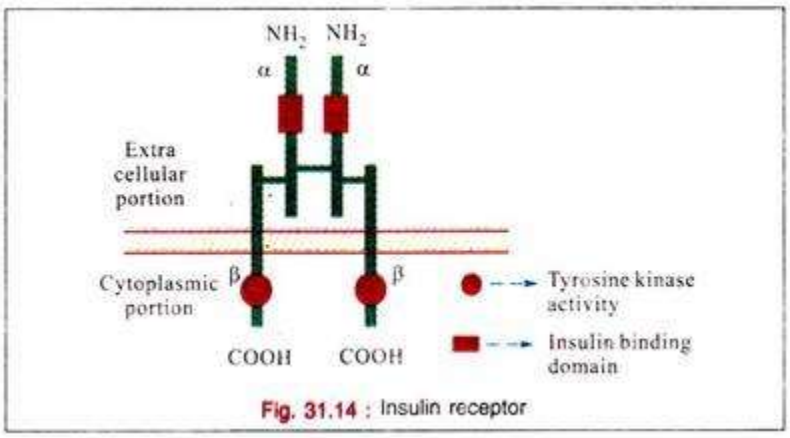


Fig. 31.14 : Insulin receptor

Insulin Secretion:

About 50 units of insulin are required per day. The human pancreas stores about 250 units.Normal concentration of insulin (fasting) in plasma: 6-126 μU/ml.

Factors Stimulating Insulin Secretion:

a. Increased blood glucose level causes an increase in insulin secretion and decreased blood glucose level depresses insulin secretion.

b. The hyperglycemia produced by glucagon enhances insulin production.

c. Since the growth hormone and glucocorticoids cause hyperglycemia they also stimulate insulin secretion.

d. Sugars which are readily metabolized— e.g., mannose and fructose—can stimulate insulin release. But non-metabolised sugars such as glactose, L-arabinose and xylose do not stimulate.

e. Many agents, such as amino acids, fatty acids and some gastro—intestinal products can stimulate insulin release only in presence of glucose.

f. Insulin secretion is enhanced by cAMP, ACTH and thyrotropin.

g. Amino acids particularly leucine and arginine can stimulate pancreas to produce insulin in both vivo and vitro. Proteins like casein also increases secretion of insulin.

h. Central nervous system indirectly influences the release of insulin. Vagal stimulation causes an increase in insulin secretion.

i. Sulfonylureas, the hypoglycemic agent, may act on insulin secretion by a different mechanism than that of glucose.

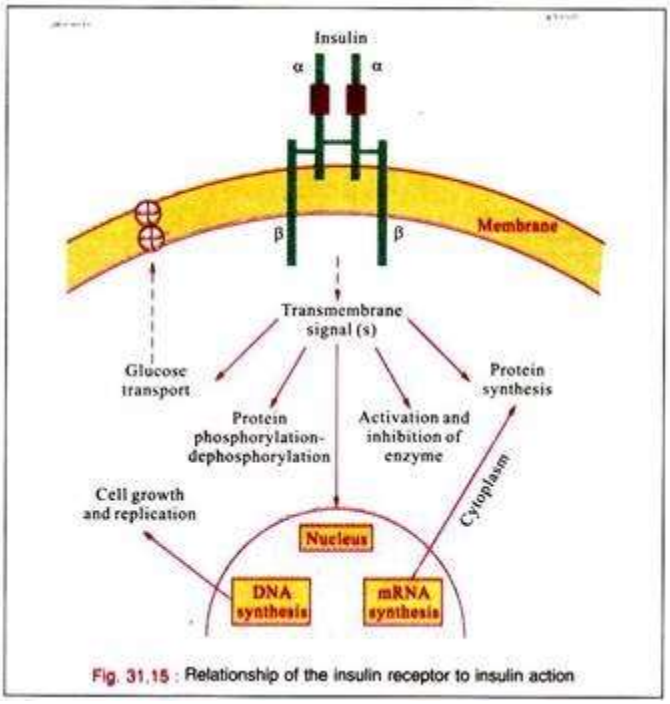


Fig. 31.15 : Relationship of the insulin receptor to insulin action

Factors Inhibiting Insulin Secretion:

a. Epinephrine is the highly effective inhibitor of insulin secretion.

b. Starvation reduces insulin secretion.

c. Magnesium also inhibits insulin secretion.

d. Vagotomy reduces insulin secretion.

Metabolism of Insulin:

a. Insulin is degraded in liver and kidney by the enzyme glutathinone insulin trans-hydrogenase which brings about reductive cleavage of the S-S bonds that connect A and B chains of the insulin molecule. Reduced glutathione acts as a coenzyme.

b. The A and B chains are further degraded by proteolysis. But when insulin is bound to antibody, it is much less sensitive to enzymic degradation.

Functions of Insulin:

a. Insulin is firmly bound to the highly specific receptor site present in the cell membrane. The receptor may probably be a glycoprotein. The biologic activities of insulin's are proportionate to their binding affinities. Insulin, thus, may carry out most of its function without entering the cell. The number of receptors declines where insulin levels are high.

b. Insulin exhibits transport at the membrane site, RNA synthesis at the nuclear site, translation at the ribosome for protein synthesis, and influence on tissue levels of cAMP. It is active in skeletal and heart muscle, adipose tissue, liver, the lens of the eye and leukocytes. It is inactive in renal tissue, red blood cells and gastrointestinal tract. The most metabolic function is centered in the muscle, adipose tissue and liver.

c. It facilitates the transport of glucose and related monosaccharides, amino acids, potassium ion, nucleosides, inorganic phosphate, and calcium ion in muscle and adipose tissue.

d. In muscle for adipose tissue, insulin increases the entry of glucose and thus leads to increased glycogen deposition, stimulation of HMP shunt resulting in increased production of NADPH, increased glycolysis, increased oxidation (Increase in oxygen uptake and CO_2 production), and increased fatty acid synthesis.

e. In adipose tissue, it increases lipid synthesis by means of fatty acid synthesis and glycerophosphate for triacylglycerol synthesis.

f. Insulin increases intracellular concentration of non-metabolized sugars such as galactose, L-arabinose, and xylose. The hormone facilitates the entry of those sugars having the same configuration at carbons, 1, 2, and 3 as D-glucose. Since fructose having a ketone group at position 2 is not transported by insulin. Intracellular transport of glucose is enhanced by anoxia indicating that glucose transport requires energy.

g. It also increases the uptake of nonmetabolizable amino acids such as alpha-aminoisobutyrate. It maintains muscle protein by decreasing protein degradation.

h. In adipose tissue, it quickly depresses the liberation of fatty acids caused by epinephrine or glucagon.

i. Insulin directly increases protein synthesis as the hormone promotes the incorporation of labelled intracellular amino acids into protein. At the ribosomal level, it increases the capacity of this organelle to translate information from messenger RNA to the protein-synthesizing machinery.

j. In the liver, it stimulates glycolysis by increasing the synthesis of glucokinase, phosphofructokinase. and pyruvate kinase. It also depresses the enzymes controlling gluconeogenesis such as pyruvate carboxylase, phosphoenolpyruvate carboxykinase, fructose-1, 6-di-phosphatase, and glucose-6-phosphatase. Enzymes which are unimportant in the control of gluconeogenesis as well as glycolysis are not affected by insulin.

Pathophysiology of Insulin:

a. About 90% of persons with diabetes have non-insulin dependent (Type II) diabetes mellitus (NIDDM). Such patients are usually obese, have elevated plasma insulin levels.

b. The other 10% have insulin dependent (Type 1) diabetes mellitus (IDDM).

c. A few individuals produce antibodies directed against their insulin receptors. These antibodies prevent insulin from binding to the receptor so that such persons develop a syndrome of severe insulin resistance.

d. Tumors of β-cell origin cause hyperinsulinism thereby hypoglycemia. Leprechaunism is caused by the role of insulin in organogenesis. The syndrome is characterized by low birth weight, decreased muscle mass, decreased subcutaneous fat, and early death.

Abnormal Metabolism in Diabetic States:

a. In diabetes, hyperglycemia occurs due to the impaired transport and uptake of glucose into muscle and adipose tissue. Transport and uptake of amino acids are also depressed causing the raised level of amino acids into the blood, particularly, alanine, which supply fuel for gluconeogenesis in the liver. The amino acid breakdown during gluconeogenesis increases the production of urea nitrogen.

b. Lipid and fatty acid synthesis is decreased due to the decrease in acetyl-CoA, ATP, NADPH and glycerophosphate in all tissues. Stored lipids are hydrolysed by increased lipolysis and the liberated fatty acids interfere the carbohydrate phosphorylation in muscle and liver developing hyperglycemia.

c. Fatty acids in high concentration reaching the liver inhibit fatty acid synthesis by a feedback inhibition at the acetyl-CoA carboxylase step. Increased acetyl-CoA from fatty acids activates pyruvate carboxylase, stimulating gluconeogenic pathway for the conversion of amino acid carbon skeletons to glucose. Fatty acids also stimulate gluconeogenesis by entering the citric acid cycle and increasing production of citrate which is an inhibitor of glycolysis (at phosphofructokinase).Thus, the fatty acid cycle at the level of citrate synthetase and pyruvate and isocitrate dehydrogenases. The acetyl CoA, which cannot enter the citric acid cycle or cannot be used for fatty acids synthesis, is utilized in the synthesis of cholesterol or ketones or both. The rise in ketone bodies concentration in body fluids and tissues leads to acidosis.

d. Glycogen synthesis is diminished due to decreased glycogen synthetase activity, increased phosphorylase activity and increased ADP: ATP ratio. The phosphorylase activity is stimulated by epinephrine or glucagon.

e. The insulin deficiency causes hormonal imbalance and favours the action of corticosteroids, growth hormone and glucagon which enhance gluconeogenesis, lipolysis, and decreased intracellular me-

tabolism of glucose. The excess glucose in the urine requires water to be excreted out causing dehydration.

f. In the degradation in insulin, both liver and kidney are required. Therefore, in renal or hepatic disease, insulin requirement is decreased. This is observed in some diabetics with associated kidney or liver disease.

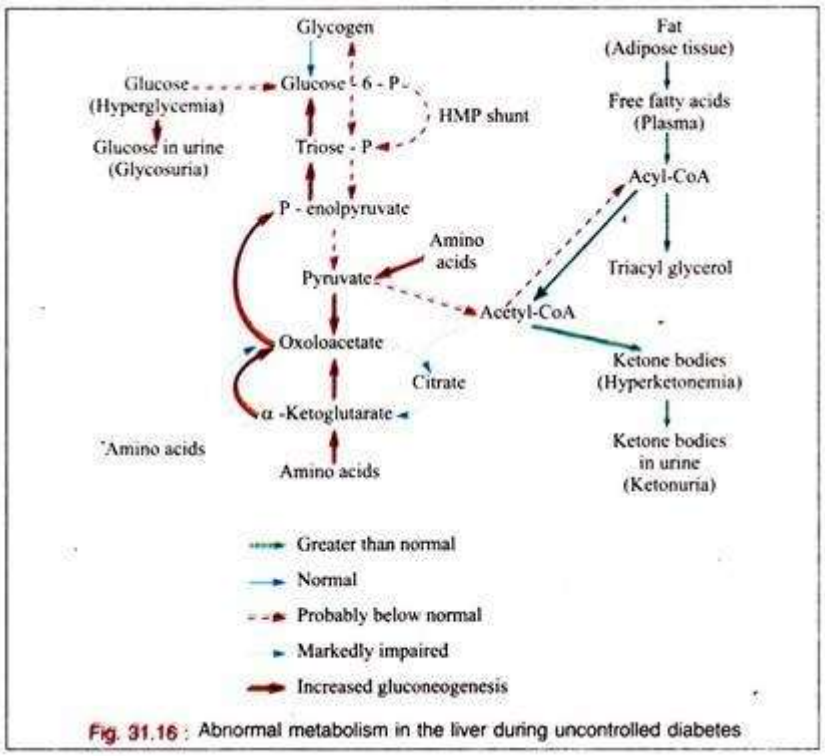


Fig. 31.16 : Abnormal metabolism in the liver during uncontrolled diabetes

Antibodies in Insulin:

a. The repeated injection of insulin produces low levels of an antibody to insulin in all subjects after 2 or 3 months of treatment.

b. The antibodies can produce lesions in the islet cells and severe diabetes.

c. Antibody-bound insulin is only slowly degraded; thus much of the insulin is actually wasted.

Experimental Diabetes:

a. Experimental diabetes can be produced by total pancreatectomy or by a single injection of alloxan, a substance related to the pyrimidine's or with streptozocin, an N-nitroso derivative of glucosamine.

b. Diabetes can also be produced by injection of diazoxide, a sulfonamide derivative which inhibits insulin secretion.

c. The injection of large amounts of antibodies to insulin is also considered to produce experimental diabetes.

d. Phlorhizin diabetes can be produced by the injection of the drug phlorhizin. This is actually a renal diabetes in which glycosuria is only produced by the failure of the reabsorption of glucose by the renal tubules.

Regulation of Insulin Secretion:

40 to 50 units of insulin is daily secreted from human pancreas. This represents about 15 to 20 per cent of the hormone stored in the gland. Insulin secretion is an energy-requiring process. Different factors are involved in insulin release.

a. Glucose:
(a) The increased concentration of glucose is the best regulator of insulin secretion.

(b) Among two ideas, one idea suggests that glucose combines "with a receptor which" is located on the B cell membrane that activates the release mechanism. The second idea suggests that intracellular metabolites pass through a pathway .such as the HMP shunt, the TCA cycle, etc.

b. Hormonal Factors:
(i) Epinephrine inhibits insulin release.

(ii) Beta adrenergic agonists stimulate insulin release by increasing intracellular cAMP.

(iii) Cortisol, estrogens, and progestin's also increase insulin secretion. Hence, insulin secretion is markedly increased during the later stages of pregnancy.

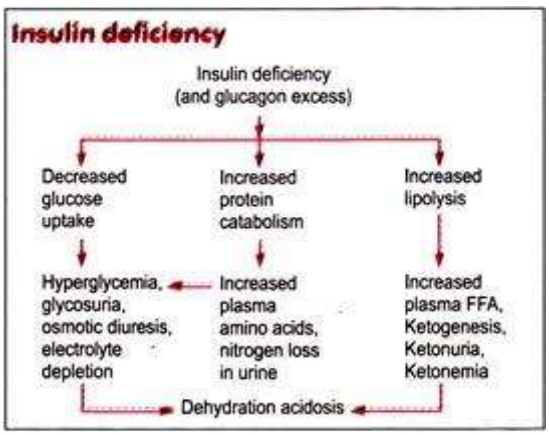
c. Pharmacologic Agents:
(i) Many drugs stimulate insulin secretion, but the sulfonyl urea compounds are used for therapy in humans.

(ii) Drugs such as tolbutamide stimulate insulin release and effectively used in the treatment of type 11 (non-insulin-dependent) diabetes mellitus. This class of drug is binded by a receptor which has been derived from the pancreatic P cells.

Effect of Insulin on Gene Expression:

(i) The actions of insulin are found to occur at the plasma membrane level or in the cytoplasm.

(ii) The synthesis of phosphoenolpyruvate carboxykinase (PEPCK) which catalyses a rate-limiting step in gluconeogenesis is decreased by insulin and hence gluconeogenesis is decreased.



(iii) Transcription is decreased due to the decreased amount of the primary transcript and of mature mRNA PEPCK which in turn is directly related to the decreased rate of PEPCK synthesis.

(iv) More than 100 specific mRNAs are affected by insulin, and a number of mRNAs in liver, adipose tissue, skeletal muscle, and cardiac muscle.

Steroid hormones:

Steroid, any of a class of natural or synthetic organic compounds characterized by a molecular structure of 17 carbon atoms arranged in four rings. Steroids are important in biology, chemistry, and medicine. The steroid group includes all the sex hormones, adrenal cortical hormones, bile acids, and sterols of vertebrates, as well as the moulting hormones of insects and many other physiologically active substances of animals and plants. Among the synthetic steroids of therapeutic value are a large number of anti-inflammatory agents, anabolic (growth-stimulating) agents, and oral contraceptives.
Different categories of steroids are frequently distinguished from each other by names that relate to their biological source—e.g., phytosterols (found in plants), adrenal steroids, and bile acids—or to some important physiological function—e.g., progesterones (promoting gestation), androgens (favouring development of masculine characteristics), and cardiotonic steroids (facilitating proper heart function).Steroids vary from one another in the nature of attached groups, the position of the groups, and the configuration of the steroid nucleus (or gonane). Small modifications in the molecular structures of steroids can produce remarkable differences in their biological activities.

Transport of Steroid Hormones:

Steroid hormones are transported through the blood by being bound to carrier proteins—serum proteins that bind them and increase the hormones' solubility in water. Some examples are sex hormone-binding globulin (SHBG), corticosteroid-binding globulin, and albumin. Most studies say that hormones can only affect cells when they are not bound by serum proteins. In order to be active, steroid hormones must free themselves from their blood-solubilizing proteins and either bind to extracellular receptors, or passively cross the cell membrane and bind to nuclear receptors. This idea is known as the free hormone hypothesis. This idea is shown in Figure 1 to the right.

One study has found that these steroid-carrier complexes are bound by megalin, a membrane receptor, and are then taken into cells via endocytosis. One possible pathway is that once inside the cell these complexes are taken to the lysosome, where the carrier protein is degraded and the steroid hormone is released into the cytoplasm of the target cell. The hormone then follows a genomic pathway of action. This process is shown in Figure 2 to the right. The role of endocytosis in steroid hormone transport is not well understood and is under further investigation.

In order for steroid hormones to cross the lipid bilayer of cells they must overcome energetic barriers that would prevent their entering or exiting the membrane. Gibbs free energy is an important concept here. These hormones, which are all derived from cholesterol, have hydrophilic functional groups at either end and hydrophobic carbon backbones. When steroid hormones are entering membranes free energy barriers exist when the functional groups are entering the hydrophobic interior of membrane, but it is energetically favorable for the hydrophobic core of these hormones to enter lipid bilayers. These energy barriers and wells are reversed for hormones exiting membranes. Steroid hormones easily enter and exit the membrane at physiologic conditions. They have been shown experimentally to cross membranes near a rate of 20 μm/s, depending on the hormone.

Though it is energetically more favorable for hormones to be in the membrane than in the ECF or ICF, they do in fact leave the membrane once they have entered it. This is an important consideration because cholesterol—the precursor to all steroid hormones—does not leave the membrane once it has

embedded itself inside. The difference between cholesterol and these hormones is that cholesterol is in a much larger negative Gibb's free energy well once inside the membrane, as compared to these hormones. This is because the aliphatic tail on cholesterol has a very favorable interaction with the interior of lipid bilayers.

Mechanisms of action and effects:

There are many different mechanisms through which steroid hormones affect their target cells. All of these different pathways can be classified as having either a genomic effect, or a non-genomic effect. Genomic pathways are slow and result in altering transcription levels of certain proteins in the cell; non-genomic pathways are much faster.

a. Genomic pathways

The first identified mechanisms of steroid hormone action were the genomic effects. In this pathway, the free hormones first pass through the cell membrane because they are fat soluble. In the cytoplasm, the steroid may or may not undergo an enzyme-mediated alteration such as reduction, hydroxylation, or aromatization. Then the steroid binds to a specific steroid hormone receptor, also known as a nuclear receptor, which is a large metalloprotein. Upon steroid binding, many kinds of steroid receptors dimerize: two receptor subunits join together to form one functional DNA-binding unit that can enter the cell nucleus. Once in the nucleus, the steroid-receptor ligand complex binds to specific DNA sequences and induces transcription of its target genes.

b. Non-genomic pathways

Because non-genomic pathways include any mechanism that is not a genomic effect, there are various non-genomic pathways. However, all of these pathways are mediated by some type of steroid hormone receptor found at the plasma membrane. Ion channels, transporters, G-protein coupled receptors (GPCR), and membrane fluidity have all been shown to be affected by steroid hormones. Of these, GPCR linked proteins are the most common. For more information on these proteins and pathways, visit the steroid hormone receptor page.

Biosysnthesis of Cholesterol:

The main steps of the biosynthesis of cholesterol are diagrammatically represented in figure 5-22. The first reaction consists of the condensation of 2 molecules of acetyl-coA. It is the reverse of the reaction which takes place during the last turn of the helix in β-oxidation.

Then a third molecule of acetyl-coA binds to the acetoacetyl-coenzyme A thus formed which gives β-hydroxy-β-methyl-glutaryl coenzyme A (HMG coA). This binding of an acetyl-coenzyme A to a carbonyl group is similar to the reaction permitting the entry of acetyl-coenzyme A in the Krebs cycle by condensation on oxaloacetic acid.The reduction of the acid group (engaged in a thioester linkage) to alcohol, catalyzed by HMG coA reductase, gives mevalonic acid. It must be noted that all the carbon atoms of cholesterol originate from acetyl- coenzyme A.

A pyrophosphate group will then bind to the primary alcohol group of mevalonic acid. Mevalonyl-pyrophosphate will react with a third molecule of ATP; this reaction gives an unstable compound which decomposes spontaneously, losing the tertiary alcohol group and the free carbonyl group.An isoprene derivative with 5 carbon atoms is formed, isopentenyl-pyrophosphate which can be isomerized to dimethyl-allyl-pyrophosphale. The condensation of 2 fragments in C_5 gives geranyl-pyrophosphate (C_{10}), and after the binding of a third fragment in C_5, farnesyl-pyrophosphate (C_{15}) is obtained. The dimerization of the latter leads to squalene (C_{30}).

In vertebrates, the cyclization of squalene by squalene-oxidocyclase takes place by a series of reactions requiring molecular oxygen and a reducing coenzyme like NADPH, and leads to lanosterol. The passage from lanosterol to cholesterol lakes place through several parallel pathways. The most

important intermediates are desmosterol and 7-dehydrocholesterol, immediate precursors of cholesterol.The isoprene derivatives with 5 carbon atoms are the precursors of dolichols, of the side chain of ubiquinone and vitamin K, of the isopentenyl group of some tRNAs.

In vertebrates, the biosynthesis of cholesterol is microsomal. It is regulated by a feedback inhibition mechanism by a metabolite of cholesterol (most probably the 25 hydroxycholesterol) acting on the HMG coA reductase. This feedback inhibition is never total to always permit the synthesis of polyisoprenoids important for other metabolisms, like the dolichols.The liver is one of the principal sites of synthesis. Cholesterol is then carried to other organs in the form of lipoproteins. It enters the cells by binding of the lipoprotein to a specific receptor. In physiological conditions the exogenous input of hepatic cholesterol to various tissues is sufficient to inhibit endogenous synthesis in these tissues.

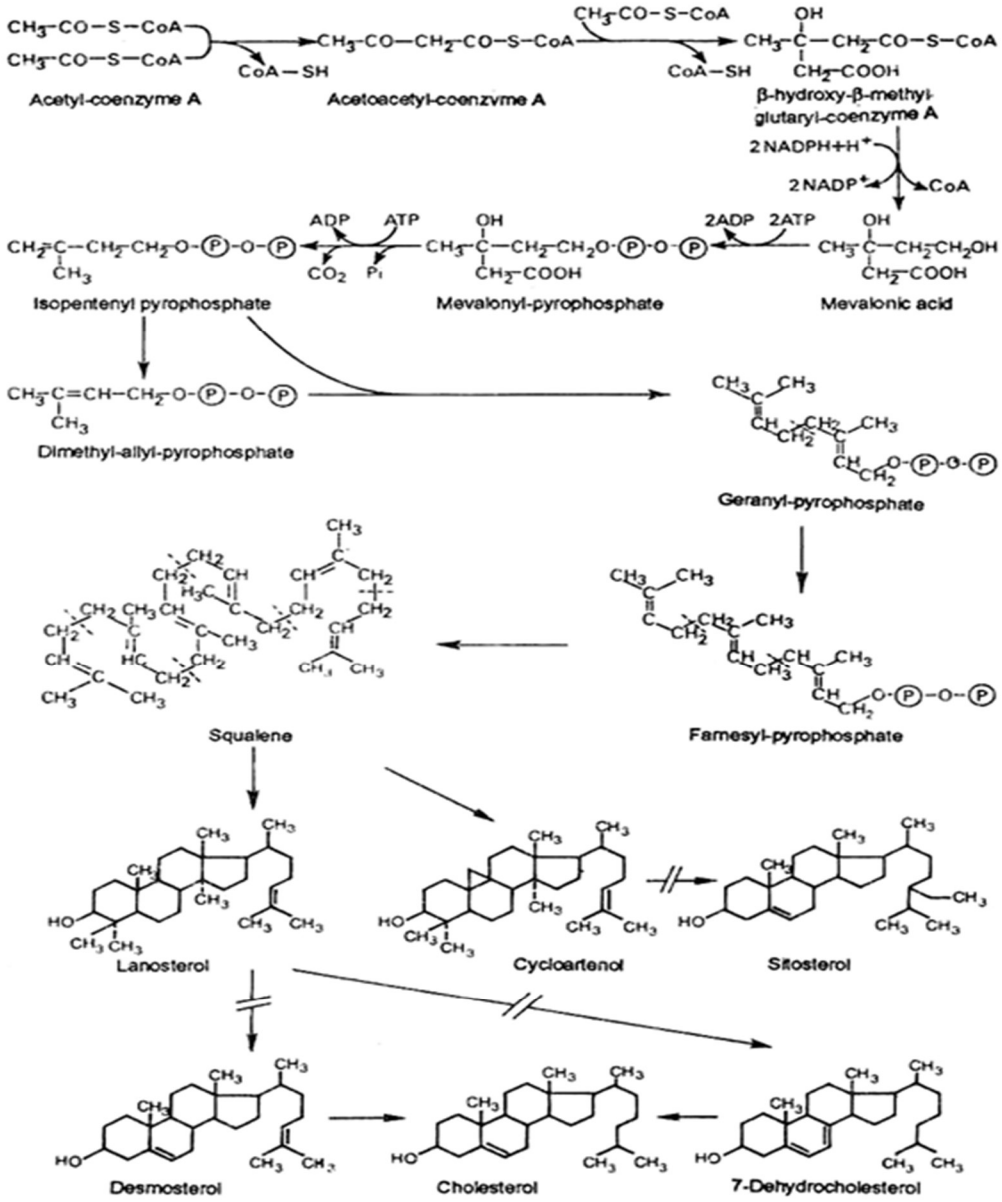


FIG. 5-22. — The principal steps of the biosynthesis of cholesterol.

Insects are capable of synthesizing squalene, but they cannot cyclize it. They use the sterols (animal or plant) present in their food and are capable of metabolizing them to cholesterol, precursor of hormonal derivatives like ecdysone.

Prokaryotes also synthesize squalene. Squalcne-hopene cyclase which carries out the cyclization is an enzyme which does not require oxygen.

Formation of Other Steroids:

Cholesterol is the starting point of the synthesis of various steroids:

1. Progesterone is secreted by the corpus luteum, the placenta and the cortex of the adrenal gland, and acts mainly in the uterus to permit implantation and gestation;

2. Aldosterone, a hormone secreted by the adrenal cortex, which permits the reabsorption of sodium (and secondarily of chlorine and water) in the kidney, hence its name, mineralocorticosteroid;

3. Cortisol and cortisone, also secreted by the adrenal cortex, sometimes called glucocorticosteroids, because they stimulate protein catabolism and neoglucogenesis in the liver (they are therefore hyperglycemic). They also act on conjunctive and limphoid tissues by depressing membrane permeability and opposing the inflammatory processes (which explains their use in therapeutics).

The synthesis of cortisol (and therefore of cortisone) is stimulated by the corticotropic hormone of the anterior lobe of the pituitary gland or ACTH (Adreno Cortico Tropic Hormone):

1. Testosterone, secreted mainly by the testicles, is responsible for the various male sexual characters;

2. Estrogenic hormones (estradiol and estrone), responsible for the various female sexual characters, synthesized mainly in the ovary and placenta, and characterized — from the structural point of view — by a phenolic ring.

It may be observed that it is relatively easy to pass (in few steps) from progesterone to other hormones having very different physiological properties; in other words, in this family of steroid hormones, small structures modifications correspond to large differences in biological activity.

We mentioned that the synthesis of Cortisol and cortisone by the adrenal glands is influenced by ACTH, a hormone of anterior lobe of the pituitary gland; we must indicate that the secretory activities of ovaries and testicles are also controlled by the hormones of the anterior lobe of the pituitary gland called gonadotropins, like FSH (Follicule Stimulating Hormone) and LH (Luteinizing Hormone), for example.

Moreover, this anterior lobe also secretes other stimulines, like the growth hormone or somatotropic hormone, and thyrotropic hormone (TSH), which stimulates the synthesis of thyroid hormones by the thyroid gland.

Chemists sometimes classify the steroid hormones according to the number of carbon atoms contained in their molecules and thus distinguish:

1. C_{18} hormones (estradiol, estrone);
2. C_{19} hormones (testosterone);

3. C_{21} hormones (progesterone and most of the hormones secreted by the cortical part of adrenal glands, the mineralocorticoids like aldosterone as well as the glucocorticoids like Cortisol and cortisone).
Vitamins D are formed by the opening of the B cycle due to ultraviolet light, either from ergosterol (vitamin D_2), or from 7-dehydro-cholesterol (which gives vitamin D_3).

Mode of Steroid Hormone Action through Intracellular Receptors :

Steroid hormones are lipid-soluble and easily pass through the cell membrane of a target cell into the cytoplasm. In the cytoplasm they bind to specific intracellular receptors (proteins) to form a hormone receptor complex that enters the nucleus.

In the nucleus, hormones which interact with intracellular receptors (e.g., steroid hormones, iodothyromines, etc.) mostly regulate gene expression or chromosome function by the interaction of hormone-receptor complex with the genome.

Biochemical actions result in physiological and developmental effects (tissue growth and differentiation, etc.). In-fact the hormone receptor complex binds to a specific regulatory site on the chromosome and activates certain genes (DNA).

The activated gene transcribes mRNA which directs the synthesis of proteins and usually enzymes in the cytoplasm. The enzymes promote the metabolic reactions in the cell. The actions of lipid soluble hormones are slower and last longer than the action of water- soluble hormones.

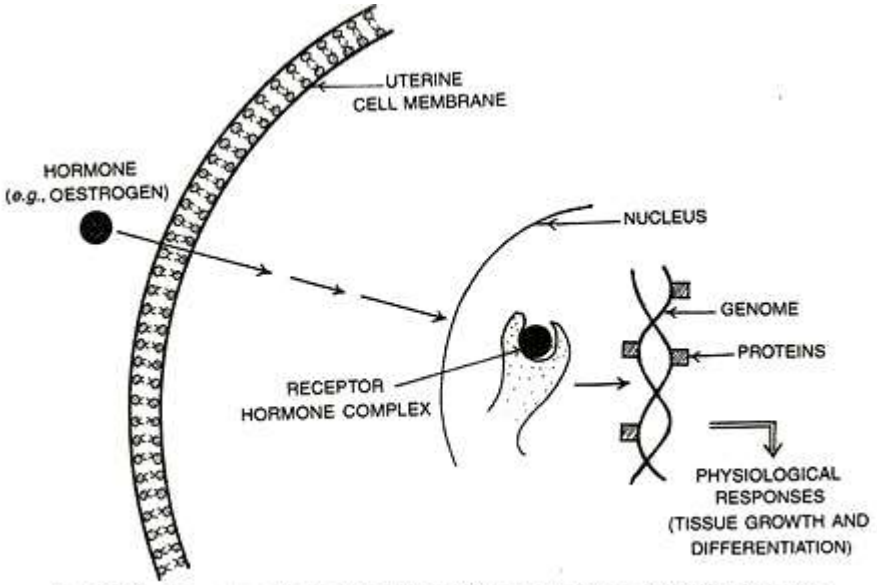


Fig. 22.20. Diagrammatic representation of the mechanism of Steroid hormone.

Thyroid Hormones:

Location and Structure of Thyroid Gland:
The thyroid gland is the largest endocrine gland located anterior to the thyroid cartilage of the larynx in the neck. The gland is well supplied with blood vessels. It is bilobed organ. The two lobes are connected by a narrow structure called the isthmus. The microscopic structure of the thyroid gland shows thyroid follicles composed of cubical epithelium and filled with a homogenous material called colloid.

Small amount of loose connective tissue forms stroma of the gland. Besides containing blood capillaries, the stroma contains small clusters of specialized Para follicular cells or 'C' cells. The thyroid gland is the only gland that stores hormones in large quantities for about two months.

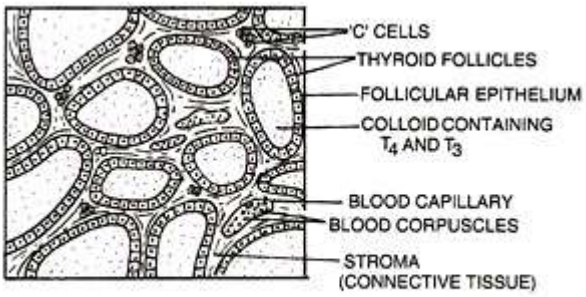


Fig. 22.2. T.S. Thyroid gland.

Hormones of Thyroid Gland:

The thyroid gland secretes three hormones. Thyroxine (tetraiodothyronine or T_4), and triiodothyronine or T_3 are secreted by the thyroid follicular cells. Thyrocalcitonin is secreted by the C-cells of the thyroid gland. This gland is stimulated to secrete its hormones by thyroid stimulating hormone (also called thyrotropin) secreted by the anterior lobe of pituitary gland.

(I) Thyroxine (T_4) and Triiodothyronine (T_3):

T_4 and T_3 contain four and three atoms of iodine respectively, therefore, they are named so. T_3 is secreted in smaller amounts but it is more active and several times more potent than T_4. T_4 is converted to T_3 by removal of one iodine in the liver, kidneys and some other tissues. Since both T_4 and T_3 have similar effects on the target cells, they are generally considered together under the name, thyroid hormone (TH).

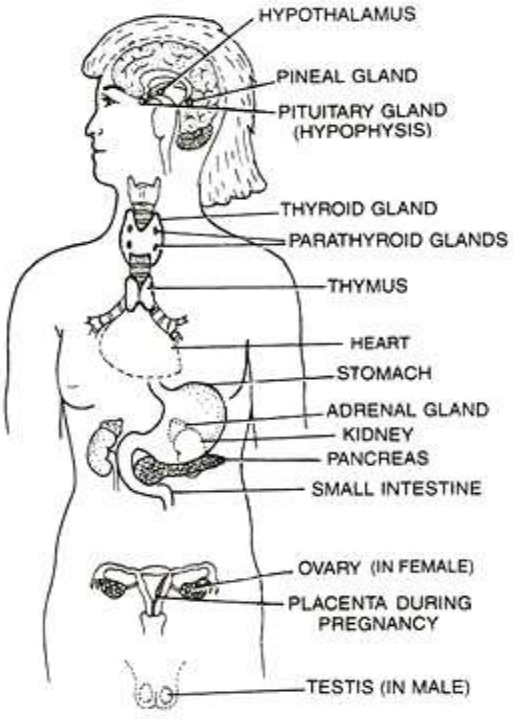


Fig. 22.3. Human endocrine glands.

The thyroid gland is the only gland that stores its hormones in large quantity. T_4 and T_3 are synthesised by attaching iodine to tyrosine amino acid.

The steps in the biosynthesis of the hormone are (Fig. 6.33):

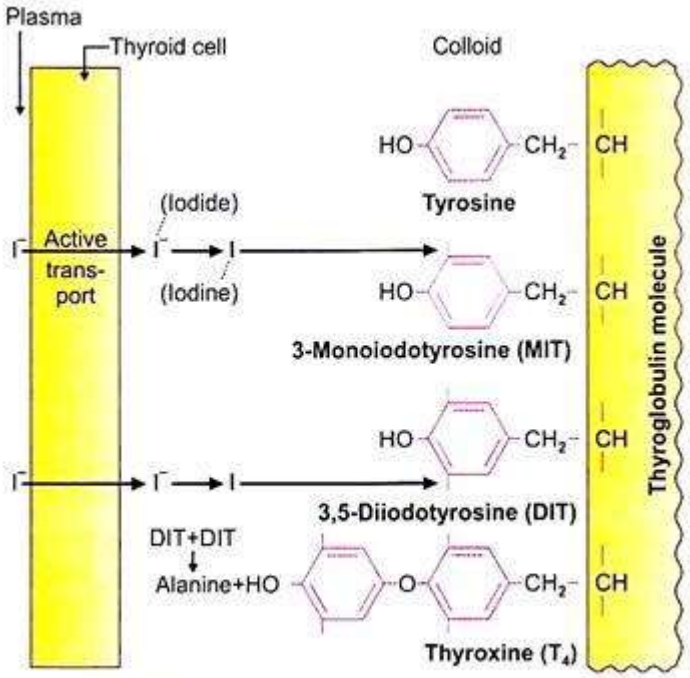


Fig. 6.33: Steps in the biosynthesis of thyroxine

i. Iodide trapping that is the uptake of iodide by the follicular cells from the plasma against the electrochemical gradient. The hormone TSH secreted by the anterior pituitary gland affects this step. Substances, like thiocyanate, pertechnetate and perchlorate that are examples of antithyroid drugs can inhibit iodide trapping.

ii. Oxidation of iodine:

Occurs inside the follicular cells by the action of the enzyme peroxidase. Drugs like thiouracil and carbimazole can inhibit this step and act as antithyroid drugs.

iii. Organification:

Iodine gets incorporated to tyrosine amino acid present in the colloid and leads to the formation of MIT (Monoiodotyrosine). On further iodination of MIT, there is formation of DIT (Di- iodotyrosine).

iv. Coupling:

Coupling of 2 DIT will lead to the formation of T_4 and 1 MIT with 1 DIT will results in T_3. After the synthesis, the hormone with thyroglobulin is stored in the colloid.There are many substances which have the ability to decrease the amount of thyroxin secreted by the gland. These drugs will be of choice when there is a necessity to decrease the amount of thyroxine secretion in certain pathological situations.

Steps involved in hormonopoiesis of thyroxine:

1. Iodide trapping (active process)

2. Conversion of iodide to molecular iodine. Peroxidase is the enzyme involved.

3. Organification of tyrosine to form MIT and DIT—iodinase.

4. Oxidative coupling of

MIT + DIT—to form T_3
DIT + DIT—to form T_4

5. Proteolytic separation of T_3 and T_4 from thyroglobulin— deiodinase

Table 6.6: Transport of thyroxine in plasma

Proteins available	*Quantity of proteins (mg/dl)*	*Affinity*	*Transported bound %*	
			T_4	T_3
TBG	2	+++++	67	46
TBPA	15	++	20	1
Albumin	3500	+	13	53

At the time of release of the hormones into circulation, the acinar cells will engulf the thyroglobulin along with the hormones by endocytosis.In the cells, the hormone will be separated by proteolysis and released into the circulation and thyroglobulin will be retained for further use.Most of the hormone in circulation is in protein bound form along with thyroid binding globulin (TBG), albumin (TBA), thyroid binding pre-albumin (TBPA) (Table 6.6).

Protein Bound Iodine (PBI) in Blood:

The term PBI in blood represents iodine present in thyroid hormones. The PBI values for normal adults are 3.5-7.5 mg/100 ml of plasma. PBI is a reliable measure of thyroxine content of plasma.

The values for PBI in hypo- and hyperthyroidism are given below:

Myxoedema (Hypothyroidism)	0.2-2.5 mg/100 ml
Grave's disease (Hyperthyroidism)	8-18 mg/100 ml

The functions of thyroxine (T_4) and tri-iodothyronine (T_3) are as follows.

(a) They regulate the metabolic rate of the body and thus maintain basal metabolic rate (BMR).

(b) They stimulate protein synthesis and, therefore, promote growth of the body tissues.

(c) They regulate the development of mental faculties.

(d) As they increase heat production, thus they maintain body temperature.

(e) They help in metamorphosis of tadpole into adult frog. If thyroid gland of the tadpole (larva) is removed, the larva fails to change into an adult.

(f) They increase action of neurotransmitters like adrenaline and noradrenaline.

(II) Thyrocalcitonin (TCT):

It is secreted when calcium level is high in the blood. It then lowers the calcium level by suppressing release of calcium ions from the bones. Thus calciton has an action opposite to that of the parathyroid hormone on calcium metabolism. Calcitonin is a peptide which contains 32 amino acids.

(f) They increase action of neurotransmitters like adrenaline and noradrenaline.

(III) Thyrocalcitonin (TCT):

It is secreted when calcium level is high in the blood. It then lowers the calcium level by suppressing release of calcium ions from the bones. Thus calciton has an action opposite to that of the parathyroid hormone on calcium metabolism. Calcitonin is a peptide which contains 32 amino acids.

Regulation of Thyroid Hormone:

It is brought about by the negative feedback mechanism. There is involvement of hypothalamo-pituitary-thyroid axis (Fig. 6.35).

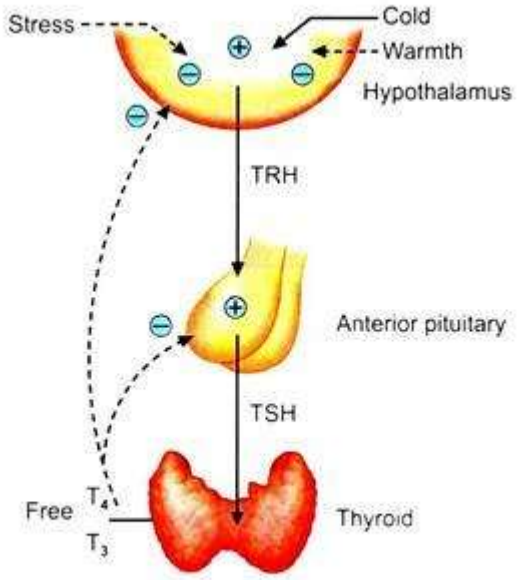


Fig. 6.35: Regulation of secretion of thyroxine (by negative feedback mechanism)

Increase in free form of hormone in circulation acts on hypothalamus and anterior pituitary gland. Acting on hypothalamus, it decreases the secretion of thyrotropin-releasing hormone (TRF/TRH) and this acts on anterior pituitary decreases secretion of TSH.Net effect will be decreased TSH from anterior pituitary gland. This decreases the secretion of thyroid hormones from the gland.

Many of the other chemical influences acting on TRH-TSH-Thyroxine (hypothalamo-pituitary-thyroid axis) secretions have been shown in Table 6.7.

Table 6.7: Thyroid hormone feedback

Hypothalamus	*Stimulatory*	*Inhibitory*
Decreased TRH	Alpha adrenergic agonists	Alpha adrenergic blockers
		Tumors
Anterior pituitary	*TRH*	*Somatostatin*
Decreased TSH	Estrogen	Dopamine
		Glucocorticoids
		Chronic illness
Thyroid gland	*TSH*	*TSH receptor blocking antibody*
Decreased T_3 and T_4	TSH receptor stimulating antibody	Iodine, lithium

Alteration in the temperature can directly act on the hypothalamus to alter the secretion of the hormone.

Disorders related to thyroid Hormones:

(A) Hyperthyroidism (Hyper secretion of thyroid hormone).

a. Exophthalmic goitre or Graves' disease or Basedow's disease or Parry's disease:

It is a thyroid enlargement (goitre) in which the thyroid secretes excessive amount of thyroid hormone. It is characterised by exophthalmia (protrusion of eye balls because of fluid accumulation behind them), loss of weight, slightly rise in the body temperature, excitability, rapid heartbeat, nervousness and restlessness.

Fig. 22.4. Graves' disease.

(B) Hypothyroidism (Hypo secretion of thyroid hormone):

(a) Cretinism:

This disorder is caused by deficiency of thyroid hormone in infants. A cretin has slow body growth and mental development of reduced metabolic rate.

Other symptoms of this disorder are slow heart beat, lower blood pressure, decrease in temperature, stunted growth, pot-belly, pigeon chest and protruding tongue and retarded sexual development. This disease can be treated by an early administration of thyroid hormones.

Fig. 22.5. Cretinism.

(b) Myxoedema or Gull's disease:

It is caused by deficiency of thyroid hormone in adults. This disease is characterized by puffy appearance due to accumulation of fat in the subcutaneous tissue because of low metabolic rate. The patient lacks alertness, intelligence and initiative. He also suffers from slow heart beat, low body temperature and regarded sexual development. This disease can be treated by administration of thyroid hormones.

Fig. 22.6. Myxoedema.

(c) Simple Goitre:

It is caused by deficiency of iodine in diet because iodine is needed for the synthesis of thyroid hormone. It causes thyroid enlargement. It may lead to cretinism or myxoedema. This disease is common in hilly areas. Addition of iodine to the table salt prevents this disease.

Fig. 22.7. Simple goitre.

(d) Hashimoto's disease:

In this disease all the aspects of thyroid function are impaired. It is an autoimmune disease in which the thyroid gland is destroyed by autoimmunity.

Probable Questions:

1. Write down the structure of Growth hormone.
2. What are the main functions of GH?
3. How hGH synthesis is regulated?
4. What is the mechanism of action of hGH?
5. state the effect of high and low secretion of hGH?
6. Write down the structure of insulin.
7. How biosynthesis is occurred of Insulin.
8. State the factors which stimulates insulin secretion.
9. State the factors which inhibits insulin secretion.
10. What are the functions of Insulin.
11. Describe the pathophysiology of insulin.
12. Describe the steps of cholesterol biosynthesis.
13. How steroid hormones are transported?
14. What are the mechanism of action of steroid hormones?
15. How secretion of steroid hormones are regulated?
16. Briefly describe the location and structure of thyroid gland.
17. How T3 and T4 hormones are synthesised in thyroid gland?
18. How thyroid hormone secretions are regulated?
19. What are the effects of thyroxine hypo secretion?
20. What are the effects of thyroxine hyper secretion?

Suggested Readings:

1. General Endocrinology. Turner and Bagnara. Sixth Edition.
2. Williams Textbook of Endocrinology. Tenth Edition.
3. Introduction to Endocrinology. Chandra S Negi. Second Edition
4. Endocrinology. Hadley and Levine. Sixth Edition

Unit-X

Physiological role of hormones: hormonal regulation of mineral metabolism and fluid volume

Objective: In this unit you will learn about role of hormones in mineral metabolism and fluid volume regulation.

Water Metabolism:

Distribution of water in the body:

Water is the major constituent of human body. The average body water is 50-70% of the body weight. Females have little less water than males.

1. The water content of intracellular fluid is 50% of total body weight.

2. The water content of extracellular fluid is 20% of the body weight, which is distributed as follows:

Plasma	4.5%
Interstitial and lymph fluid	8%
Dense connective tissue, cartilage and bone	6%
Transcellular fluids(found in salivary glands, liver, pancreas, thyroid gland, gonads, skin, mucous membranes of the respiratory and gastrointestinal tracts, kidneys, fluid spaces in eye, CSF etc)	1.5%

Factors influencing the distribution of body water:
The distribution of water is continuously changing. Osmotic forces are the principal factors for controlling the amount of fluid in various compartments of the body. These are maintained by the solutes of the body. Solutes are of three types.

1. Organic molecules of small molecular size (glucose, urea, amino acids etc.):
Since these diffuse freely across the cell membrane, they are not important in the distribution of water. If they are present in large quantities, they can help retaining water.

2. Organic substances of large molecular size {proteins):
These substances can throw effect in the transport of fluids from one compartment to the other.

3. The inorganic electrolytes:
These inorganic electrolytes are the most important both in the distribution and in the retention of body water.

Intake and Loss of Body Water:

A. Water intake:

Water is supplied to the body by the following processes:

1. Water taken orally.

2. Along with food.

3. Oxidation of food stuffs i.e. fats, proteins and carbohydrates yield water after combustion.

B. Water loss:

Water is lost from the body by 4 routes

1. Evaporation from lungs.

2. Kidneys eliminate water as urine.

3. The intestines excrete in the feces.

4. Perspiration.

C. Additional water loss in diseases:

1. Water loss is more in diarrhea and vomiting. These losses can be fatal in infants.

2. In kidney disease, renal water loss is more.

3. In fever, insensible losses may rise much higher than normal.

4. Patients in high environmental temperatures sustain extremely high external water loss.

Water Balance:

An equilibrium persists between the intake and output of water in the body. In addition to other factors, certain hormones such as ADH, vasopressin, oxytocin and aldosterone influence the regulatory mechanism.

Balance sheet of water

Water intake (ml/day)		Water loss (ml/day)	
Drinks	1350	Urine	1500
Solid food	900	Lungs	500
Oxidation of food	450	Skin	600
		Feces	100
TOTAL	2700	TOTAL	2700

There is a continuous excretion of water in the form of digestive juices from the body into the alimentary canal. This water (except 100 ml) is reabsorbed with the water of the food and drinks. The amount of this internal secretion is 7 to 10 liters/day.

Physiological Functions of Water:

1. Specific heat:

Heat is required to raise the temperature of 1 gm. of water through one degree Celsius is more than for almost any other solid or liquid. The high specific heat of water helps in minimizing the rise in body temperature due to the heat emitted out of chemical reactions.

2. Latent heat of evaporation:

Water has the highest latent heat of evaporation than any other liquid. A certain amount of water can cause maximum cooling by evaporation, so that body temperature does not rise.

3. Solvent power:

Water forms true solutions as well as colloidal solutions. Even water insoluble substances are made water soluble by the hydrotropic action. Therefore, it is the most suitable solvent for cellular components; water thus brings various substances in contact for chemical reactions to proceed.

4. Dielectric constant:

Oppositely charge particles can coexist in water. Therefore, it is a good ionizing medium. This stimulates the chemical reactions.

5. Catalytic action:

A large number of chemical reactions in the body are accelerated by water due to its ionizing power. All chemical reactions in the body proceed in presence of water only.

6. Lubricating action:

Water acts as a lubricant in the body to prevent friction in joints, pleura, conjunctiva and peritoneum.

Regulation of Passage of Water:

1. If capillary pressure is increased, more water will flow into the tissues.

2. A fall in blood pressure helps in passage of water form the tissues to the blood.

3. If the plasma proteins are decreased, water will flow into the tissues.

4. Dilution of blood by excessive ingestion of water can lower the osmotic pressure of the plasma proteins and thus may increase capillary pressure.

Dehydration:

When the loss of water exceeds the intake, the body's water content is reduced. This means that the body is in negative water balance and the condition is known as dehydration.

Causes:

1. Primary dehydration:
(a) Deprivation of water during desert travel, extreme weakness and mental patients refraining to drinking water causes dehydration. Occurs more quickly in fever and in high environmental temperatures.

(b) Excessive water loss due to vomiting, prolonged diarrhoea, excretion of large quantities of urine and sweat. In water depletion, the concentration of extracellular fluid increases. Water is drawn from the cells and both extracellular and intracellular compartments shrink. Extreme thirst results; individual complains of hot and dry body. The tongue becomes dry.

2. Secondary dehydration:
Concentration of electrolytes of the body fluids is maintained constant through the elimination or retention of water. The reduction or increase in the total electrolytes which affects, chiefly the basic radical Na (extracellular) or K (intracellular) and the acid radicals HCO_3 and CI is accompanied by a corresponding increase or decrease in the volume of body water. This causes intracellular edema, slowing of circulation and impairment of urinal function. The individual becomes weak.

3. Dehydration due to injection of hypertonic solution:
When a highly concentrated sugar or salt solution is injected into the body, the osmotic pressure of blood will increase. This results in the flow of fluid from the tissues into the blood until equilibrium sets in. Consequently, the blood volume increases. This increased blood volume soon returns to normal by the loss of excess material through urination. This causes a net loss of body water producing dehydration.

Effects of dehydration:
1. Loss of weight due to the reduction in tissue water.

2. Disturbances in acid-base balance.

3. Rise in the non-protein nitrogen of blood.

4. Rise in the plasma protein concentration and of chloride.

5. Rise in body temperature due to reduction in circulating fluid.

6. Increased pulse rate and reduced cardiac output.

7. Dryness, wrinkling and looseness of skin.

8. Exhaustion and collapse.

Correction of dehydration:

1. Ordinary NaCl solution may be given parenterally to repair the losses.

2. In case of excretion of fluid high in Na and HCO_3 resulting in fluid and electrolyte loss, a mixture of 2/3 isotonic saline and 1/3 Na lactate should be administered intravenously.
3. Dehydration in diabetes mellitus, Addison's disease, uremia, extensive burns and shock cannot be corrected by the above methods.

Water intoxication:
Caused by excessive water retention due to renal failure, hyper secretion of ADH, excessive administration of fluids parenterally

Symptoms:
Headache, nausea and muscular weakness

Hormonal Control of water volume:

While the kidneys operate to maintain osmotic balance and blood pressure in the body, they also act in concert with hormones. Hormones are small molecules that act as messengers within the body. Hormones are typically secreted from one cell and travel in the bloodstream to affect a target cell in another portion of the body. Different regions of the nephron bear specialized cells that have receptors to respond to chemical messengers and hormones. Table 22.1 summarizes the hormones that control the osmoregulatory functions.

Table 22.1.Hormones That Affect Osmoregulation

Hormone	Where produced	Function
Epinephrine and Norepinephrine	Adrenal medulla	Can decrease kidney function temporarily by vasoconstriction
Renin	Kidney nephrons	Increases blood pressure by acting on angiotensinogen
Angiotensin	Liver	Angiotensin II affects multiple processes and increases blood pressure
Aldosterone	Adrenal cortex	Prevents loss of sodium and water
Anti-diuretic hormone (vasopressin)	Hypothalamus (stored in the posterior pituitary)	Prevents water loss
Atrial natriuretic peptide	Heart atrium	Decreases blood pressure by acting as a vasodilator and increasing glomerular filtration rate; decreases sodium reabsorption in kidneys

Epinephrine and Norepinephrine

Epinephrine and norepinephrine are released by the adrenal medulla and nervous system respectively. They are the flight/fight hormones that are released when the body is under extreme stress. During stress, much of the body's energy is used to combat imminent danger. Kidney function is halted temporarily by epinephrine and norepinephrine. These hormones function by acting directly on the smooth muscles of blood vessels to constrict them. Once the afferent arterioles are constricted, blood flow into the nephrons stops. These hormones go one step further and trigger the **renin-angiotensin-aldosterone** system.

Renin-Angiotensin-Aldosterone

The renin-angiotensin-aldosterone system, illustrated in Figure 22.15 proceeds through several steps to produce **angiotensin II**, which acts to stabilize blood pressure and volume. Renin (secreted by a part of the juxtaglomerular complex) is produced by the granular cells of the afferent and efferent arterioles. Thus, the kidneys control blood pressure and volume directly. Renin acts on angiotensinogen, which is made in the liver and converts it to **angiotensin I**. **Angiotensin converting enzyme (ACE)** converts angiotensin I to angiotensin II. Angiotensin II raises blood pressure by constricting blood vessels. It also triggers the release of the mineralocorticoid aldosterone from the adrenal cortex, which in turn stimulates the renal tubules to reabsorb more sodium. Angiotensin II also triggers the release of **anti-diuretic hormone (ADH)** from the hypothalamus, leading to water retention in the kidneys. It acts directly on the nephrons and decreases glomerular filtration rate. Medically, blood pressure can be controlled by drugs that inhibit ACE (called ACE inhibitors).

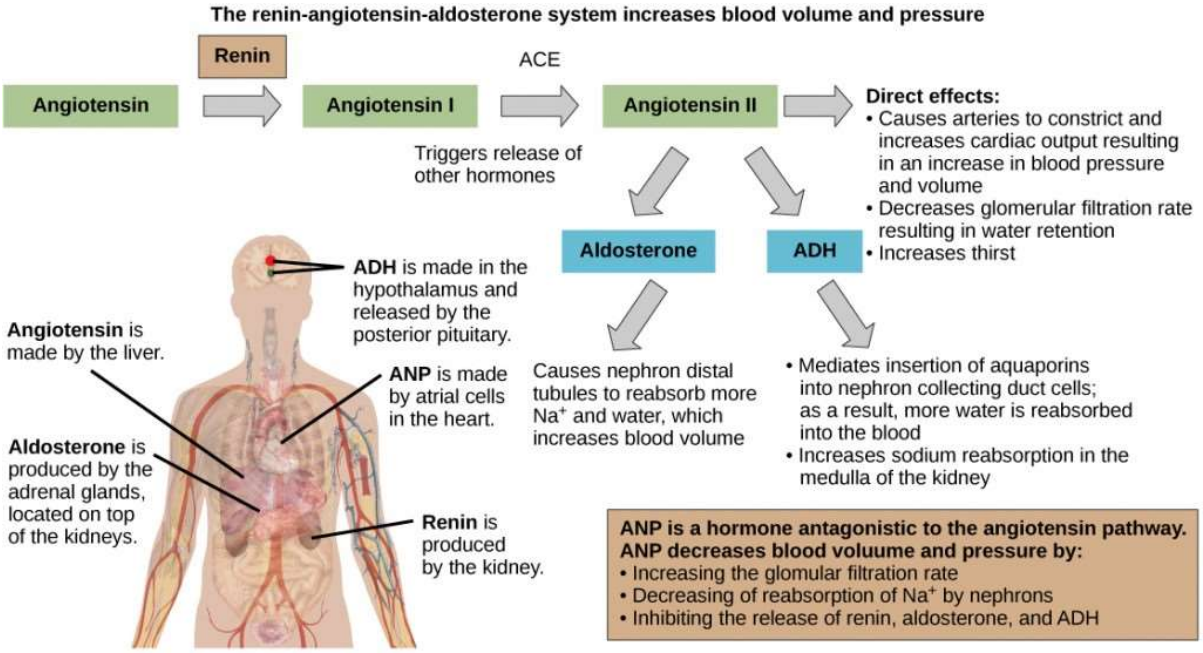


Figure 22.15. The renin-angiotensin-aldosterone system increases blood pressure and volume. The hormone ANP has antagonistic effects. (credit: modification of work by Mikael Häggström)

Mineralocorticoids

Mineralocorticoids are hormones synthesized by the adrenal cortex that affect osmotic balance. Aldosterone is a mineralocorticoid that regulates sodium levels in the blood. Almost all of the sodium in the blood is reclaimed by the renal tubules under the influence of aldosterone. Because sodium is

always reabsorbed by active transport and water follows sodium to maintain osmotic balance, aldosterone manages not only sodium levels but also the water levels in body fluids. In contrast, the aldosterone also stimulates potassium secretion concurrently with sodium reabsorption. In contrast, absence of aldosterone means that no sodium gets reabsorbed in the renal tubules and all of it gets excreted in the urine. In addition, the daily dietary potassium load is not secreted and the retention of K^+ can cause a dangerous increase in plasma K^+ concentration. Patients who have Addison's disease have a failing adrenal cortex and cannot produce aldosterone. They lose sodium in their urine constantly, and if the supply is not replenished, the consequences can be fatal.

Antidiurectic Hormone:

As previously discussed, antidiuretic hormone or ADH (also called **vasopressin**), as the name suggests, helps the body conserve water when body fluid volume, especially that of blood, is low. It is formed by the hypothalamus and is stored and released from the posterior pituitary. It acts by inserting aquaporins in the collecting ducts and promotes reabsorption of water. ADH also acts as a vasoconstrictor and increases blood pressure during hemorrhaging.

Atrial Natriuretic Peptide Hormone:

The atrial natriuretic peptide (ANP) lowers blood pressure by acting as a **vasodilator**. It is released by cells in the atrium of the heart in response to high blood pressure and in patients with sleep apnea. ANP affects salt release, and because water passively follows salt to maintain osmotic balance, it also has a diuretic effect. ANP also prevents sodium reabsorption by the renal tubules, decreasing water reabsorption (thus acting as a diuretic) and lowering blood pressure. Its actions suppress the actions of aldosterone, ADH, and renin.

Mineral Metabolism

Living beings have organic and inorganic types of chemical constituents. The organic constituents i.e. proteins, carbohydrates, fats etc. are made up of C, H, O and N. The inorganic constituents described as 'minerals' comprise of the elements present in the body other than C, H, O and N. Although they constitute a relatively small amount of the total body tissues, they are essential for many vital processes.

There are 31 elements present in the body.

They are divided into two classes:

(1) Essential elements and

(2) Non-essential elements.

Essential elements:

Those which are essential to maintain the normal living state of a tissue.

They are again divided into two sub groups:

Macro elements:
They are required to be present in the diet, more than 1 mg.

Ex. Ca, P, Mg, Na, K, CI and S.

Micro elements:
They are 8 in number and utilized in trace quantities (in microgram or Nano-gram). Hence they are called trace elements. These are Fe, Cu, Zn, Co, Mo, F, I and Mn.

Non-essential elements:
They are 8 in number. They are present in tissues but their functions if any are not clearly defined. They include Al, B, Se, Cr, Br, As, Ti and Pb. Four additional elements, Ni, Tin, Vanadium and Silicon have been suggested as essential trace elements in nutrition but their implications for human nutrition are unknown.

The mineral elements present in the body are supplied in the diet. In poor diets consumed by a large majority of people, calcium and iron deficiency occur commonly. Iodine deficiency occurs in people living in certain hilly tracts, where the soil and water are deficient in iodine. In tropical countries, addition of sodium chloride in the diet is of great importance, because of the loss of NaCl in sweat. The deficiencies of other minerals do not occur normally in average diets.

i. Sodium, potassium and chlorine are involved mainly in the maintenance of acid-base balance and osmotic control of water metabolism.

ii. Calcium, phosphorus and magnesium are constituents of bone and teeth.

iii. Phosphorus is the constituent of body cells of the tissues, such as muscle, liver etc.

iv. Sulphur is present in cysteine, methionine, thiamine, biotin, lipoic acid and coenzyme A.

I. Calcium:

Source:
Milk (0.2 gm./100 ml) and cheese are important dietary sources. Other sources-are egg yolk, lentils, nuts, cabbage, cauliflower and asparagus, etc.

Requirement:
(1) Men and women after 18 years of age require 800 mg/day.

(2) During lactation and in pregnancy of 2^{nd} and 3^{rd} term 1.2 gm./day is required.
(3) Infants under 1 year require-360-540 mg/day.

(4) Children of 1-18 years need 800-1200 mg/day.

Absorption:

Calcium is taken in the diet as calcium phosphate, carbonate, tartarate and oxalate. Calcium is absorbed actively in the upper small intestine. The active process is regulated by 1,25 dihyrocholecalciferol, a metabolite of vitamin D which is produced in the kidney in response to low plasma Ca^{++} concentrations. Absorption of calcium by the intestine is never complete. Ca is absorbed by an active transport process occurring mainly in the upper small intestine.

Calcium absorption is influenced by the following factors:

1. Vitamin D promotes absorption of Ca.

2. Acidic pH favous calcium absorption because Ca salts (phosphate and carbonates) are quite soluble in acid solution arid are relatively insoluble in alkaline solutions. Hence an increase in acidophilic flora, e.g. lactobacilli is recommended to lower pH which favours the absorption of Calcium.

3. Organic acids, lactose and basic amino acids in the diet favour calcium absorption.

4. Higher levels of proteins in the diet help to increase the absorption of calcium. On a high protein diet, about 15% of the dietary calcium is absorbed, compared with 5% absorption on a low protein diet. Certain calcium salts are much more soluble in aqueous solution of amino acids than in water and thus absorption of calcium is increased in presence of amino acids.

5. If calcium: phosphorus ratio is much high, $Ca_3(PO_4)_2$ will be formed and absorption of calcium is reduced. The optimal ratio for both elements is about 1:1 (1:2 to 2:1) and with ratios outside these limits, absorption is decreased. This is because of formation of insoluble calcium phosphate.

6. When fat absorption is impaired much free fatty acids are formed due to hydrolysis. These fatty acids react with free calcium to form insoluble calcium soap and then Ca is lost in faeces.

7. Absorption of calcium is inhibited by a number of dietary factors that cause formation of insoluble calcium salts, i.e. phytate (cereal grain), oxalate, phosphate and iron, etc.

8. High concentration of Mg in the diet decreases absorption of Ca.

9. Presence of excess fibre in the diet interferes with the absorption of Ca.

10. Percentage of calcium absorption decreases as its intake increases.

11. Parathyroid hormone increases the intestinal absorption of calcium.

12. Adrenal glucocorticoids diminish intestinal transport of Ca.

13. After the age of 55 to 60 there is gradual diminution of intestinal transport of calcium. During menopause many women develop negative calcium-phosphorus balance leading to a type of osteoporosis. This is usually accompanied by pain and fractures. The negative balance of calcium and phosphorous are markedly improved by administration of estrogen or by androgens such as testosterone. A combination of estrogen and androgen is more effective.

14. Kidney threshold regulates the blood calcium level. In a normal adult any extra calcium absorbed from the intestine is readily excreted in the urine. In hypocalcaemia kidney threshold also becomes abnormal.

15. Excess of iron also dis-favours absorption of calcium and phosphorus, as ferric phosphate is highly insoluble. The net result is an upset in the Ca:P ratio.

16. Oxalate in certain foods precipitate calcium in the intestine as insoluble calcium oxalate. The phytic acids of food form insoluble salt with calcium and reduce calcium absorption.

17. Vitamin D increases calcium and phosphorus absorption from the intestine. Vitamin D promotes synthesis of specific calcium binding protein which participates in the active transport of calcium across the small intestinal mucosa. Lack of vitamin D, excess of phytates, low Ca/P ratio in diet, increased pH of upper intestine and malabsorption syndromes influence the amount of calcium absorption adversely.

Biological role:

Calcium is involved in the following biological processes:

1. Constituent of bones and teeth:
Calcium along with phosphate constitutes the mineral part of the skeleton and teeth where it is present to the extent of 99% of the total calcium present in the body. It is primarily in the form of crystals of hydroxyapatite, while some is in combination with phosphate (calcium phosphate) in the form of amorphous crystals.

2. Neuromuscular functions:
This involves excitability of nerve function, neural transmission, and contractility of cardiac and skeletal muscle. Normal concentration of calcium ions is required for the normal excitability of heart muscle.

3. Blood coagulation:
It plays a vital role in blood clotting process since it activates the enzymic conversion of prothrombin into thrombin and production of thromboplastin. The removal of calcium from the blood can prevent blood coagulation and because of this reason EDTA, oxalates, citrates are used as anticoagulant because these ions can precipitate calcium into the respective insoluble salts.

4. Membrane function:
It controls the permeability of all membranes and is often bound by lecithine in the membrane, i.e. it decreases the permeability and balances the opposite action of sodium and potassium capillary permeability. This involves transfer of inorganic ions across cell membranes and release of neurotransmitters at synaptic junction.

5. Selected enzymatic reactions:
Calcium acts as activator for number of en2ymes like ATPase, succinic dehydrogenase, lipase, etc. It also antagonizes the effect of magnesium on many enzymes. It releases cellular enzymes such as amylase from the parotid and increases the level of activity of intracellular enzymes such as—Isocitric dehydrogenase, phosphorylase and phosphofructokinase.

6. Regulation of secretion of certain peptide hormones:
Pituitary hormones, parathyroid hormone, calcitonin and vasopressin are regulated through calcium ionic concentration. Calcium along with zinc plays a vital role in release of insulin from pancreas. Calcium homeostasis: Normal blood values are 9.5-10.5 mg/100 ml. 35-45% of this is bound to proteins, mostly to the albumin fraction. In the extracellular fluid nearly all the calcium is in ionized form (55-65%). 0.5 (5-10%) mg is complexed to organic acids, phosphate, citrate, etc., while in renal failure, it may be complexed to other organic ions as well.

The skeleton is in a dynamic state of equilibrium to maintain calcium homeostasis. 4-8 gm. of calcium in bone is rapidly exchangeable with that in plasma and is present on the surface of the bone crystals—labile calcium storage pool. The remaining 99% of bone calcium is more firmly fixed in bone tissue and exchanges at a very slow rate.

Metabolism:

The blood cells contain very little amount of calcium, most of the blood calcium is therefore, in the plasma, where it is present in 3 fractions:

(1) Ionized about 2 mg/100 ml.

(2) Non-diffusible (protein bound) above 3.5 mg/100 ml.

(3) A small amount as calcium complex of citrate and phosphate.

All these forms of calcium in the serum are in equilibrium with one another. A decrease in ionized calcium in the serum causes tetany. This may be due to an increase in the pH of blood or lack of calcium because of poor absorption from the intestine, decreased dietary intake, increased renal excretion as in nephritis or parathyroid deficiency.

Factors influencing blood calcium level:

1. Parathyroid hormone:
In fasting condition or state there is no absorption from the intestine, the normal plasma Ca concentration is maintained by its rate of excretion and its mobilization from bones through the action of the parathyroid hormone.

2. Vitamin D:
It enhances absorption of Ca from the intestine and thus maintains normal Ca concentration.

3. Plasma proteins:
Half of the blood Ca (non-diffusible) is bound to plasma proteins and thus any decrease in these proteins will be accompanied by a decrease in the total calcium level.

4. Plasma phosphate:
A reciprocal relationship exists between the concentration of Ca and phosphate ions in plasma. The marked increase in serum phosphate causes a fall in serum calcium concentration.

5. Calcitonin:
An increase in the ionized Ca levels in the plasma is the stimulus for the production of calcitonin which then causes a deposition of Ca in bone.

Excretion:

Calcium is excreted in the urine, bile and digestive secretion. About 75% of dietary calcium is absorbed and rest is excreted as fecal calcium. Nearly 10 g of Ca is filtered by the renal glomeruli in 24 hours. But only 200 mg appear in the urine, which is in the ionic state as well as in the complexes with citrate and other organic anions. A very small amount of Ca is excreted into the intestine after absorption. About 15 mg of Ca is excreted in the sweat. Vigorous physical exercise increases the loss of Ca by way of sweat.

Disease state:

Calcium metabolism is highly influenced by parathyroid hormones. In hyperparathyroidism serum calcium rises (12-22 mg/100 ml) (hypercalcaemia), phosphatase activity is increased, urinary calcium is decreased and phosphorus rises in serum. The calcium, phosphorus ratio is important in

ossification. In the serum the product of calcium and phosphorus (in mg/100 ml) is normally 50 in children and may be below 30 during rickets.

The following are the diseases related to calcium in the body:

(a) Effects of parathyroid:

1. In hyperparathyroidism, the following changes occur:

(i) Hypercalcemia (12-22 mg/dl).

(ii) Decrease in serum phosphate.

(iii) Diminished renal tubular reabsorption of phosphate.

(iv) Increased phosphatase activity.

(v) Renal urinary Ca and phosphorus found from bone decalcification and dehydration.

(vi) Extra Ca and P are lost from soft tissue and bones by increased bone destroying activity.

2. In hypoparathyroidism, the following changes occur:

(i) The concentration of serum Ca may drop below 7 mg/100 ml.

(ii) Increased serum phosphate and decreased urinary excretion of calcium and phosphorus.

(iii) Normal or occasionally raised serum phosphatase activity.

(iv) Normal acid-base equilibrium.

(v) Probably increased bone density.

(b) Tetany:

Decreased ionized fraction of serum Ca causes tetany.

This may be due to:
1. Increase in the pH of blood.

2. Poor absorption of Ca from the intestine.

3. Decreased dietary intake of Ca.

4. Increased excretion of Ca as in hepatitis.

5. Parathyroid deficiency.

6. Increased retention of phosphorus as in renal tubular disease.

Symptoms:

Muscles lose tone and become flabby.

Affects the face, hands and feet.

(c) Rickets:

This is characterized by faulty calcification of bones in children showing serum phosphate values of 1 to 2 mg/100 ml.

This may be due to:

1. Vitamin D deficiency.

2. A deficiency of Ca and P in the diet or a combination of both.

3. Poor absorption of Ca from the intestine.

4. Parathyroid deficiency.

5. Increased alkaline phosphatase activity.

(d) Osteoporosis:

This disease occurs in adults due to the following causes:

1. Decalcification of bones as a result of Ca deficiency in the diet.

2. Hypoparathyroidism.

3. Low vitamin D content of the body.

Symptoms:

Fractures of the brittle bones occur even after minor accidents.

Pain due to fracture of vertebrae (may radiate round the trunk, to the buttocks or down the legs).

Renal rickets:

It is a hereditary disease. It is called familial hypophosphatemia rickets. Affected persons show severe rickets with hypophosphatemia.

The causes are:

(i) Defective transport of phosphate by the intestine and the renal tubules

(ii) Lowered serum phosphorus and hyperphosphaturia

(iii) Reduced intestinal absorption of calcium and phosphorus. Vitamin D in ordinary doses does not relieve the disease. Hence, it is referred to as vitamin D resistant rickets.

II. Phosphorus:

Source:
Phosphorus is present in nearly all foods therefore a dietary deficiency is not known to occur in man. Dairy products, cereals, egg yolk, meat, beans and nuts are usually rich sources. The daily average intake is 800-1000 mg and is about twice that of calcium.

Absorption:
Like calcium, phosphorus is also absorbed by upper small intestine and factors influencing the absorption are also similar. The normal range for plasma inorganic phosphorus is 3.0-4.5 mg/dl. In children values are higher (5-6 mg/dl) and remain so up-till puberty.

Distribution:
Phosphorus is distributed more widely than calcium. 15% is found in muscle and other soft tissues and 85% in the inorganic mineral phase of bone. It is an integral part of many macromolecules. Ex. Phospholipids, phosphoproteins and nucleic acids.

Functions:

It has no physiological effects comparable to that of calcium but it has many other functions which are as follows:

1. Formation of bone and teeth.

2. Formation of phospholipids essential to every cell.

3. Formation of nucleic acids and derivatives.

Ex. Adenylic acid and is thus significant in (RNA and DNA) protein synthesis and from genetics point of view.

4. Formation of organic phosphates as intermediate in metabolic processes.

Ex. In glycolysis, Glucose + ATP → G-6-P + ADP.

5. Formation of energy rich phosphate compounds.

Ex. ATP (energy currency of the cell).

6. Both inorganic and organic phosphates can take part in buffering the cell.

Ex, Sodium-potassium-phosphates.

7. Formation of coenzymes.

Ex. TPP, NADP.

8. Formation of phosphoprotein.

Ex. Casein.

Excretion:

Urinary excretion is equivalent to dietary phosphate intake. It varies diurnally, more being excreted at night. The usual daily loss is 600-800 mg, tubular resorption being 85-95%. Renal loss of phosphate can be of significant magnitude to lower serum phosphorus values and enhance osteoid demineralization.

Homeostasis:

There is a greater fluctuation observed in blood phosphate values due to easy shift between extracellular fluid and intracellular compartments. Thus it is quite dependent on dietary phosphorus. Inorganic phosphate affects the net movement of calcium into and out of bone.

Raised phosphate will lead to depression of the solubility of the calcium of bone crystals and thus shift equilibrium towards bone. In this manner it opposes the effect of the parathyroids. Ingestion of heavy dose of phosphate can lower serum calcium and increase excretion of calcium in urine. Lowered phosphorus on the other hand will make parathyroid activity more apparent.

Hormonal factors are not directly linked. However renal phosphate clearance is very vital in homeo-stasis and seems to be secondarily involved in certain endocrinopathies, e.g. involving parathormone, growth hormone and corticosteroids.

Disease state:

The following are the disease states of phosphorus in the body:

1. In rickets, serum phosphate is as low as 1-2 mg/100 ml (There is a temporary decrease in serum P during absorption of carbohydrates and some fats).

2. Organic P content is low but inorganic content is high in the serum in diabetes.

3. P retention causes acidosis in severe renal diseases. This results in increase of serum P.

4. Serum P levels are increased in hypoparathyroidism and decreased in hyperparathyroidism and celiac disease.

5. In renal rickets, blood P is very low with an increased alkaline phosphatase activity.

6. The deficiency of vitamin D is the cause of low serum P and the defects in the calcification of bones (referred to as vitamin D resistant rickets).

III. Magnesium:

Source:
Magnesium is present in milk, egg, cabbage, cauliflower etc.

Daily requirement:
Infants—100-150 mg; Children—150-200 mg and Adults—200-300 mg.

Absorption:
A greater part of the daily ingested Mg is not absorbed. A very high intake of fat, phosphate, calcium and alkalies diminish its absorption. Parathyroid hormone increases its absorption.

Distribution:
Whole blood it is 2-4 mg/dl, CSF it is 3 mg/100 ml and muscle it is 2 mg/100 ml.

Functions:
1. 70% of the total magnesium content (21g) of the body is combined with calcium and phosphorus in the complex salts of bone. The remainder is in the soft tissues and body fluids. It is the principal cation of the soft tissue.

2. Magnesium ions act as activators for many of the phosphate group transfer enzymes.

3. It is found in certain enzymes, such as co-carboxylase.

4. It functions as a cofactor for oxidative phosphorylation.

Disease state:

The following are the disease states of magnesium in the body:
1. Magnesium deficiency causes depression, muscular weakness and liability to convulsions. Its deficiency has also been observed in chronic alcoholics with low serum mg and muscular weakness.

2. Low in Kwashiorkor, causing weakness.

Low levels of Mg are reported in uremia, normal and abnormal pregnancy, rickets, growth hormone treatment, hypercalcemia and recovery phase of diabetic coma.

IV. Sodium, Potassium, Chloride:

Substances whose solutions conduct an electric current are called 'electrolytes'. They are about 11 in general. Na, K, Ca and Mg are cations whereas CI, HCO_3, HPO_4, SO_4, organic acids and proteins are anions. Among these sodium, potassium and chloride are important in the distribution and the retention of body water, thus have close relationship among them. Hence these three elements appear as a single question in the university exams.

Source:
The most important source of Na and CI in the diet is common table salt (NaCl). The good source of K are chicken, calf flesh, beef liver, dried apricot, dried peaches, bananas, the juice of orange and pineapple, potatoes etc.

Absorption:
Normally Na, K and CI are completely absorbed from the gastro-intestinal tract. About 95% of sodium which leaves the body is excreted in the urine.

Distribution:
In the tissues both Na and K occur in a relatively large amount as compared to chloride and other inorganic salts as well as protein and organic salts. Sodium is present in extra cellular fluid and in a very low concentration inside the cells whereas potassium is mainly found inside the cells and in a very low concentration in the extracellular fluid.

Functions of sodium and potassium:

These electrolytes maintain normal osmotic pressure in the body and protect the body against excessive loss of fluid.

1. They maintain the acid base balance in the body. Sodium bicarbonate, sodium phosphate, potassium phosphate form the buffer system of extracellular and intracellular fluids.

2. They maintain normal water balance.

3. Na also functions in the preservation of normal excitability of muscle and the permeability of the cells. K inhibits 'muscular contraction' in general.

4. High intracellular potassium concentrations are essential for several important metabolic functions, including protein biosynthesis by ribosomes.

5. Sodium and Potassium chlorides maintain the viscosity of blood. A number of enzymes including glycolytic enzymes, such as pyruvate kinase, require K^+ for maximal activity.
6. Na helps in the formation of the gastric juice. NaCl takes part in the series of reactions as a result of which HC1 is manufactured by the stomach.

7. K of KHb in the red cells helps in carbon dioxide transport.

8. K ions inhibit cardiac contraction and prolong relaxation.

9. K ions exert important effect on the function of nervous system.

Functions of chloride:

1. It provides 2/3rd of the anion of plasma and is the main factor for regulating body reactions.
2. NaCl and KCl are important agents in regulation of osmotic pressure in the body.

3. HCl of gastric juice is ultimately derived from the blood chlorides.

4. Chloride ions are essential for the action of ptyalin and pancreatic amylase.

5. It is essential in acid-base regulation. Chloride plays a role in the body by chloride shift mechanism.

Metabolism:

The metabolism of these elements is influenced by the following factors:

Hormones:
Mainly adrenocortical steroids and some of the sex hormones facilitate the retention of sodium and chloride in the body and excretion of potassium by kidneys in the urine. In adrenocortical deficiency, serum sodium decreases because excretion increases.

Temperature:
When atmospheric temperature is high as in summer, large amounts of sodium and chloride are lost in perspiration (sweating) and this loss may be checked when temperature is low (in winter).

Renal function:
In renal disease, with acidosis, Na and CI ion excretion in urine is increased due to poor tubular reabsorption of sodium whereas that of K ion is decreased leading to hyponatraemia and hypochloraemia but hyperkalaemia.

Average requirement of Na and K in human body is 5-15 and 4 gm. per day, respectively.

Disorders:

Hyponatraemia:
On sodium deficient diet, young ones grow slowly, lack fat deposit, there is muscle and testicular atrophy, lung infection and deficiency of osteoid tissues. There will also be loss of water, which will be evident by rapid weight loss.

Hypokalaemia:
Extreme potassium depletion in circulating blood causes hypokalemia in young one, they grow slowly and both sexes become sterile. The heart rate is slow, muscle weakness, irritability and paralysis are seen. Bone growth is retarded and in becomes excessively fragile and kidney hypertrophy is exhibited.

Hyperkalemia:

Hyperkalemia paralysis occurs due to excessive amount of potassium in blood. The disease is characterized by periodical attacks of weakness or paralysis. The symptoms of hyperkalaemia are chiefly cardiac and central nervous system depression. They are related to the elevated plasma potassium level and not to increase in intracellular potassium levels.

A dietary chlorine deficiency produces no symptom except a subnormal growth rate. Under normal dietary condition human beings are not subject to a deficiency of sodium, potassium or chlorine. However excessive diarrhoea, vomiting or extreme sweating over long period may bring about a NaCl deficiency. Sometimes the metabolism of individual minerals is asked as a separate question in the university exams. Hence each one is described separately in detail, hereunder.

V. Sodium:

Physiological functions:

1. Major component of extracellular fluids and exists in the body in association with anions chloride, bicarbonate, phosphate and lactate.

2. In association with chloride and bicarbonate it plays a role in acid base equilibrium.

3. Maintains osmotic pressure of the body fluids and thus protects the body against excessive fluid loss.

4. Plays an important role in the absorption of glucose and galactose from small intestine.

5. Maintains normal water balance and distribution.

6. Maintains the normal neuromuscular function.

7. Functions in permeability of cells.

Distribution:
About 1 /3rd of the total sodium content of the body is present in the inorganic portion of the skeleton. Most of the sodium is present in the extracellular fluid.
Plasma — 330 mg/100 ml

Muscles — 60 to 160 mg/100 gm.

Cells — 85 mg/100 gm.

Nerve — 312 mg/100 gm.

Daily requirement:
Adults require 5-15 gms/day. In temperate region, NaCl intake is less. In tropical region, NaCl intake is more. Hypertension patients should not take more than 1 gm. of Na per day.

Absorption:
Normally, Na is completely absorbed from gastro-intestinal tract. Less than 2% is eliminated in feces. In persons suffering from diarrhoea, large amounts are lost in feces.

Excretion:
Urine — 5-35 gm.

Skin — 25-50 mg

Stool — 10-125 mg

Excessive loss of Na by sweating causes heat arrays.

Disease state:
1. Adrenal cortical steroids regulate the metabolism of Na. Insufficiency of adrenal cortical steroids decreases serum Na level with an increase in sodium excretion.

2. In chronic renal disease when acidosis exists, Na depletion occurs due to poor tubular reabsorption of Na as well as to the loss of Na in the buffering acids.

3. In persons not adapted to high environmental temperature large amount of Na is lost in the sweat, developing muscular cramps of extremities, oedema, headache, nausea and diarrhoea.

4. Hyponatremia causes dehydration and reduced blood pressure, decreased blood volume and circulatory failure.

This may be due to:

(a) Prolonged vomiting and diarrhoea resulting in excessive loss of digestive fluid.

(b) Chronic renal disease with acidosis due to poor tubular reabsorption of Na.

(c) Adrenocortical insufficiency.

(d) Loss of weight due to loss of water.

5. In Hypernatremia, serum Na is high.

This occurs in:

(a) Hyperactivity of adrenal cortex as in Cushing's syndrome.

(b) Prolonged treatment with cortisone and ACTH as well as sex hormones, this results in—

(i) Increased retention of water in the body.

(ii) Increase in blood volume,

(iii) Increase in blood pressure.

6. Steroid hormones cause retention of Na and water in pregnancy.

VI. Potassium:

Physiological junctions:
1. Potassium is largely present in intracellular fluid and it is also present in small amounts in the extra cellular fluid because it influences the cardiac muscle activity.

2. It plays an important role in the regulation of acid-base balance in the cell.

3. It maintains osmotic pressure.

4. It functions in water retention.

5. It is essential for protein biosynthesis by ribosomes.

6. The glycolytic enzyme pyruvate kinase requires K^+ for maximal activity.

Sources:
High content of potassium is found in chicken, beef, liver, bananas, orange juice, pineapple, yam, potatoes etc.

Distribution:
Plasma — 20 mg/100 ml

Cells — 440 mg/100 gm.

Muscles — 250-400 mg/100g

Nerves — 530 mg/100g.

Daily requirement:
Normal intake of K^+ in food is about 4 gm. It is so widely distributed that its deficiency is rare except in pathological condition.

Blood potassium:
Normal level of serum K is 14-20 mg/100 ml. Erythrocytes contain large amounts of K which avoids hemolysis. Serum K decreases during increased carbohydrate utilization following glucose or insulin administration. Aldosterone decreases serum K.

Absorption:
Normally, K is practically completely absorbed from gastrointestinal tract and less than 10% of K is eliminated in the feces. In subjects with diarrhea large amounts are lost in feces.

Excretion:

K is normally eliminated almost entirely in urine and a small amount in the feces. Aldosterone exerts an influence on potassium excretion. In normal kidney function; K is very promptly and efficiently removed from the blood.

Disease state:
1. K is not only filtered by the kidney but is also secreted by the renal tubules. Excretion of K is greatly influenced by changes in acid-base balance and also by adrenal cortex. The capacity of kidney to excrete K is very great and therefore hyperkalaemia does not occur even after ingestion of K, if kidney function is impaired K should not be given intravenously unless, circulatory collapse and dehydration are corrected.

2. Hyperkalaemia occurs in patients in the following conditions.

(a) Renal failure

(b) Severe dehydration

(c) Addison's disease due to decreased excretion of K by the kidney

K deficiency occurs in chronic wasting diseases like malnutrition, prolonged negative nitrogen balance, gastrointestinal losses and metabolic alkalosis.

VII. Chlorine:

Physiological functions:
1. As a component of sodium chloride, chloride ion is essential in acid-base balance.

2. As Cl^- it is also essential in water balance and osmotic pressure regulation.
3. It is also important in the production of HCl in the gastric juice.

4. Cl^- ion is an activator of amylase.

Sources:
Mainly as NaCl salt (table salt).

Distribution:
Plasma — 365 mg/100ml

Cells — 190 mg/ 100mg

CSF — 440 mg/100ml

Muscle — 40 mg/100g

Nerve — 171 mg/100g

Daily requirement:
5-20 gms. Excess consumption of NaCl increases blood pressure in hypertensive patients. Causes edema in protein deficiency.

Absorption:
Normally CI is practically completely absorbed from the GI tract.

Excretion:
CI is chiefly eliminated in the urine, also in sweat. Its concentration in sweat is increased in hot climates and decreased by aldosterone.

Diseases state:
1. CI deficit also occurs when losses of Na are excessive in diarrhoea, sweating and certain endocrine disturbances.

2. Loss of CI due to loss of gastric juice by vomiting or pyloric or duodenal obstruction.

3. Hypochloremia alkalosis may develop in Cushing's syndrome or after administration of ACTH or cortisone.

VIII. Sulphur:

Sources:
Sulphur is taken mainly as cysteine and methionine present in proteins. Other compounds in the diet contribute small amounts of sulphur.

Absorption:
Inorganic sulphate is absorbed as such from intestine into the portal circulation. Small amount of sulphide may be formed in the bowel by the action of bacteria, but if absorbed into the blood stream, it is rapidly oxidized to sulphate.

Sulphur in blood (serum):
Inorganic — 0.5-1.1 mg/100 ml

Ethereal sulphate — 0.1-1.0 mg/100 ml

Neutral sulphur — 1.7-3.5 mg/100 ml

Physiological functions:
1. Sulphur is present primarily in the cell protein in the form of cysteine and methionine.

2. Cysteine plays important part in the protein structure and enzyme activity.

3. Methionine is the principal methyl group donor in the body. The 'activated' form of methionine, s-adenosyl methionine is the precursor in the synthesis of a large number of methylated compounds which are involved in intermediary metabolism and detoxification mechanism.

4. Sulphur is a constituent of coenzyme A and lipoic acid which are utilized in the synthesis of acetyl-CoA, malonyl CoA, Acyl-CoA and S-acetyl lipoate (involved in fatty acid oxidation and synthesis).

5. It is a component of a number of other organic compounds such as heparin, glutathione, thiamine, pantothenic acid, biotin, ergothionine, taurocholic acids, sulphocyamides, indoxyl sulphate, chondroitin sulphate, insulin, penicillin, anterior pituitary hormones and melanin.

Excretion:
Excreted in urine in 3 forms. Total sulphate excretion may be diminished in renal function impairment and is increased in condition accompanied by excessive tissue breakdown as in high fever and increased metabolism.

Disease state:
Serum sulphate is increased in renal function impairment, pyloric and intestinal obstruction and leukemia.

Marked sulphate retention in advanced glomerulo-nephritis causes the development of acidosis.

Increase in blood indica (indoxyl potassium sulphate) may occur in uremia.

IX. Iron:
Iron is present in all organisms and in all the cells. It does not exist in the free state, instead is always present in organic combination, usually with proteins. It exists in two forms i.e. Fe^{2+} (ferrous) and Fe^{3+} (ferric). It serves as an oxygen and electron carrier and is incorporated into redox enzymes and substances which carry out the function of oxygen transport such as haemoglobin and cytochromes.
Total iron content in normal adult is 4 to 5 grams. 60-70% is present in hemoglobin, 3% in myoglobin and 0.1% in plasma combined with β-globulin transport protein transferrin. Hemoprotein and flavoprotein make up to less than 1% of total iron. Rest is stored as ferritin.

Source:
Rich – Liver, heart, kidney, spleen.

Good – Egg yolk, fish, nuts, dates, beans, spinach, molasses, apples, bananas, etc.

Poor — Milk, wheat flour, polished rice, potatoes etc.

Daily requirement:
Only about 10% of ingested iron is absorbed.

i. Infants – 10-15 mg..

ii. Children – 1-3 years 15 mg.

iii. 4-10 years – 10 mg.

iv. Older children and adults of 11 to 18 years — 18 mg.

v. 19 years and above — 10 mg.

vi. Females between 11 and 50 years of age and during pregnancy or lactation – 18 mg.

vii. After 51 years of age — 10 mg.

viii. In adult women the average loss of iron with blood during menstrual period is 16-32 mg per month or an additional loss of 0.5 to 1.0 mg per day. This amount is easily obtained from diet.

ix. In excessive menstrual blood loss and in chronic iron-deficiency anemia, a supplement of 100 mg of iron per day is sufficient to replenish.

x. During growth, pregnancy and lactation iron demand is more.

xi. In healthy adult male or post menopause women dietary iron requirement is negligible unless any deficiency or loss of iron occurs.

xii. Iron deficiency occurs as a result of malabsorption from gastro-intestinal tract.

xiii. A defect in hemoglobin synthesis in anemia is commonly found in copper deficiency.

Biologically active compounds that contain iron:

1. Haemic compounds:
In these compounds the protoporphyrin is combined with iron to form haem (divalent iron) and haematin.

Ex. Hemoglobin, myoglobin, cytochromes, catalases and peroxidases.

2. Non-haemic compounds:
These include Transferrin (siderophilin) to transport iron, ferritin and haemosiderin which are the stored forms of iron and miscellaneous compounds like enzymes.

Absorption:
Very little (less than 10%) of dietary iron is absorbed. Excretion in the urine is minimal. Infants and children absorb more iron as compared to adults. Iron deficiency in infants is due to dietary deficiency. Iron deficient children absorb approximately twice as much as normal children do. Absorption mainly occurs in the duodenum and the proximal jejunum.

(a) Most of the iron in food occurs in the ferric form (Fe^{3+}), ex. either as ferric hydroxide or as ferric organic compounds. Acidic pH of the gastrointestinal tract favours the absorption whereas alkaline pH decreases it. In an acid medium, these compounds are broken down into free ferric ions or loosely bound organic iron, reducing substances such as —SH groups ex. cysteine and ascorbic acid which convert ferric iron into the reduced (ferrous) state, in this form iron is more soluble and should therefore be more readily absorbed.

(b) A diet high in phosphate, phytic acid and oxalic acid decreases iron absorption since these substances form the insoluble compounds with iron. Conversely, a diet very low in phosphate markedly increases iron absorption.

(c) The extent of absorption depends on the degree of saturation of the tissue, ex. anemic individuals absorb more than normal individuals.

(d) Iron absorption is enhanced by protein, possibly as a result of the formation of low molecular weight digestive products (peptides, amino acids) which can form soluble iron chelates.

(e) It is also increased in pernicious anaemia and in hypo plastic anaemia.

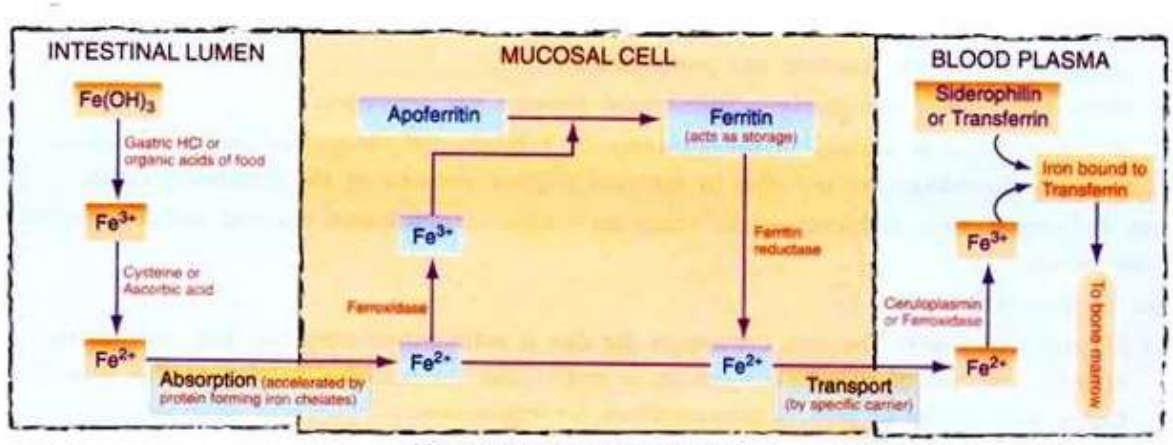
(f) Impaired absorption takes place in patients who have total removal of stomach or a removal of considerable amount of the intestine.

(g) Achlorhydria, administration of alkali, copper deficiency decrease iron absorption.

(h) Alcohol ingestion favours iron absorption.

Mechanism of Iron Absorption:
Ferrous ion on entering the mucosal cells is oxidized to ferric state and then combines with apoferritin forming ferritin which contains 23% of iron by weight. When apoferritin gets saturated with iron no

further iron can be taken up by the mucosal cells to store it in the form of ferritin. Heme enters the mucosal cells without being released from the porphyrin ring. Heme is broken down in the mucosa and iron appears in the plasma transferrin.



Mechanism of iron absorption

Transport:
In the plasma, the iron is bound to transferrin which is only partially saturated. Plasma iron is also in exchange with interstitial and intra-cellular compartments. The iron in these compartments is generally referred to as 'labile iron pool' and is estimated to be in the order of 80 to 90 mg. Here the iron may stay briefly on the cell membrane before its incorporation into haem or storage compounds. Nearly all the iron released from the mucosal cell enter the portal blood mostly in the ferrous state (Fe^{2+}). In the plasma, Fe^{2+} is oxidized rapidly to the ferric state (Fe^{3+}) and then incorporated into a specific protein.

Storage:
Stores of iron are maintained chiefly in the liver, spleen and bone marrow in the form of ferritin and haemosiderin. Women have lower stores than men and therefore, develop anaemia much more frequently than men. Iron stores are increased in haemochromatosis, severe haemolytic anaemias, aplastic anaemia and in persons receiving multiple blood transfusions, prolonged oral or parenteral iron therapy.The normal content of protein bound iron (FBI) in plasma of males is 120-140 jig/100 ml; in females it is 90-120 pg/100ml. However, the total iron binding capacity (TIBC) is about the same in both sexes i.e. 300-360 pg/100 ml.

Excretion:
Physiological excretion of iron is minimal. The normal routes of excretion are urine, bile, faeces, cellular desquamation, and sweat. Daily excretion in an adult male is estimated to be about 1 mg. In women of reproductive age, additional loss through menstruation averages to 1 mg per day.

Abnormal iron metabolism:
Ferritin and hemosiderin, the storage forms of iron act as internal iron reserve to protect against sudden loss of iron by bleeding. Ferritin is present not only in the intestine but also in liver (about 700 mg) spleen and bone marrow. If excess iron is administered parenterally exceeding the capacity of the body to store it as ferritin, it accumulates in the liver as hemosiderin, a form of colloidal iron oxide in association with protein.

Iron metabolism is disturbed mainly by the following causes:
(a) Decreased formation of hemoglobin.

(b) Decrease in circulating hemoglobin.

(c) Abnormalities in the serum iron concentration

(d) Abnormal deposition of iron-combining pigments in the tissues.

Physiological functions:

1. Iron functions mainly in the transport of oxygen to the tissues.

2. Involved in the process of cellular respiration.

3. Essential component of hemoglobin, myoglobin, cytochromes and the respiratory enzyme systems (cytochrome oxidase, catalase and peroxidase).

4. Non-heme iron is completely protein-bound (storage and transport).

5. Non-heme iron is utilized in the structure of xanthine dehydrogenase (xanthine oxidase) and succinate dehydrogenase and also in the iron sulphur proteins of the respiratory chain.

Iron deficiency:

Iron deficiency is the commonest cause of nutritional anaemia and is prevalent all over the world. Causes of iron deficiency:

(1) Dietary deficiency:
The iron content in the diet is sufficient to meet the daily requirements, but excessive amount of phytates in cereals, is responsible for non-absorbability of this iron. Hence higher daily intake of iron is recommended for vegetarians.

(2) Lack of absorption:
This may be seen in malabsorptive syndromes.

(3) Increased demand:
This occurs during rapid growth in infancy and pregnancy.

(4) Poor stores at birth:
These are found in premature birth and twin pregnancy.

(5) Pathological blood loss:
With loss of 1g of haemoglobin 3.4 mg of iron is lost. Hook-worm infestation is the most important factor responsible for blood loss. Other sources of blood loss are bleeding piles, peptic ulcer, hiatus hernia, cancer of gastrointestinal tract, chronic aspirin ingestion, and oesophageal varies.

(6) Iron deficiency anemia:
Iron deficiency anemia is widely prevalent among children, adolescent girls and nursing mothers. The hemoglobin content of the blood during iron deficiency anemia is 5 to 9 g/100 ml.

(a) Women of child bearing age:
The clinical symptoms are breathlessness on exertion, giddiness and pallor of the skin. In severe cases, there may be edema of the ankles.

(b) Weaned infants and young children:
The hemoglobin level is 5 to 9 g/100 ml of blood. The children are dull and inactive and show pallor of the skin. The appetite is poor and growth and development are retarded.

Treatment of iron deficiency anaemia:
Anaemia responds to oral iron therapy. The commonly used preparations are ferrous sulphate, ferrous fumarate and ferrous gluconate. Iron dextran can be administered both intramuscularly and intravenously, iron sorbitex is given intramuscularly, and saccharide iron oxide is given intravenously.

Anemic women should take ferrous sulphate tablet. For a child below 12 months, a mixture of ferrous ammonium citrate sweetened with glycerine and for children of 1 to 5 years ferrous ammonium citrate mixture should be given for curing.

Iron overload:
Hypersiderosis may occur as a primary disorder (Idiopathic haemochromatosis) or secondary with excessive entry of exogenous, iron into the body.

1. Siderosis:
When excessive amounts of iron are released in or introduced into the body beyond the capacity for its utilization, the excess is deposited in various tissues, mainly in the liver. This may occur due to repeated blood transfusions, excessive breakdown of erythrocytes in hemolytic types of anaemia and inadequate synthesis of haemoglobin as in pernicious anaemia.

2. Nutritional siderosis:
This disorder is found among Bantus in South Africa. Bantus cook their food in large iron pots and consume iron-rich food. The absorption of iron appears to be high, leading to the development of nutritional siderosis. Livers of the Bantus contain large amounts of iron.

Hemochromatosis:
Hemochromatosis is a rare disease in which large amounts of iron are deposited in the tissues, especially the liver, pancreas, spleen and skin producing various disorders. Accumulation of iron in the liver, pancreas and skin produces hepatic cirrhosis, bronze diabetes and bronze-state pigment respectively.

X. Copper:

Source:

Richest sources:
Liver, kidney, other meats, shell fish, nuts and dried legumes.

Poor sources:
Milk and milk products. The concentration of copper in the fetal liver is 5-10 times higher than that in liver of an adult.

Daily requirements:
Infants and children – 0.05 mg/kg body weight

Adults – 2.5 mg

A nutritional deficiency of copper has never been demonstrated in man, although it has been suspected in case of nephrosis.

Absorption:
About 30% of the normal daily diet of copper is absorbed in the duodenum.

Blood copper:
The normal concentration of copper in serum is 90 µg/100 ml. Both RBC and serum contain copper. 80% of RBC copper is present as superoxide dismutase (erythrocuperin), Plasma copper occurs as firmly bound form and loosely bound forms. The firmly bound copper consists of ceruloplas-min. Loosely bound copper is called 'directly reacting copper' and is bound to serum albumin. The plasma copper levels increase in pregnancy because of their estrogen content. Oral contraceptives have a similar effect.

Physiological functions:

1. It has important role in hemoglobin synthesis.

2. It is required for melanin formation, phospholipids synthesis and collagen synthesis.

3. It has a role in bone formation and in maintenance of the integrity of myelin sheath.

4. It is a constituent of several enzymes such as tyrosinase, cytochrome oxidase, ascorbic acid oxidase, uricase, ferroxidase I (ceruloplasmin), ferroxidase II, superoxide dismutase, amino oxidase and dopamine hydroxylase.

5. Three copper containing proteins namely cerebrocuperin, erythrocuperin and hepatocuperin are present in brain, RBC and liver respectively.

Excretion:

Only 10 to 60 mg of copper is excreted in the urine. 0.5 to 1.3 mg is excreted through bile and 0.1 to 0.3 mg is excreted by intestinal mucosa into the bowel lumen.

Effects of copper deficiency:

1. Although iron absorption is not disturbed but the release of iron into the plasma is prevented due to the decreased synthesis of ceruloplasmin. As a result, hypoferremia occurs which leads to the depressed synthesis of heme developing anemia in severe deficiency of copper.

2. The experimental animals on a copper deficient diet lose weight and die.

3. In copper deficient lambs, low cytochrome oxidase activity results in neonatal ataxia.

4. Copper deficiency produces marked skeletal changes, osteoporosis and spontaneous fractures.

5. Elastin formation is impaired in the deficiency of copper. Because a copper containing enzyme plays an important role in the connective tissue metabolism, especially in the oxidation of lysine into aldehyde group which is necessary for cross linkage of the polypeptide chains of elastin and collagen.

6. Copper deficiency results in myocardial fibrosis in cows. It is suggested that reduction in cyto-chrome oxidase activity may lead to cardiac hypertrophy.

Disorders of copper metabolism:

Wilson's disease (hepatoreticular degeneration):
Wilson's disease is a rare hereditary disorder of copper metabolism.

The following disorders have been observed in this disease:

(a) The absorption of copper from the intestine is very high (about 50 percent); whereas 2 to 5 percent copper is absorbed in normal subjects.

(b) Ceruloplasmin formation is very less. Hence a greater part of serum copper remains loosely bound to serum protein-notably albumin and therefore, copper can be transported to the tissues, such as brain and liver or to the urine.

(c) Excessive deposition of copper in the liver and the kidney causes hepatic cirrhosis and renal tubular damage respectively. The renal tubular damage results in the increased urinary excretion of amino acids, peptides and glucose.

XI. Iodine:

Source:
Rich sources are sea water, marine vegetation and vegetables as well as fruits grown on the sea board. Plants grown at high altitudes are deficient in iodine because of its low concentration in the water. In such regions, iodide is commonly added to the drinking water or table salt in concentrations of 1:5000 to 1:200000.

Daily requirement:
Adults – 100 to 150 μg

In adolescence and in pregnancy – 200 μg

Distribution:
Normal iodine content of body is 10 to 20 mg. 70 to 80% of this is present in thyroid gland. Muscles contain large amount of iodine. The concentration of iodine in the salivary glands, ovaries, pituitary gland, brain and bile is greater than that in muscle. Iodine in saliva is inorganic iodide, while most of the iodine present in tissue is in the organic form.

Blood Iodine:
Practically all the iodine in the blood is in the plasma. The normal concentration in plasma or serum is 4 to 10 μg/100 ml. 0.06 to 0.08 μg/100 ml is in inorganic form, 4 to 8 μg/100 ml is in the organic form bound to protein, precipitated by protein precipitating agents. 90% of the organic form consists of thyroxine and the remainder tri and di-iodothyronine. About 0.05% of thyroxine is in the free state. RBC contains no organic iodine.

Absorption:
Iodine and iodide are absorbed most readily from the small intestine. Organic iodide compounds (di-iodothyronine and thyroxine) are partly absorbed as such and a part is broken down in the stomach and intestines with the formation of iodides. Absorption also takes place from outer mucus membrane and skin.

Storage:
90% of the iodine of the thyroid gland is in organic combination and stored in the follicular colloid as ‘thyroglobulin’ a glycoprotein containing thyroxine, di-iodothyronine and smaller amounts of triiodothyronine.

On demand these substances are mobilized and thyroxine as well as triiodothyronine is passed into the systemic circulation. They undergo metabolic degradation in the liver.

Excretion:
1. Inorganic iodine is mostly excreted by the kidney, liver, skin, lungs and intestine and in milk.

2. About 10% of circulating organic iodine is excreted in feces. This is entirely unabsorbed food iodine.

3. 40 to 80 % is usually excreted in the urine, 20 to 70 μg daily in adults, 20 to 35 μg in children. The urinary elimination is largest when the intake is lowest.

4. Urinary iodine is increased by exercise and other metabolic factors.

Physiological functions:
Iodine is required for the formation of thyroxine and triiodothyronine hormones of the thyroid gland. These thyroid hormones are involved in cellular oxidation, growth, reproduction and the activity of the central and autonomic nervous systems. Triiodothyronine is more active than thyroxine in many respects.

Iodine deficiency:

1. In adults the thyroid gland is enlarged producing goiter. If treatment is started very early, the thyroid becomes normal. If treatment is delayed, the enlargement persists.

2. In children, severe iodine deficiency results in the extreme retardation of growth causing cretinism.

Prevention of goiter:

Goiter can be prevented by the regular use of iodized salt or iodine added to the drinking water.

Goitrogenic substances in foods:
Cabbage, cauliflower and radish contain substance like vinyl-2- thiooxazolidone which makes iodide present in the food unavailable by reacting with it. Such substances are called ‘goitrogenic’ substances.

Selenium:
i. Good dietary sources are kidney cortex, pancreas, pituitary and liver.

ii. It is rapidly absorbed mainly in duodenum.

iii. It is distributed in liver 0.44 μg/gm in skin 0.27 μg/gm and in muscle 0.37 μg/gm.

iv. In the cells it is present as selenocystinenadselenomethionine.

v. Selenium along with Vitamin E plays an important role in tissue respiration.

vi. Selenium is involved in biosynthesis of coenzyme Q (ubiquinone), which is involved in respiratory chain.

vii. Selenium acts as an antioxidant providing protection against peroxidation in tissues and membrane.

viii. It is an essential component of glutathione peroxidase, an enzyme which catalyzes the conversion of reduced glutathione to its oxidized form.

ix. Selenium is excreted in faeces, urine and via exhalation.

x. It causes toxic effect called selenosis.

Probable Questions:

1. State the distribution of water in the body. What factors affect distribution of water?
2. state the procedure of water intake and water loss from the body.
3. What are the physiological functions of water?
4. What is primary and secondary dehydration?
5. Write down the effect of dehydration.
6. How hormones regulate water balance in the body.
7. What is macro elements and micro elements. Give examples.
8. How calcium balance is regulated by hormones.
9. What factors control calcium absorption?
10. What factor control calcium level in blood.
11. State the diseases associated with problems in calcium metabolism.
12. Write down the physiological role of Phosphorous in the body.
13. How hormone controls phosphorous metabolism.
14. How iron get absorbed in the body.

Suggested Readings:

1. General Endocrinology. Turner and Bagnara. Sixth Edition.
2. Williams Textbook of Endocrinology. Tenth Edition.
3. Introduction to Endocrinology. Chandra S Negi. Second Edition
4. Endocrinology. Hadley and Levine. Sixth Edition

Unit-XI

GI tract hormone source, composition and function

Objective: In this unit you will know about source , composition and function of different gastro intestinal hormones.

Introduction:

The gastrointestinal hormones (or gut hormones) constitute a group of hormones secreted by enteroendocrine cells in the stomach, pancreas, and small intestine that control various functions of the digestive organs. Later studies showed that most of the gut peptides, such as secretin, cholecystokinin or substance P, were found to play a role of neurotransmitters and neuromodulators in the central and peripheral nervous systems.

Enteroendocrine cells do not form glands but are spread throughout the digestive tract. They exert their autocrine and paracrine actions that integrate gastrointestinal function.

The primary function of the gastrointestinal tract is to supply nutrients to our bodies via the processes of ingestion, motility, secretion, digestion, and absorption; this occurs through complex coordination of digestive processes that are regulated by intrinsic endocrine and nervous systems. Although the nervous system exerts influence on many digestive processes, the GI tract is the largest endocrine organ in the human body and produces numerous mediators that play an integral role in regulating functions of the GI tract.

Types of GI Hormones:

I.**Gastrin:**
This hormone is secreted by gastrin cells (= G-cells) in the pyloric region of the stomach. It stimulates gastric glands to secrete and release the gastric juice. It also stimulates gastric mobility.

II.Enterogastrone:
(= Gastric Inhibitory Peptide— GIP). It is secreted by the duodenal epithelium. It inhibits gastric secretion and motality. It slows gastric contraction, hence it is also called gastric inhibitory peptide.

III. Secretin:
It was the first hormone to be discovered by scientists. It is secreted by the epithelium of duodenum. It releases bicarbonates in the pancreatic juice. It increases secretion of bile. It decreases gastric secretion and motality.

IV. Cholecystokinin pancreozymin (CCK-PZ):
The word cholecystokinin is derived from three roots: Chol meaning bile, cyst meaning bladder and kinin meaning to remove. The word pancreozymin is derived from pancreas and zymin, which means enzyme producer. This hormone is secreted by the epithelium of entire small intestine. It stimulates the gall bladder to release bile and pancreas to secrete and release digestive enzymes in the pancreatic juice.

V. Duocrmin:
It is secreted by the duodenal epithelium and stimulates the Brunner's glands to release mucus and enzymes into the intestinal juice.

VI. Enterocrinin:
It is secreted by the epithelium of entire small intestine. It stimulates the crypts of Lieberkuhn to release enzymes into the intestinal juice.

VII. Vasoactive Intestinal Peptide (VIP):
It is secreted by the epithelium of entire small intestine. It dilates peripheral blood vessels of the gut. It also inhibits gastric acid secretion.

VIII. Villikinin:
It is secreted by the epithelium of entire small intestine. It accelerates movement of villi.

IX. Somatostatin (SS):
Somatostatin secreted by the Delta cells of islets of Langerhans of the pancreas inhibits the secretion of glucagon by aplha cells and insulin by beta cells Somatostatin produced by argentaffin cells of gastric and intestinal glands suppresses the release of hormones from the digestive tract.

X. Pancreatic Polypeptide (PP):
It is secreted by the pancreatic polypeptide cells (also ailed PP cells or F-cells) of islets of Langerhans. It inhibits the release of pancreatic juice from the pancreas.

Bio synthesis of GI Hormones:

The GI hormones classify as endocrines, paracrine, or neurocrine based on the method by which the molecule gets delivered to its target cell(s). Endocrine hormones are secreted from enteroendocrine cells directly into the bloodstream, passing from the portal circulation to the systemic circulation, before being delivered to target cells with receptor-specificity for the hormone. The five GI hormones that qualify as endocrines are **gastrin, cholecystokinin (CCK), secretin, glucose-dependent insulinotropic peptide (GIP), and motilin**. Enteroendocrine cells also secrete paracrine hormones, but they diffuse through the extracellular space to act locally on target tissues and do not enter the systemic circulation. Two examples of paracrine hormones are somatostatin and histamine. Additionally, some hormones may operate via a combination of endocrine and paracrine mechanisms. These "candidate" hormones are glucagon-like peptide-1 (GLP-1), pancreatic polypeptide, and peptide YY. Lastly, neurocrine hormones get secreted by postganglionic non-cholinergic neurons of the enteric nervous system. Three neurocrine hormones with significant physiologic functions in the gut are vasoactive intestinal peptide (VIP), gastrin release peptide (GRP), and enkephalins.

Gastrointestinal hormones undergo synthesis in specialized cells of the GI tract mucosa known as enteroendocrine cells. Enteroendocrine cells are specialized endoderm-derived epithelial cells that originate from stem cells located at the base of intestinal crypts. These cells are dispersed throughout the GI mucosa, sprinkled in between epithelial cells from the stomach all the way through to the colon. Also, these enteroendocrine cells possess hormone-containing granules concentrated at the basolateral membrane, adjacent to capillaries, that secrete their hormones via exocytosis in response to a wide range of stimuli related to food intake. These stimuli include small peptides, amino acids, fatty acids, oral glucose, distension of an organ, and vagal stimulation.

G cells secrete gastrin in the antrum of the stomach and the duodenum in response to the presence of breakdown products of protein digestion (such as amino acids and small peptides), distention by food, and vagal nerve stimulation via GRP. More specifically, phenylalanine and tryptophan are the most potent stimulators of gastrin secretion among the protein digestion products. The vagal nerve stimulation of gastrin secretion is unique because gastrin and motilin are the only hormones released directly by neural stimulation.

CCK is secreted from I cells in the duodenum and jejunum in response to acids and monoglycerides (but not triglycerides), as well as the presence of protein digestion products.Secretin is secreted from S cells in the duodenum in response to H+ and fatty acids in the lumen. Specifically, a pH less than 4.5 signals arrival of gastric contents, which initiates the release of secretin.

GIP is secreted by K cells in the duodenum and jejunum in response to glucose, amino acids, and fatty acids. GIP is the only GI hormone with a response to all three macronutrient types, and newer studies suggest that changes in intraluminal osmolarity may be what stimulates GIP secretion.GLP-1 is also produced in the small intestine and secreted from L cells. The presence of hexose and fat stimulate its release. Pancreatic polypeptide and peptide YY are secreted by protein and fat, respectively, although their functions are still relatively unknown.

Organ Systems Involved:

The digestive system is the primary site of action for most GI hormones and related polypeptides. The stomach is the primary site of gastrin production with some D-cells also populating the duodenum. Somatostatin and histamine are also produced in the stomach by enterochromaffin-like (ECL) cells, which is an enteroendocrine cell subtype. The small intestines, namely the duodenum and jejunum handle secretion of CCK, secretin, GIP, and motilin.

Function

The two gastrointestinal hormone families discussed above are responsible for most of the regulation of gastrointestinal function. The main actions of the gastrin-CCK family and the secretin family of hormones are listed below.

a. Gastrin

- Stimulates H+ (acid) secretion by parietal cells in the stomach
- Trophic (growth) effects on the mucosa of the small intestine, colon, and stomach
- Inhibits the actions of Secretin and GIP
- Inhibited by H+

b. CCK

- Contraction of the gallbladder with simultaneous relaxation of the sphincter of Oddi
- Inhibits gastric emptying
- Stimulates secretion of pancreatic enzymes: lipases, amylase, and proteases
- Secretion of bicarbonate from the pancreas
- Trophic effects on the exocrine pancreas and gallbladder

c. Secretin

- Inhibits gastrin, H+ secretion, and growth of stomach mucosa
- Stimulates biliary secretion of bicarbonate and fluid
- Secretion of bicarbonate from the pancreas

- Trophic effect on the exocrine pancreas

d. GIP

- Stimulation of insulin secretion
- Induces satiety
- In large doses, decreases gastric acid secretion
- In large doses, decreases the motor activity of the stomach and therefore slows gastric emptying when the upper small intestine is already full of food products.
- Stimulates the activity of lipoprotein lipase in adipocytes
- Protects beta-cells of the pancreas from destruction by apoptosis

e. GLP-1

- Decreases gastric emptying
- Induces satiety
- Increases sensitivity of pancreatic beta-cells to glucose.

f. Motilin

- Increases gastrointestinal motility by stimulating the "migrating motility" or "myoelectric complex" that moves through the fasting stomach and small intestines every 90 minutes. This cyclical release and action get inhibited by the ingestion of food. Not much is known about this peptide, except for this essential function.

Mechanism of action of GI hormones:

The release of GI hormones is in response to input from G-protein-coupled receptors that detect changes in luminal contents. Some of these receptors only respond to selective luminal substances and subsequently release GI hormones from their respective enteroendocrine cells through unknown mechanisms. Overall, gastrointestinal hormones manage a diverse set of actions in the body including:

- Contraction and relaxation of smooth muscle wall and sphincters
- Secretion of enzymes for digestion
- Secretion of fluid and electrolytes
- Trophic (growth) effects on tissues of GI tract
- Regulating secretion of other GI peptides (i.e., somatostatin inhibits secretion of all GI hormones)

To better understand how these actions are carried out by GI hormones, it is best to use gastrin's functions as an example. Gastrin is an interesting hormone because it acts through two mechanisms that ultimately increase the secretion of gastric acid (hydrogen ions) into the stomach. The first mechanism involves gastrin binding to CCK-2 receptors on parietal cells, causing increased expression of K/H ATPase enzymes that are directly responsible for

increased hydrogen ion secretion into the stomach. The second mechanism is mediated by enterochromaffin-like cells, which secrete histamine in response to activation by gastrin. Histamine then binds H2 receptors on nearby parietal cells, which further stimulates secretion of hydrogen ions. In addition to stimulating ECL cells to produce acid, gastrin also stimulates these parietal cells and ECL cells to proliferate.

Hormone	Major Activities	Stimuli for Release
Gastrin	Stimulates gastric acid secretion and proliferation of gastric epithelium	Presence of peptides and amino acids in gastric lumen
Cholecystokinin	Stimulates secretion of pancreatic enzymes, and contraction and emptying of the gall bladder	Presence of fatty acids and amino acids in the small intestine
Secretin	Stimulates secretion of water and bicarbonate from the pancreas and bile ducts	Acidic pH in the lumen of the small intestine
Ghrelin	Appears to be a strong stimulant for appetite and feeding; also a potent stimulator of growth hormone secretion.	Not clear, but secretion peaks prior to feeding and diminishes with gastric filling
Motilin	Apparently involved in stimulating housekeeping patterns of motility in the stomach and small intestine	Not clear, but secretion is associated with fasting
Gastric Inhibitory Peptide	Inhibits gastric secretion and motility and potentiates release of insulin from beta cells in response to elevated blood glucose concentration	Presence of fat and glucose in the small intestine

Possible Questions:

1. Describe source and function of any five GI hormones.
2. How GI hormones are synthesized in the body.
3. State the mechanism of actions of GI hormones.
4. Describe stimuli of release of any five GI hormones.

Suggested readings:

1. General Endocrinology. Turner and Bagnara. Sixth Edition.
2. Williams Textbook of Endocrinology. Tenth Edition.
3. Introduction to Endocrinology. Chandra S Negi. Second Edition
4. Endocrinology. Hadley and Levine. Sixth Edition.

Unit-XII

Neuroendocrine system and neurosecretion: neural control of glandular secretion; hypothalamic pituitary unit, neuroendocrine feedback

Objective: In this unit you will learn about Neuroendocrine system and neurosecretion: neural control of glandular secretion; hypothalamic pituitary unit, neuroendocrine feedback

Neuroendocrine system and neurosecretion:

Neuroendocrine system: The nervous system in association with endocrine system that serves as the primary control centre of the body is called neuroendocrine system.

Example: The hypothalamus (releasing factors) stimulates the pituitary gland to release various hormones that control various metabolic activities of the body.

Neurohormone: Any hormone that is produced by a specialized nerve cell (but not by endocrine gland) and is secreted from the nerve endings into the blood stream or tissues to exert its function is called neurohormone.

Example: ADH, noradrenaline, ecdyson, juvenile hormone etc.

Neurosecretion: The synthesis, storage & secretion of (hormones) neurohormones by neurosecretory cells (possess both nerve & endocrine functions) is called neurosecretion.

Example: In the hypothalamus, neurosecretory cells receive nerve impulses from the other parts of the brain or body which signal is transmitted to the pituitary gland by means of neurohormones.

Neurotransmitters: The chemicals that mediate the transmission of nerve impulse across a synapse or neuromuscular junction.
Exam: Acetylcholine, adrenaline, noradrenaline, dopamine, serotonin, GABA etc.

Hypothalamus: It is a part of vertebrate forebrain situated below the thalamus & cerebrum that mainly regulates body temperature & neuroendocrine functions.

The neuroendocrine system:
The neuroendocrine system is made up of special cells called neuroendocrine cells. They are spread throughout the body. Neuroendocrine cells are like nerve cells (neurons), but they also make hormones like cells of the endocrine system (endocrine cells). They receive messages (signals) from the nervous system and respond by making and releasing hormones. These hormones control many body functions.

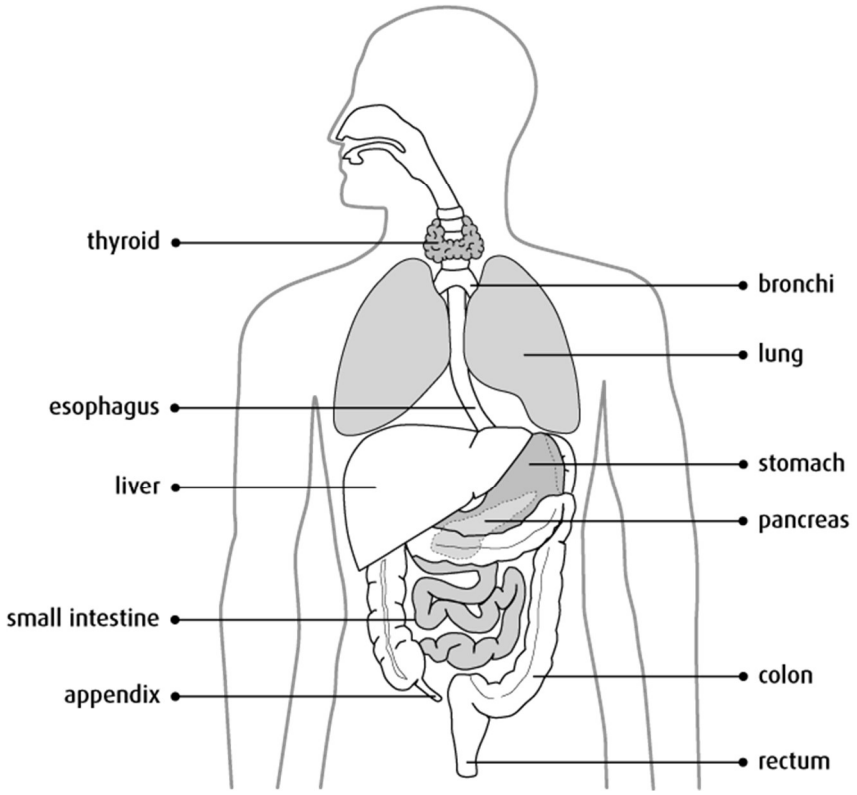
Location of neuroendocrine cells:
Neuroendocrine cells are found in almost every organ of the body. They are mainly found scattered in the gastrointestinal (GI) tract (including the small intestine, rectum, stomach, colon, esophagus and appendix), the gallbladder, the pancreas (islet cells) and the thyroid (C

cells). Neuroendocrine cells are also commonly found in the lungs or airways into the lungs (bronchi), as well as the respiratory tract of the head and neck. The neuroendocrine cells scattered throughout these organs are often referred to as the diffuse neuroendocrine system.

The pituitary gland, the parathyroid glands and the inner layer of the adrenal gland (adrenal medulla) are almost all made up of neuroendocrine cells.

Other sites of neuroendocrine cells include the thymus, kidneys, liver, prostate, skin, cervix, ovaries and testicles.

Part of the Neuroendocrine System



Function of neuroendocrine cells :
Neuroendocrine cells make and release hormones and similar substances (peptides) in response to neurological or chemical signals. The hormones then enter the blood and travel throughout the body to other cells (target cells). The hormones attach to specific receptors on target cells, which cause changes in the cells and what they do.

Neuroendocrine cells have many functions, which include controlling:

- the release of digestive enzymes to break down food
- how fast food moves through the GI tract
- air and blood flow through the lungs
- blood pressure and heart rate
- the amount of sugar (glucose) in the blood
- bone and muscle growth and development

The following are examples of hormones or peptides released by neuroendocrine cells and what they do.

- Serotonin (5-HT or 5-hydroxytryptamine) is a chemical released by nerve cells (neurotransmitter) that helps with digestion. A lot of the body's serotonin is found and made in the neuroendocrine cells of the GI tract where it controls the movement of food through the GI tract.
- Gastrin tells the stomach to release acid and enzymes to help with digestion.
- Insulin is made by pancreatic islet cells. It lowers the level of sugar (glucose) in the blood when it's high. It controls when cells absorb (take up) sugar for energy.
- Epinephrine (adrenaline) is made by neuroendocrine cells of the adrenal gland. It is released during times of stress, like when you feel fear, and increases heart rate and blood pressure.
- Growth hormone is made in the pituitary gland. It promotes the growth and development of bones and muscles.

Major Neuroendocrine Systems:

Various endocrine glands are intimately associated with hypothalamus and pituitary to controlthe various physiological function of the body by means of various axes which are:

1. Hypothalamic-pituitary-thyroid (HPT) axis
2. Hypothalamic-pituitary-gonodal (HPG) axis
3. Hypothalamic-pituitary-adrenal (HPA) axis
4. Hypothalamic-neurohypophyseal axis.

Any of the systems of dual control of certain activities in the body of some higher animals by nervous and hormonal stimulation is the neuroendocrine system. For example, the posterior pituitary gland and the medulla of the adrenal gland receive direct nervous stimulation to secrete their hormones, whereas the anterior pituitary gland is stimulated by releasing hormones from the hypothalamus.

A substantial volume of scientific evidence has been accumulated demonstrating that biological
aging is associated with functional deficits at the cellular, tissue, organ, and system levels. Although several theories have been proposed to explain these changes, as well as the increased risk of disease with age, no single explanation has adequately accounted for the diversity of physiological changes associated with age.

The concept that deficiencies in the neuroendocrine system contribute to aging evolved from studies indicating that-
(1) the endocrine system has an important role in developmental processes,
(2) hormones have an important trophic & integrative role in maintaining tissue function, and
(3) hormone deficiency results in deterioration of tissue function.

The neuroendocrine system is composed of the hypothalamus and pituitary gland and is under the influence of neurotransmitters and neuropeptides that regulate hypothalamic releasing and hypothalamic release inhibiting hormones secreted into the blood vessels that

connect the hypothalamus and pituitary gland. The release of these hypothalamic hormones influences the
secretion of anterior pituitary hormones that subsequently regulate tissue function. The hypothalamus and pituitary gland have the capacity to detect humoral secretions (hormones secreted) from target tissues and adjust hormone production to maintain an optimal internal "milieu" appropriate for normal function.

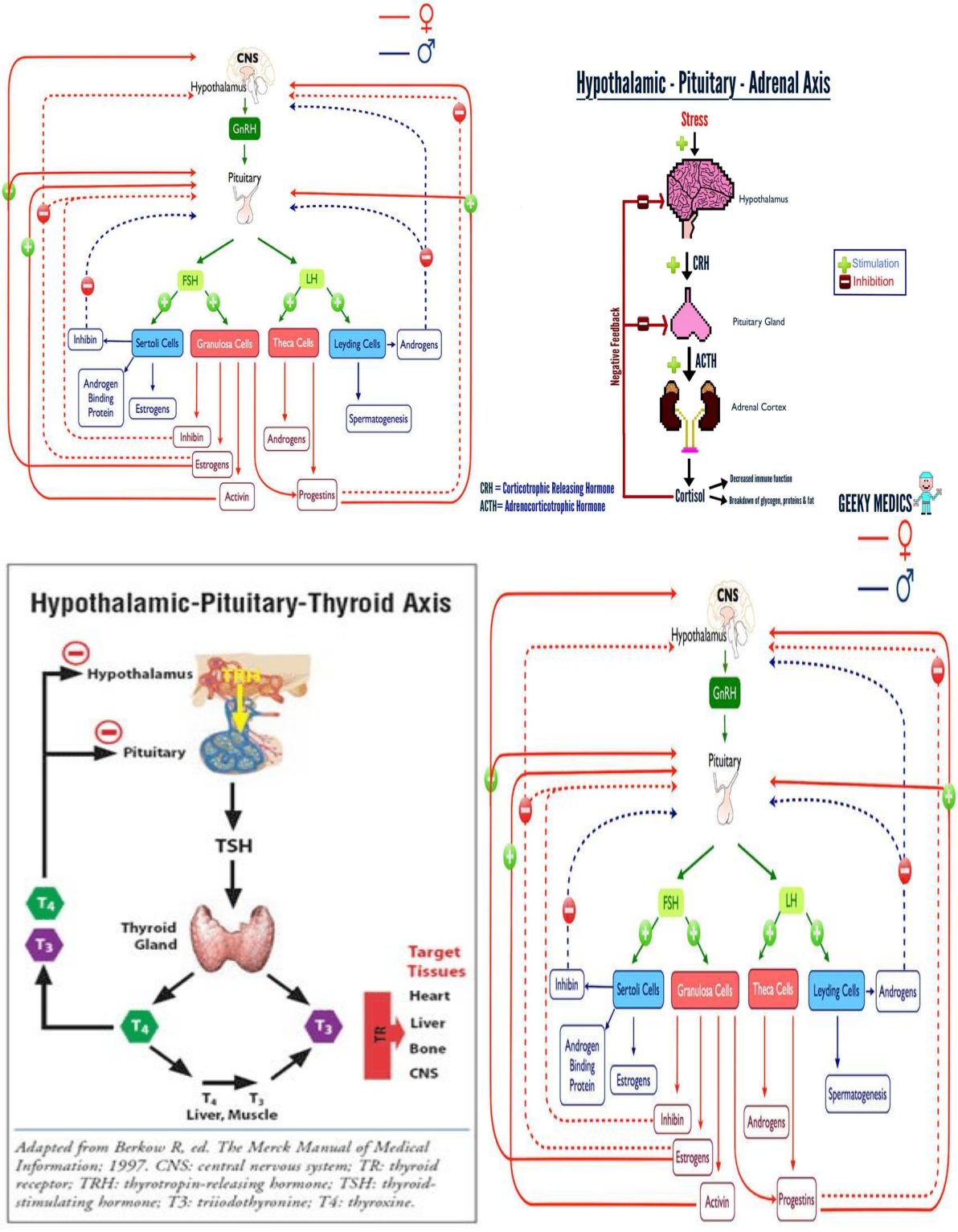


Figure: Various hypothalamic-hypophyseal axes:

It is well-established that the neuroendocrine system has a critical role in integrating biological responses and influencing:

(1) cellular protein synthesis and general metabolism through the release of growth hormone andthyroid-stimulating hormone (TSH), respectively,

(2) reproductive function through the release of luteinizing hormone (LH), follicle-stimulating
hormone (FSH), prolactin, and oxytocin, and

(3) plasma electrolytes and responses to stress through regulation of the hormones vasopressin
(antidiuretic hormone, or ADH) and adrenocorticotropin (ACTH).

In addition, the hypothalamus also has an important role in the integration of parasympathetic and sympathetic nervous system activity, and can thereby influence a wide variety of functions, including heart rate, blood pressure, vascular responses, and glucose metabolism. Thehypothalamus has been implicated in the regulation of biological rhythms by its interactions withhypothalamic nuclei. More recently, the regulation of fat metabolism and food intake has beenshown to be regulated through the hypothalamus by its response to the protein, leptin, and itssynthesis of neuropeptide Y. It should be noted that the classification of hormones and their primary function presented here is an overly simplistic view of the neuroendocrine system, sincecritical interactions occur among these hormones that contribute to the coordinated regulation ofcellular and tissue function.

Although the specific etiology of age-related changes in the neuroendocrine system is unknown,it has been proposed that cellular and molecular alterations in specific subpopulations of neuronswithin the hypothalamus and pituitary, and/or supporting structures within the brain, contributeto the decrease in tissue function. Some of the alterations may be related to loss of neurons orsynapses, genetic errors, and/ or the production of free radicals, all of which lead to progressive aberrations in neurons and contribute to neuroendocrine aging. As a result, the neuroendocrine theory of aging is unique when compared to other theories of aging in that the neuroendocrine alterations are, in many cases, not considered the primary causative factors of biological aging, but rather are considered to be mediators of aging that are initiated by cellular changes in specific subpopulations of neurons or systems that closely interact with hypothalamic neurons.

Three classic examples of age-associated changes in neuroendocrine regulation, and the resulting consequences for tissue function, help emphasize the importance of this system in the development of the aging phenotype.

First, with increasing age there is a decline in growth-hormone secretion that results in a decrease in insulin-like growth factor-1 (IGF-1) production in the liver and other tissues. The loss of these anabolic hormones contributes to the general decline in cellular protein synthesis,
skeletal muscle mass, immune function, and cognitive ability in rodents, nonhuman primates, and humans. The decrease in growth-hormone release from the pituitary gland results from impaired release of growth-hormone-releasing hormone and increased release of somatostatin

(an inhibitor of growth hormone) from hypothalamic neurons.

Second, decreased secretion of gonadotropin-releasing hormone (GnRH) from hypothalamic neurons results in a decline in luteinizing hormone. This is the primary factor in the loss of reproductive cycles in the female rodent, and, in conjunction with the loss of ovarian follicles,
contributes to the decline in oestrogen levels in women. These latter changes result in atrophy of secondary reproductive tissues and have been implicated in the post-menopausal loss of bone and cognitive function. Decreased GnRH secretion in the male also contributes to a decrease in LH and androgen levels and to the corresponding loss of skeletal muscle mass and reproductive function.

Finally, increased secretion of ACTH and the adrenal hormone, cortisol, in response to stress have been reported to contribute to atrophy and/or loss of neurons, as well as age-related decline in cognitive function. These latter findings have contributed to the hypothesis that increased levels of glucocorticoids contribute to brain aging.

Although other mechanisms are possible, the alterations in the secretion of hypothalamic hormones with age have been traced to deficiencies in the secretion of brain neurotransmitters. For example, the activity of dopamine and norepinephrine decreases with age, and both acute and chronic procedures used to increase levels of these neurotransmitters in aged animals have been shown to restore some aspects of neuroendocrine function. Studies have shown an increase in growth hormone release and a restoration of some aspects of reproductive function in older animals in response to the L-Dopa, dopamine and norepinephrine precursor. These findings have led investigators to conclude that a decline in neurotransmitter activity is a contributing factor in the neuroendocrine decline that accompanies aging. Nevertheless, the possibility that interactions with other hypothalamic peptides, the loss of neurons, or intracellular changes within hypothalamic neurons contribute to the loss of function cannot be excluded.
In fact, the inability of hypothalamic neurons to compensate for the age-related alterations in circulating levels of hormones supports the concept that the normal feedback mechanisms that occur within the hypothalamus are impaired in aged animals. Whether these altered feedback mechanisms are related to the deficiencies in neurotransmitters or result from other aberrations within the aging neuroendocrine system remain to be established. Nevertheless, deficits in the regulation of these critical hormonal systems contribute to deterioration of tissue function and undoubtedly are an important factor in age-related disease and disability.

Probbable Questions:

1. Define neurohormone. Give examples.
2. Define neuroendocrine system. State the location of neuroendocrine cells.
3. What are the functions of neuroendocrine cells?
4. Name four major neuroendocrine system.

Suggested readings:

1. General Endocrinology. Turner and Bagnara. Sixth Edition.
2. Williams Textbook of Endocrinology. Tenth Edition.
3. Introduction to Endocrinology. Chandra S Negi. Second Edition
4. Endocrinology. Hadley and Levine. Sixth Edition.

Disclaimer

The study materials of this book have been collected from various books, e-books, journals and other e sources.

Post-Graduate Degree Programme (CBCS)

in

ZOOLOGY

SEMESTER-III

SOFT CORE THEORY PAPER

HUMAN MOLECULAR GENETICS

ZST-303

SELF LEARNING MATERIAL

DIRECTORATE OFOPEN AND DISTANCE LEARNING

UNIVERSITY OF KALYANI

KALYANI, NADIA,

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Director's Message

Satisfying the varied needs of distance learners, overcoming the obstacle of distance and reaching the unreached students are the threefold functions catered by Open and Distance Learning (ODL) systems. The onus lies on writers, editors, production professionals and other personnel involved in the process to overcome the challenges inherent to curriculum design and production of relevant Self Learning Materials (SLMs). At the University of Kalyani a dedicated team under the able guidance of the Hon'ble Vice-Chancellor has invested its best efforts, professionally and in keeping with the demands of Post Graduate CBCS Programmes in Distance Mode to devise a self-sufficient curriculum for each course offered by the Directorate of Open and Distance Learning (DODL), University of Kalyani.

Development of printed SLMs for students admitted to the DODL within a limited time to cater to the academic requirements of the Course as per standards set by Distance Education Bureau of the University Grants Commission, New Delhi, India under Open and Distance Mode UGC Regulations, 2017 had been our endeavour. We are happy to have achieved our goal.

Utmost care and precision have been ensured in the development of the SLMs, making them useful to the learners, besides avoiding errors as far as practicable. Further suggestions from the stakeholders in this would be welcome.

During the production-process of the SLMs, the team continuously received positive stimulations and feedback from Professor (Dr.) Sankar Kumar Ghosh, Hon'ble Vice-Chancellor, University of Kalyani, who kindly accorded directions, encouragements and suggestions, offered constructive criticism to develop it within proper requirements. We gracefully, acknowledge his inspiration and guidance.

Sincere gratitude is due to the respective chairpersons as well as each and every member of PGBOS (DODL), University of Kalyani. Heartfelt thanks is also due to the Course Writers-faculty members at the DODL, subject-experts serving at University Post Graduate departments and also to the authors and academicians whose academic contributions have enriched the SLMs. We humbly acknowledge their valuable academic contributions. I would especially like to convey gratitude to all other University dignitaries and personnel involved either at the conceptual or operational level of the DODL of University of Kalyani.

Their persistent and co-ordinated efforts have resulted in the compilation of comprehensive, learner-friendly, flexible texts that meet the curriculum requirements of the Post Graduate Programme through Distance Mode.

All the Self Learning Materials are self writing and collected from e-book, journals and websites.

Prof Manas Mohan Adhikary
Director
Directorate of Open and Distance Learning
University of Kalyani

SOFT CORE THEORY PAPER (ZST-303)

HUMAN MOLECULAR GENETICS

Module	Unit	Content	Credit	Class	Time (h)	Page No.
ZST - 303 (HUMAN MOLECULAR GENETICS)	I	Human population genetics and evolution: Basic attributes and polymorphic structures in human protein coding genes. Mitochondrial DNA polymorphism. Y-chromosome polymorphism and Single nucleotide polymorphism (SNP), Basic concept in Molecular phylogenetics	1.0	1	1	
	II	Genetics in forensic science: DNA comparisons, RFLPs, genetic finger-printing, VNTRs, and Single nucleotide polymorphism (SNP). Genetic profiles. Protein comparisons.		1	1	
	III	Sociobiology, Altruism, Kin selection and inclusive fitness, Haplodiploidy, Imprinting phenomena		1	1	
	IV	Human genome project and the age of genomics		1	1	

Unit-I

Human population genetics and evolution: Basic attributes and polymorphic structures in human protein coding genes. Mitochondrial DNA polymorphism. Y-chromosome polymorphism and Single nucleotide polymorphism (SNP), Basic concept in Molecular phylogenetics

Objective: In this unit you will know about polymorphism of protein coding genes, Mitochondrial DNA polymorphism. Y-chromosome polymorphism and Single nucleotide polymorphism (SNP) as well as basic concept in molecular phylogenetics.

Definition of Genetic Polymorphism:

Genetic polymorphism refers to the regular occurrence of several phenotypes in the genetic population. The term genetic polymorphism was coined by Ford in 1940. It has been reported that two third of the loci in a population exhibit polymorphism. The genetic polymorphism is usually maintained due to superiority of heterozygotes over both the homozygotes.

When polymorphism is maintained as a result of heterozygote advantage, it is known as balanced polymorphism. Polymorphism can be detected on the basis of morphological, biochemical and molecular traits or markers.

Genetic polymorphism increases the buffering capacity of a population by providing increased diversity of genotypes in a population. Genetic polymorphism broadens the genetic base of a population and thus enhances the adaptability of the population.

Types of Genetic Polymorphism:

There are six types of genetic polymorphism which are as follows:

i. Balanced Polymorphism:

The genetic polymorphism which is maintained due to superiority of heterozygote over both the homozygotes is referred to as balanced polymorphism. This leads to regular occurrence of several phenotypes in a population. The term balanced polymorphism was first used by Ford in 1940.

Main features of balanced polymorphism are given below:

(a) The polymorphism is maintained due to heterozygote advantage.

(b) This was first reported by Ford in 1940.

(c) This is the most common type of polymorphism observed in plant breeding populations.

ii. Transient Polymorphism:

A genetic polymorphism that is limited to a particular ‘period is called transient polymorphism. Sometimes one allele undergoes replacement by a superior allele. The genetic polymorphism during

such period is known as transient polymorphism. It is not a regular phenomenon like balanced polymorphism.

Thus there are three main features of transient polymorphism:

(a) It is for a limited period,

(b) It is not a regular feature, and

(c) It was also reported by Ford in 1940.

iii. Neutral Polymorphism:

It refers to the genetic polymorphism that is dependent on a gene action which is almost neutral in its effect on the survival of the genotype in which it is contained. In other words, the effect on the career genotype is neutral. It results due to neutral mutations.

The main features of neutral polymorphism are given below:

(a) It was coined by Ford in 1940.

(b) It results due to neutral mutation.

(c) Neutral mutations take long time in contributing to polymorphism.

iv. Regional Polymorphism:

This refers to occurrence of two or more phenotypes in a population in different regions of the habitat. It results due to adaptation of different individuals in different environment.

Main features of regional polymorphism are given below:

(a) It results due to adaptive variation of alleles.

(b) It is not related to superiority of heterozygotes.

(c) It is also known as geographical polymorphism.

v. Unisexual Polymorphism:

If refers to the genetic polymorphism that is confined to one sex only. It results due to sex limited manifestation of genes. However, such gene can recombine in both sexes.

vi. Cryptic Polymorphism:

It refers to genetic polymorphism in which the genetically different alleles cannot be identified on the basis of their phenotype. It may include chromosomal polymorphism.

Causes of Genetic Polymorphism:

The possible causes of genetic polymorphism include:

i. Heterozygote Advantage:

The natural selection usually favours heterozygotes than homozygotes because heterozygotes are more adaptable than homozygotes. In other words, heterozygotes have more buffering capacity to environmental changes than homozygotes. The heterozygotes maintain genetic polymorphism in a population.

ii. Frequency Dependent Selection:

The frequency dependent selection also leads to maintenance of polymorphism in a population. Generally selection favours those alleles that have low frequency but produce rare phenotype. The selection goes against the alleles that have high frequency. This type of frequency dependent selection maintains balanced polymorphism in a population.

iii. Heterogeneous Environment:

The environment differs from region to region and season to season. The balanced polymorphism is maintained when one allele is advantageous in one environment and another in different environment. In such situation stable polymorphism can be maintained even without heterozygote advantage.

iv. Transition:

In the evolutionary process, sometimes one allele is replaced by another which is more advantageous for adaptation. This may lead to polymorphism in a population. However, such polymorphism is for a limited period and hence is called as transitional polymorphism.

v. Neutral Mutation:

In a population, mutations do arise. However, the majority of mutants are harmful and deleterious. Such mutants are lost only few mutants will survive and replace the original allele. The changes in gene frequency depend on chance. Thus spread of a mutant through the population is erratic.

The frequency of a mutant is sometimes increasing and sometimes decreasing. Only those mutants that have selective advantage will survive and contribute to polymorphism in a population. The surviving few mutants take a very long time to spread in the population but contribute to the polymorphism.

Methods of Detecting Genetic Polymorphism:

In a plant breeding population, the genetic polymorphism can be detected in three main ways, viz.:

(i) On the basis of phenotype

(ii) Biochemical markers and

(iii) Molecular markers.

There are briefly discussed below:

i. On the basis of Phenotype:

The best way of detection of genetic polymorphism is the average heterozygosity at various loci. The higher the heterozygosity, the higher the polymorphism will be. The regular occurrence of several phenotypes in a population is an indication of genetic polymorphism. The polymorphism can be detected on the basis of plant characters such as shape, colour, surface, size of various plant characters. The polymorphism is observed for both oligogenic and polygenic traits.

ii. Biochemical Markers:

Sometimes it is difficult or impossible to identify the polymorphic alleles by visual observations. In such situation the best way of detecting the polymorphic alleles is the isozyme studies or gel electrophoretic studies. Sometimes mutations give rise to protein polymorphism and the variant forms of protein differ only at few amino acid sites. This type of polymorphism can be easily detected by gel electrophoretic studies which throw light on amino acid banding pattern.

iii. Molecular Markers:

Sometimes polymorphic differences are at molecular or DNA level. In other words, the differences are in nucleotide sequences in the DNA. These can be observed by restriction fragment length polymorphisms (RFLPs), amplified fragment length polymorphisms (AFLPs), random amplified polymorphic DNA (RAPD), single sequence repeat (SSR) etc. The molecular or DNA markers are very accurate in detecting the level of polymorphism in a population.

Theories of Genetic Polymorphism:

Two theories have been put forth to explain the wide spread existence of polymorphic variation. These are selectionist theory and neutralist theory.

A brief account of these theories is presented below:

i. Selection Theory:

This theory states that polymorphism is balanced or stable and the stable equilibrium is maintained by selective forces. For example, the balanced polymorphism is the selection in favour of heterozygotes.

ii. Neutral Mutation Theory:

This theory was proposed by Kimura (1983) and further elaborated by Crow (1986). According to this theory some polymorphisms are due to presence of mutant alleles that are nearly neutral with regard to fitness. Such alleles were mutated in distant past and are still present in the population contributing to polymorphism. This theory is widely accepted.

Advantages of Genetic Polymorphism:

There are several advantages of genetic polymorphism which are briefly presented below:

i. Genetic Diversity:

Polymorphic population has greater genetic diversity than pure lines and inbred lines. The genetic diversity avoids danger of uniformity and provides protection from biotic and abiotic stresses to the population.

ii. Broad Genetic Base:

Polymorphic population has broad genetic base due to presence of several phenotypes. Such population has greater buffering capacity to environmental changes.

iii. Adaptation:

Genetic polymorphism enhances the adaptive value of a population by providing increased diversity of genotypes in a population. It also enhances adaptability of a population, because heterozygotes are more adaptable than homozygotes. Genetic polymorphism gives rise to variation of quantitative characters.

Disadvantages of Genetic Polymorphism:

There are some demerits or disadvantages of genetic polymorphism which are briefly discussed below:

i. Difficult to get Purelines:

It is difficult to get purelines from a polymorphic population. Inbreeding does not have much effect in polymorphic population. It is difficult to control the number of loci that have to be kept in polymorphic state.

ii. Less Uniform:

The polymorphic populations are less uniform due to presence of genetic diversity. The produce of such population is also less uniform and less attractive.

iii. Low Yield:

The yield of polymorphic population is poorer than the best genotype present in the polymorphic population.

Modern Concept of Gene:

A gene can be described as a polynucleotide chain, which is a segment of DNA. It is a functional unit controlling a particular trait such as eye colour.

Beadle and Tatum concluded by various experiments that gene is a segment of DNA that codes for one enzyme. They proposed one gene-one enzyme hypothesis. But as some genes code for proteins that are not enzymes, the definition of gene was changed to one gene-one protein hypothesis.

Protein Hypothesis:

The concept of gene has undergone further changes as the new facts came to light. Since proteins are polypeptide chains of amino acids translated by mRNA, gene was defined as one gene-one polypeptide relationship.

Some proteins have two or more different kinds of polypeptide chains, each with a different amino acid sequence. They are products of different genes. For example, haemoglobin has two kinds of chains a andβ chains, which differ in amino acid sequence and length. They are encoded by different genes. Thus, gene is defined as one gene-one polypeptide relationship.

Structural and Regulatory Genes:

Even the one gene-one polypeptide definition is not complete as it does not include gene which codes for rRNA and tRNA. Only mRNA is translated into proteins. Therefore genes which code for polypeptides and RNAs are called structural genes.

In addition to structural genes, DNA also contains some sequences that have only regulatory function. These regulatory genes constitute signals, which "turn on" and "turn off" the transcription of structural genes and perform various other regulatory functions. In this way the definition of gene includes structural genes as well as regulatory genes. Benzer coined terms for the gene, they are Cistron which is the unit of function, Recon which is the unit of recombination and Muton which is the unit of mutation.

Molecular Definition of a Gene:

Gene is defined as the entire nucleic acid sequence that is necessary for the synthesis of a functional gene product, which may be polypeptide or any type of RNA. In addition to structural genes (coding genes) it also includes all the control sequences and non-coding introns. Most prokaryotic genes transcribe polycistronic mRNA and most eukaryotic genes transcribe monocistronic mRNA.

Number of Genes on a Single Chromosome:

Total number of genes on a single chromosome is different in different organisms. Bacteriophage virus R17 consists of only three genes, SV40 consists of 5-10 genes. E. coli bacteria have more than 3000 genes on single 1 mm long chromosome.

Size of a Gene:

In E. coli there are more than four million pairs of nucleotides (4638858 base pairs). It has been estimated that there are about 3000 genes in E. coli.

The minimum size of a gene that encodes a protein can be directly estimated, Each amino acid of a polypeptide chain is encoded by a sequence of three consecutive nucleotides in a single strand of DNA. Therefore by measuring the size of the polypeptide chain, the size of a gene can be directly measured.

The average polypeptide chain has about 450 amino acids, which are encoded by 1350 nucleotides. Therefore, in E. coli the number of genes will be around 3000 (4000000/1350 = 3000). Human genome contains about 30000 genes, (Source : International Human genome sequencing consortium led in the United States by National Human Genome Research Institute (NHGRI) have estimated the number of human protein coding genes to be less than 30000. Simple round worm C. elegans has about 20000 genes).

A single copy of chromosome is composed of more than 3 billion base pairs. Coding regions of these genes take up only 3% of the genome.

Fine Structure of a Gene:

A gene is present only in one strand of DNA, which is a double stranded helix. A gene consists of several different regions. The main region is the coding sequence which carries information regarding amino acid sequence of polypeptides. The region on the left side of coding sequence (upstream or

minus region) and on the right side (downstream or plus region) consists of fairly fixed regulatory sequences.

Regulatory sequences consist of promoters which are different in prokaryotes and eukaryotes.

Types of Genes:

1. Simple Genes:

Simple genes have a coding sequence of bases in one DNA strand. Upstream the coding region, the promoter is present. Downstream, the termination region is present.

2. Split Genes:

In most of eukaryotes, many non-coding sequences are present between coding sequences. The coding sequences of DNA of the genes are called exons. In between exons are present non-coding sequences called introns. Exons alternate with introns. Normally introns do not possess any genetic information and are not translated. Such genes are called split genes or interrupted genes.

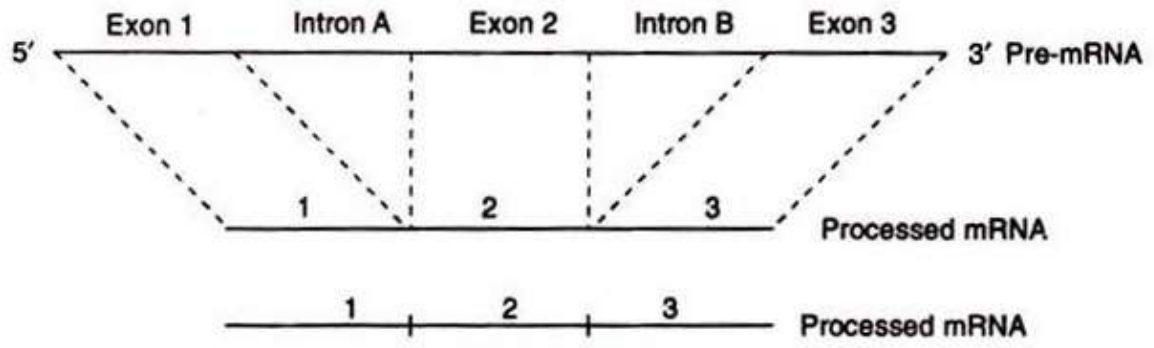

Fig. 16.1. Splicing.

The mRNA transcribed from this DNA is called precursor mRNA (pre-mRNA) and contains exons as well as introns. The introns are removed by excision and discarded. This process is known as splicing. The remaining segments or exons are joined together to form the mature mRNA which takes part in translation. The mature mRNA is much smaller than the pre-mRNA for example α-globin has two introns, ovalbumin has seven introns and α-collagen has 52 introns.

3. Overlapping Genes:

Most genes consist of DNA sequences that code for one protein. But there are some sequences that code for more than one protein. Fredrick Sanger discovered this phenomenon in bacteriophage φ x 174. Overlapping genes are common in many viruses. Here the small length of viral DNA is exploited for synthesizing different proteins.

This is achieved in different ways. In some cases, one gene generates two proteins by having different starting points. Similarly, the same gene generates two proteins by terminating the expression at different points.In other cases, a sequence of DNA makes no distinction between exons and introns. This sequence of DNA, which uses only exons for expression, also uses adjoining introns at other times for expression. The differential splicing of a single stretch of mRNA leads to overlapping and therefore different proteins. In this way, multiple proteins can be generated from a single stretch of DNA.

4. Jumping Genes or Transposons:

Earlier it was thought that genes are static and have definite and fixed locus. However, recently it has been discovered that segments of DNA can jump to new locations in the same or different chromosome. First of all it was discovered by Barbara MClintock in Indian maize corn. It has cobs with kernels of different colours. The light coloured kernels were caused by segments of DNA that move into genes coding for pigmented kernels, thereby inactivating pigmented kernels.

These mobile genes are called transposable elements or transposons. They can jump within the genome, thus affecting the gene expression. Transposable elements are components of moderately repetitive class of DNA.

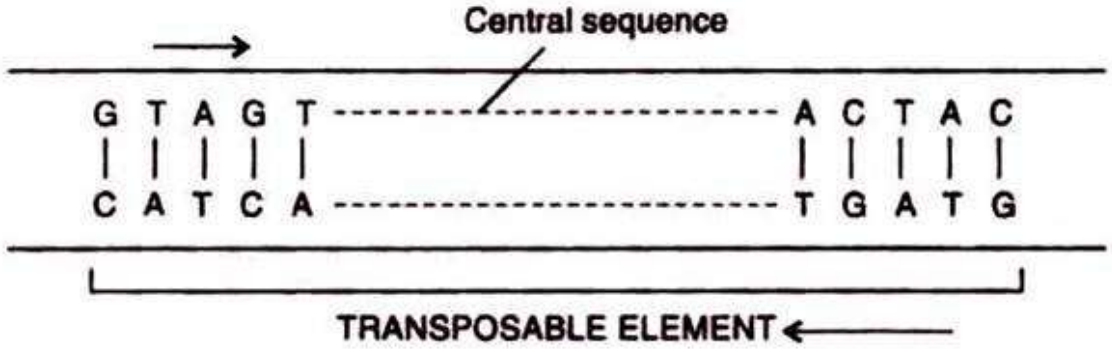

Fig. 16.2.

A transposon has well defined ends. It consists of a long central portion. On either end each transposon has specific sequence of bases which are inverted repeats or palindromes on opposite strands. These terminal repeats help in identifying transposons. The site where a transposon is inserted is called target site or recipient site.

Transposable elements can lead to change in the expression of genes. They can also cause mutations. In bacteria, they are present on plasmids.

5. Variable Genes:

Certain polypeptides are coded not by one gene but they are coded by more than one gene present on the same or different chromosomes.

Open Reading Frame:

A gene is a segment of genome which is transcribed into RNA. If the RNA is a transcript of a protein coding gene then it is called messenger RNA or mRNA. This is translated into protein. If the RNA is non-coding as ribosomal RNA (rRNA) or transfer RNA (tRNA) it is not translated.

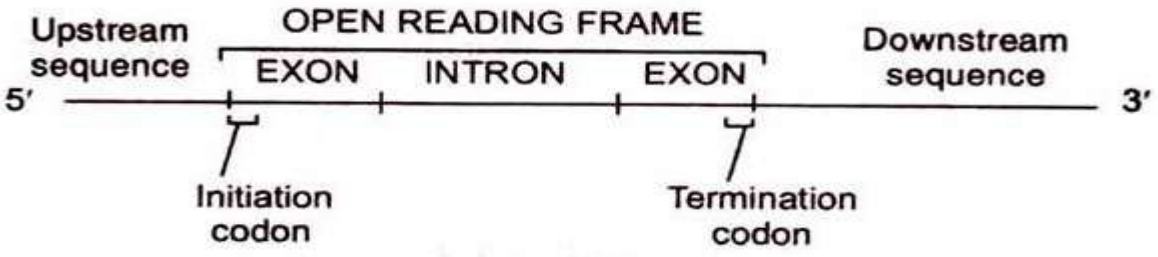

Fig. 16.3.

The part of the protein coding gene which is translated into protein is called open reading frame. It has triplet nucleotide codons. Open reading frame starts with an initiation codon and ends with a termination codon. The region of DNA before a gene is called up-ream region denoted with a minus (-) sign while region after the gene is called downstream denoted with a plus (+) sign. Many genes are split between exons and introns. The introns are removed by splicing to produce a functional RNA before translation.

6. Pseudogenes:

There are some DNA sequences, especially in eukaryotes, which are non-functional or defective copies of normal genes. These sequences do not have any function. Such DNA sequences or genes are known as pseudogenes. Pseudogenes have been reported in humans, mouse and Drosophila.

The main features of pseudogenes are given below:

1. Pseudogenes are non-functional or defective copies of some normal genes. These genes are found in large numbers.

2. These genes being defective cannot be translated.

3. These genes do not code for protein synthesis, means they do not have any significance.

4. The well-known examples of pseudogenes are alpha and beta globin pseudogenes of mouse.

Classification of Genes:

Genes can be classified in various ways. The classification of genes is generally done on the basis of:

(1) Dominance.

(2) Interaction,

(3) Character controlled,

(4) Effect on survival,

(5) Location,

(6) Movement,

(7) Nucleotide sequence,

(8) Sex linkage,

(9) Operon model, and

(10) Role in mutation.

A brief classification of genes on the basis of above criteria is presented in Table 13.4.

TABLE 13.4. Classification and brief description of genes

Classification of genes	*A brief description*
1. Based on Dominance	
Dominant genes	Genes that express in the F_1.
Recessive genes	Genes whose effect is suppressed in F_1.
2. Based on Interaction	
Epistatic gene	A gene that has masking effect on the other gene controlling the same trait.
Hypostatic gene	A gene whose expression is masked by another gene governing the same trait.
3. Based on Character Controlled	
Major gene	A gene that governs qualitative trait. Such genes have distinct phenotypic effects.
Minor gene	A gene which is involved in the expression of quantitative trait. Effect of such genes cannot be easily detected.
4. Based on Effect on Survival	
Lethal gene	A gene which leads to death of its carrier when in homozygous condition. It may be dominant or recessive.
Semilethal gene	A gene that causes mortality of more than 50% of its carriers.
Sub-vital gene	A gene that causes mortality of less than 50% of its carriers.
Vital gene	A gene that does not have lethal effect on its carriers.
5. Based on Location	
Nuclear genes	Genes that are found in nuclear genome in the chromosomes.
Plasma genes	Genes that are found in the cytoplasm in mitochondria and chloroplasts. Also called cytoplasmic or extranuclear genes.
6. Based on Position	
Normal genes	Genes that have a fixed position on the chromosomes. Most of the genes belong to this category.
Jumping genes	Genes which keep on changing their position on the chromosome of a genome. Such genes have been reported in maize.
7. Based on Nucleotide Sequence	
Normal genes	Genes having continuous sequence of nucleotides which code for a single polypeptide chain.
Split gene	A gene having discontinuous sequence of nucleotides. Such genes have been reported in some eukaryotes. The intervening sequences do not code for amino acids.
Pseudo genes	Genes having defective nucleotides which are non-functional. These genes are defective copies of some normal genes.
8. Based on Sex Linkage	
Sex linked genes	Genes which are located on sex or X-chromosomes.
Sex limited genes	Genes which express in one sex only.

Polymorphism of Mitochondrial DNA:

Mitochondrial DNA is a double stranded circular molecule, which is inherited from the mother in all multi-cellular organisms, though some recent evidence suggests that in rare instances mitochondria may also be inherited via a paternal route. Typically, a sperm carries mitochondria in its tail as an energy source for its long journey to the egg. When the sperm attaches to the egg during fertilization, the tail falls off. Consequently, the only mitochondria the new organism usually gets are from the egg its mother provided. There are about 2 to 10 transcripts of the mt-DNA in each mitochondrion. Compared to chromosomes, it is relatively smaller, and contains the genes in a limited number.

The size of mitochondrial genomes varies greatly among different organisms, with the largest found among plants, including that of the plant Arabidopsis, with a genome of 200 kbp in size and 57 protein-encoding genes. The smallest mtDNA genomes include that of the protist Plasmodium falciparum, which has a genome of only 6 kbp and just 2 protein- encoding genomes. Humans and other animals have a mitochondrial genome size of 17 kbp and 13 protein genes.

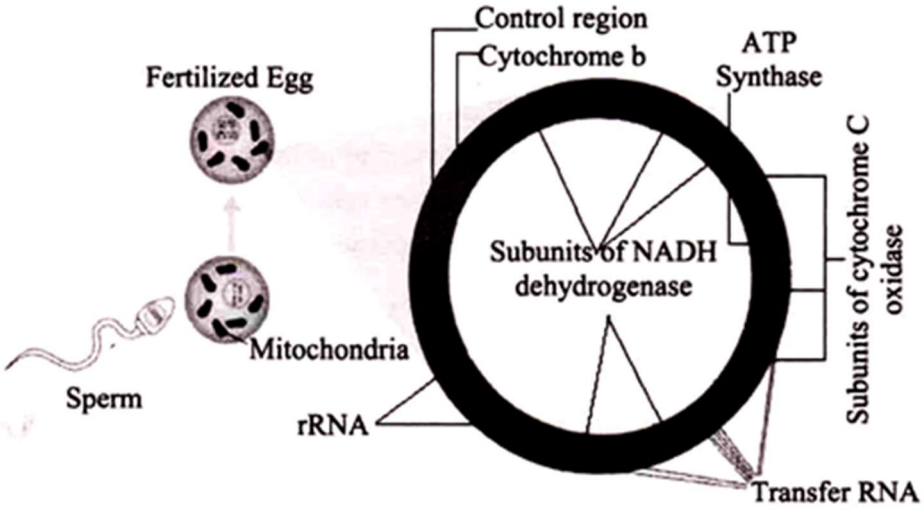

Figure 4.56: Mitochondrial DNA

Mitochondrial DNA consists of 5-10 rings of DNA and appears to carry 16,569 base pairs with 37 genes (13 proteins, 22 t-RNAs and two r-RNA) which are concerned with the production of proteins involved in respiration. Out of the 37 genes, 13 are responsible for making enzymes, involved in oxidative phosphorylation, a process that uses oxygen and sugar to produce adenosine tri-phosphate (Fig. 4.56). The other 14 genes are responsible for making molecules, called transfer RNA (t-RNA) and ribosomal RNA (r-RNA). In some metazoans, there are about 100 – 10,000 separate copies of mt-DNA present in each cell.

Unlike nuclear DNA, mitochondrial DNA doesn't get shuffled every generation, so it is presumed to change at a slower rate, which is useful for the study of human evolution. Mitochondrial DNA is also used in forensic science as a tool for identifying corpses or body parts and has been implicated in a number of genetic diseases, such as Alzheimer's disease and diabetes. Changes in mt-DNA can cause maternally inherited diseases, which leads to faster aging process and genetic disorders.

Mitochondria convert the potential energy of food molecules into ATP by the Krebs cycle, electron transport and oxidative phosphorylation in presence of oxygen. The energy from food molecules (e.g., glucose) is used to produce NADH and $FADH_2$ molecules, via glycolysis and the Krebs cycle. The protein complexes in the inner membrane (NADH dehydrogenase, cytochrome c reductase, cytochrome c oxidase) use the released energy to pump protons (FT) against a gradient

Mitochondrial DNA:

Each human cell contains hundreds of mitochondria each containing multiple copies of mitochondrial DNAs (mtDNA). Mitochondria generate cellular energy through the process of oxidative phosphorylation. As a by-product they produce most of the endogenous toxic reactive oxygen species Mitochondria are also the central regulators of apoptosis or programmed cell death.

These interrelated functional systems involve activities of about 1000 genes distributed in the nuclear genome and the mitochondrial genome. Due to their dependence on the nuclear genome, mitochondria are considered as semi-autonomous. This has been shown by experiments in which mitochondria and

mtDNA could be transferred from one cell to another. The donor cell was enucleated and its mitochondria-containing cytoplast fused with a recipient cell (technique of cybrid transfer).

The genomes of mitochondria show wide variation particularly among plants and protists. Most mitochondrial DNAs (mtDNA) consist of a closed circular double stranded supercoiled DNA molecules located in multiple nucleoid regions (similar to those in bacterial cells); some protists however, have varying lengths or multiple circular molecules of DNA as in the trypanosomes. mtDNA in the protist *Amoebidium parasiticum* consists of several distinct types of linear molecules with terminal and sub-terminal repeats. Although most mtDNAs are in the size range of 15 to 60 kb, mtDNA in malarial parasite (Plasmodium spp) is only 6 kb long, while that of rice (Oryza sativa) is 490 kb, and cucurbits 2 Mb. There are about 40 to 50 coding genes in mitochondrial DNA, Plasmodium being an exception with 5 coding genes.

The large size of mitochondrial genomes in plants are due to noncoding inter-genic regions and their content of tandem repeats. Introns are present in many mtDNAs, and in some unusual cases, the genes are split into as many as 8 regions that are dispersed in the genome, and located on both strands of the DNA. Transcription takes place separately in portions of the split genes producing discrete pieces of RNA that are held together by base pairing of complementary sequences. The mtDNA contains information for a number of mitochondrial compounds such as tRNAs, rRNA, and some of the polypeptide subunits of the proteins cytochrome oxidase, NADH- dehydrogenase and ATPase. Most of the other proteins found in mitochondria are encoded by the nuclear genome and transported into mitochondria.These include DNA polymerase and other proteins for mtDNA replication, RNA polymerase and other proteins for transcription, ribosomal proteins for ribosome assembly, protein factors for translation, and the aminoacyl-tRNA synthetases.T he mitochondrial oxidative phosphorylation complexes are composed of multiple polypeptides, mostly encoded by the nuclear DNA (nDNA). However, 13 polypeptides are encoded by mtDNA. The mtDNA also codes for 12S and 16S rRNAs and 22 tRNAs required for mitochondrial protein synthesis. The mtDNA also contains a control region consisting of approximately 1000 base pairs constituting the promoter region and the origin of replication.

The mRNAs synthesised within the mitochondria remain in the organelle and are translated by mitochondrial ribosomes that are assembled within mitochondria. Mitochondrial ribosomes have two subunits. Mitochondria in human cells have 60S ribosomes consisting of a 45S and a 35S subunit.There are only two rRNAs in mitochondrial ribosomes of most organisms, that is, 16S rRNA in large subunit and 12S rRNA in small subunit of most animal ribosomes. There is usually one gene for each rRNA in a mitochondrial genome. The proteins in mitochondrial ribosomes are encoded by the nuclear genome and transported into mitochondria from the cytoplasm.

Mitochondrial ribosomes are sensitive to most of the inhibitors of bacterial ribosome function such as streptomycin, neomycin and chloramphenicol. For protein synthesis, mitochondria of most organisms use a genetic code that shows differences from the universal genetic code. Only plant mitochondria use the universal nuclear genetic code. Transcription of mammalian mtDNA is unusual in that each strand is transcribed into a single RNA molecule that is then cut into smaller pieces. In the large RNA transcripts that are produced, most of the genes encoding the rRNAs and the mRNAs are separated by tRNA gene.

The tRNAs in the transcript are recognised by specific enzymes and are cut out, leaving only the mRNAs and the rRNAs. A poly (A) tail is then added to the 3'end of each mRNA and CCA is added to the 3'end of each tRNA. There are no 5′ caps in mitochondrial mRNAs. Mitochondrial DNA replication is semi-conservative and uses DNA polymerases that are specific to the mitochondria. The mtDNA replicates throughout the cell cycle, independently of nuclear DNA synthesis which takes place in S phase of cell cycle. Observations on mtDNA replication in animal mitochondria in vivo have resulted in a model referred to as the displacement loop (D loop) model as follows (Fig. below).

The two strands of mtDNA in most animals have different densities because the bases are not equally distributed on both strands, called H (heavy) and L (light) strands. The synthesis of a new H strand starts at the replication origin for the H strand and forms a D-loop structure (Fig. below).As the new H strand extends to about halfway around the molecule, initiation of synthesis of a new L strand takes place at a second replication origin. Synthesis continues until both strands are completed. Finally, each circular DNA assumes a supercoiled form.

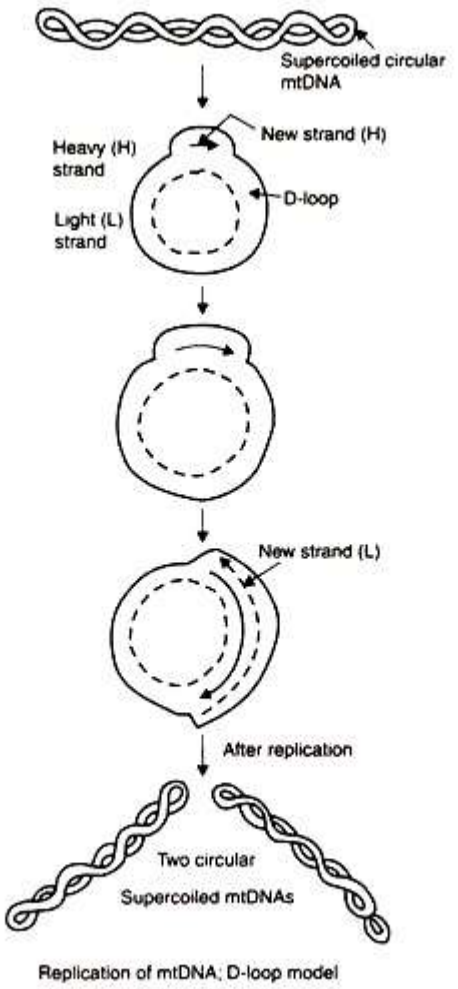

Fig. 17.6 Model for mitochondrial DNA replication by formation of a D-loop structure.

The mtDNA is maternally inherited and has a very high mutation rate. When a new mtDNA mutation occurs in a cell, a mixed intracellular population of mtDNAs is generated, known as heteroplasmy. During replication in a heteroplasmic cell, the mutant and normal molecules are randomly distributed into daughter cells.

When the percentage of mutant mtDNAs increases, the mitochondrial energy producing capacity declines, production of toxic reactive oxygen species increases, and cells become more prone for apoptosis. The result is mitochondrial dysfunction. Tissues most sensitive to mitochondrial dysfunction are brain, heart, kidney and skeletal muscle.The mtDNA mutations are associated with a variety of neuromuscular disease symptoms, including various ophthalmological symptoms, muscle degeneration, cardiovascular diseases, diabetes mellitus, renal function and dementias.

The mtDNA diseases can be caused either by base substitutions or rearrangement mutation. Base substitution mutations can either alter protein (missense mutation) or rRNAs and tRNAs (protein synthesis mutations). Rearrangement mutations generally delete at least one tRNA and thus cause

protein synthesis defects. Missense mutations are associated with myopathy, optic atrophy, dystonia and Leigh's syndrome. Base substitution mutations in protein synthesizing genes have been associated with a wide spectrum of neuromuscular diseases, and the more severe typically include mitochondrial myopathy. Mitochondrial diseases are also associated with a number of different nuclear DNA mutations. Mutations in the RNA component of the mitochondrial RNase have been implicated in metaphyseal chondrodysplasia or cartilage hair hypoplasia which is an autosomal recessive disorder resulting from mutation in nuclear chromosome 9 short arm position (9p13).

Y-Chromosome Polymorphism:

The Y chromosome is one of two sex chromosomes (allosomes) in mammals, including humans, and many other animals. The other is the X chromosome. Y is the sex-determining chromosome in many species, since it is the presence or absence of Y that determines the male or female sex of offspring produced in sexual reproduction. In mammals, the Y chromosome contains the gene SRY, which triggers testis development. The DNA in the human Y chromosome is composed of about 59 million base pairs. The Y chromosome is passed only from father to son. With a 30% difference between humans and chimpanzees, the Y chromosome is one of the fastest-evolving parts of the human genome. To date, over 200 Y-linked genes have been identified. All Y-linked genes are expressed and (apart from duplicated genes) hemizygous (present on only one chromosome) except in the cases of aneuploidy such as XYY syndrome or XXYY syndrome.

The following are some of the gene count estimates of human Y chromosome. Because researchers use different approaches to genome annotation their predictions of the number of genes on each chromosome varies (for technical details, see gene prediction). Among various projects, the collaborative consensus coding sequence project (CCDS) takes an extremely conservative strategy. So CCDS's gene number prediction represents a lower bound on the total number of human protein-coding genes. In human genetics, a human Y-chromosome DNA haplogroup is a haplogroup defined by mutations in the non-recombining portions of DNA from the Y-chromosome (called Y-DNA). Mutations that are shared by many people are called single-nucleotide polymorphisms (SNPs). Schematic illustration of Y-DNA haplogroups naming convention. Haplogroups are defined through mutations (SNPs).

The human Y-chromosome accumulates roughly two mutations per generation. Y-DNA haplogroups represent major branches of the Y-chromosome phylogenetic tree that share hundreds or even thousands of mutations unique to each haplogroup.

The Y-chromosomal most recent common ancestor (Y-MRCA, informally known as Y chromosomal Adam) is the most recent common ancestor (MRCA) from whom all currently living men are descended patrilineally. Y-chromosomal Adam is estimated to have lived roughly 236,000 years ago in Africa. By examining other bottlenecks most Eurasian men are descended from a man who lived 69,000 years ago. Other major bottlenecks occurred about 5,000 years ago and subsequently most Eurasian men can trace their ancestry back to a dozen ancestors who lived 5,000 years ago.

Y-DNA haplogroups are defined by the presence of a series of Y-DNA SNP markers. Subclades are defined by a terminal SNP, the SNP furthest down in the Y-chromosome phylogenetic tree. The Y Chromosome Consortium (YCC) developed a system of naming major Y-DNA haplogroups with the capital letters A through T, with further subclades named using numbers and lower case letters (YCC longhand nomenclature). YCC shorthand nomenclature names Y DNA haplogroups and their subclades with the first letter of the major Y-DNA haplogroup followed by a dash and the name of the defining terminal SNP. Y-DNA haplogroup nomenclature is changing over time to accommodate the increasing number of SNPs being discovered and tested, and the resulting expansion of the Y-chromosome phylogenetic tree. This change in nomenclature has resulted in inconsistent nomenclature beingused in different sources. This inconsistency, and increasingly cumbersome longhand nomenclature, has prompted a move towards using the simpler shorthand nomenclature.

Recent innovations have included the creation of primers targeting polymorphic regions on the Y-chromosome (Y-STR), which allows resolution of a mixed DNA sample from a male and female or cases in which a differential extraction is not possible. Y-chromosomes are paternally inherited, so Y-STR analysis can help in the identification of paternally related males. Y-STR analysis was performed in the Sally Hemings controversy to determine if Thomas Jefferson had sired a son with one of his

slaves. The analysis of the Y-chromosome yields weaker results than autosomal chromosome analysis. The Y male sex-determining chromosome, as it is inherited only by males from their fathers, is almost identical along the patrilineal line. This leads to a less precise analysis than if autosomal chromosomes were testing, because of the random matching that occurs between pairs of chromosomes as zygotes are being made

Probable Questions:

1. What do you mean by polymorphism? Give suitable examples.
2. Define balanced polymorphism. What are the main features of it?
3. Define transient polymorphism. What are the main features of it?
4. Define neutral polymorphism. What are the main features of it?
5. Define regional polymorphism. What are the main features of it?
6. What are the causes of genetic polymorphism?
7. Define neutral mutation theory and selection theory.
8. What are the advantages of genetic polymorphism?
9. What are the disadvantages of genetic polymorphism?
10. Define split genes and overlapping genes.
11. Define pseudogenes and jumping genes.
12. Write a brief note on mitochondrial DNA polymorphism.
13. Write a brief note on Y chromosome DNA polymorphism.

Suggested Readings:

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7th Edition.
6. Gene cloning and DNA Analysis by T. A. Brown, Sixth Edition.
7. Genetics . Verma and Agarwal

Unit-II

Genetics in forensic science: DNA comparisons, RFLPs, genetic finger-printing, VNTRs, and Single nucleotide polymorphism (SNP). Genetic profiles. Protein comparisons.

Objective: In this unit you will learn about Protein comparisons, DNA comparisons, RFLPs, genetic finger-printing, VNTRs, and Single nucleotide polymorphism (SNP).

Meaning of DNA Polymorphisms:

Different alleles of a gene produce different phenotypes which can be detected by making crosses between parents with different alleles of two or more genes. Then by determining recombinants in the progeny, a genetic map can be deduced.

These are low resolution genetic maps that contain genes with observable phenotypic effects, all mapped to their respective loci. The position of a specific gene, or locus can be found from the map. However, measurements showed that the chromosomal intervals between the mapped genes would contain vast amounts of DNA.

These intervals could not be mapped by the recombinant progeny method because there were no markers in those intervening regions. It became necessary to find additional differential markers or genetic differences that fall in the gaps. This need was met by exploitation of various polymorphic DNA markers.A DNA polymorphism is a DNA sequence variation that is not associated with any observable phenotypic variation, and can exist anywhere in the genome, not necessarily in a gene. Polymorphism means one of two or more alternative forms (alleles) of a chromosomal region that either has a different nucleotide sequence, or it has variable numbers of tandemly repeated nucleotides.

Thus, it is a site of heterozygosity for any sequence variation. Many DNA polymorphisms are useful for genetic mapping studies, hence they are referred to as DNA markers. DNA markers can be detected on Southern blot hybridisation or by PCR. The alleles of DNA markers are co-dominant, that is they are neither dominant nor recessive as observed in alleles of most genes. DNA polymorphisms constitute molecularly defined differences between individual human beings.

DNA Fingerprinting:

DNA fingerprinting is the present day genetic detective in the practice of modern medical forensics. The underlying principles of DNA fingerprinting are briefly described. The structure of each person's genome is unique. The only exception being monozygotic identical twins (twins developed from a single fertilized ovum).

The unique nature of genome structure provides a good opportunity for the specific identification of an individual. It may be remembered here that in the traditional fingerprint technique, the individual is identified by preparing an ink impression of the skin folds at the tip of the person's finger. This is based on the fact that the nature of these skin folds is genetically determined, and thus the fingerprint

is unique for an individual. In contrast, the DNA fingerprint is an analysis of the nitrogenous base sequence in the DNA of an individual.

History and Terminology:

The original DNA fingerprinting technique was developed by Alec Jaffrey's in 1985. Although the DNA fingerprinting is commonly used, a more general term DNA profiling is preferred. This is due to the fact that a wide range of tests can be carried out by DNA sequencing with improved technology.

Applications of DNA Fingerprinting:

The amount of DNA required for DNA fingerprint is remarkably small. The minute quantities of DNA from blood strains, body fluids, and hair fiber or skin fragments are enough. Polymerase chain reaction is used to amplify this DNA for use in fingerprinting. DNA profiling has wide range of applications—most of them related to medical forensics.

Some important ones are listed below:

i. Identification of criminals, rapists, thieves etc.

ii. Settlement of paternity disputes.

iii. Use in immigration test cases and disputes.

In general, the fingerprinting technique is carried out by collecting the DNA from a suspect (or a person in a paternity or immigration dispute) and matching it with that of a reference sample (from the victim of a crime, or a close relative in a civil case).

DNA Markers in Disease Diagnosis and Fingerprinting:

The DNA markers are highly useful for genetic mapping of genomes. There are four types of DNA sequences which can be used as markers.

1. Restriction fragment length polymorphisms (RFLF).

2. Minisatellites or variable number tandem repeats (VNTR).

3. Microsatellites or simple tandem repeats (STRs).

4. Single nucleotide polymorphisms (SNPs, pronounced as snips).

The general aspects of the above DNA markers are described along with their utility in disease diagnosis and DNA fingerprinting.

Restriction Fragment Length Polymorphisms (RFLPs):

A RFLP represents a stretch of DNA that serves as a marker for mapping a specified gene. RFLPs are located randomly throughout a person's chromosomes and have no apparent function. A DNA molecule can be cut into different fragments by a group of enzymes called restriction endonucleases .These fragments are called polymorphisms (literally means many forms).

An outline of RFLP is depicted in Fig. 14.5. The DNA molecule 1 has three restriction sites (R_1, R_2, R_3), and when cleaved by restriction endonucleases forms 4 fragments. Let us now consider DNA 2 with an inherited mutation (or a genetic change) that has altered some base pairs. As a result, the site (R_2) for the recognition by restriction endonuclease is lost. This DNA molecule 2 when cut by restriction endonuclease forms only 3 fragments (instead of 4 in DNA 1).

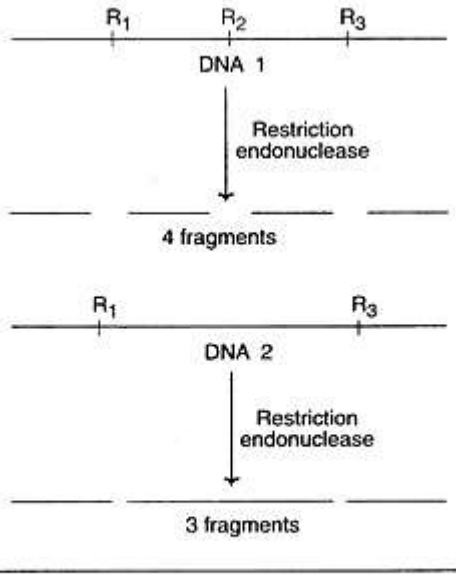

Fig. 14.5 : An outline of the restriction fragment length polymorphism (RFLP) (R_1, R_2, R_3 represent the sites for the action of restriction endonucleases).

As is evident from the above description, a stretch of DNA exists in fragments of various lengths (polymorphisms), derived by the action of restriction enzymes, hence the name restriction fragment length polymorphisms.

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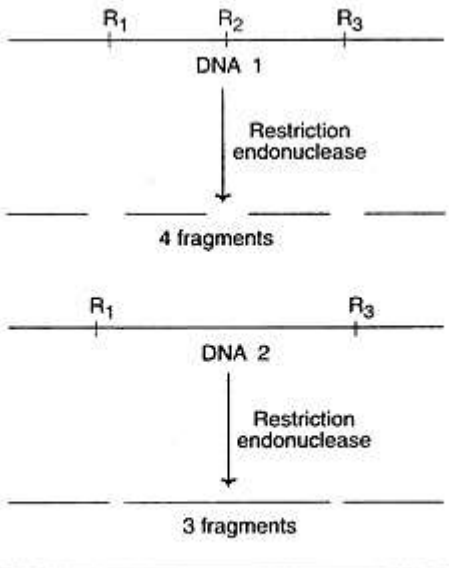


Fig. 14.5 : An outline of the restriction fragment length polymorphism (RFLP) (R_1, R_2, R_3 represent the sites for the action of restriction endonucleases).

As is evident from the above description, a stretch of DNA exists in fragments of various lengths (polymorphisms), derived by the action of restriction enzymes, hence the name restriction fragment length polymorphisms.

RFLPs in the Diagnosis of Diseases:

If the RFLP lies within or even close to the locus of a gene that causes a particular disease, it is possible to trace the defective gene by the analysis of RFLP in DNA. The person's cellular DNA is isolated and treated with restriction enzymes. The DNA fragments so obtained are separated by electrophoresis.

The RFLP patterns of the disease suspected individuals can be compared with that of normal people (preferably with the relatives in the same family). By this approach, it is possible to determine whether the individual has the marker RFLP and the disease gene. With 95% certainty, RFLPs can detect single gene-based diseases.

Methods of RFLP scoring:

Two methods are in common use for the detection of RFLPs (Fig. 14.5).

1. Southern hybridization:

The DNA is digested with appropriate restriction enzyme, and separated by agarose gel electrophoresis. The so obtained DNA fragments are transferred to a nylon membrane. A DNA probe that spans the suspected restriction site is now added, and the hybridized bands are detected by autoradiograph. If the restriction site is absent, then only a single restriction fragment is detected. If the site is present, then two fragments are detected (Fig. 14.6A).

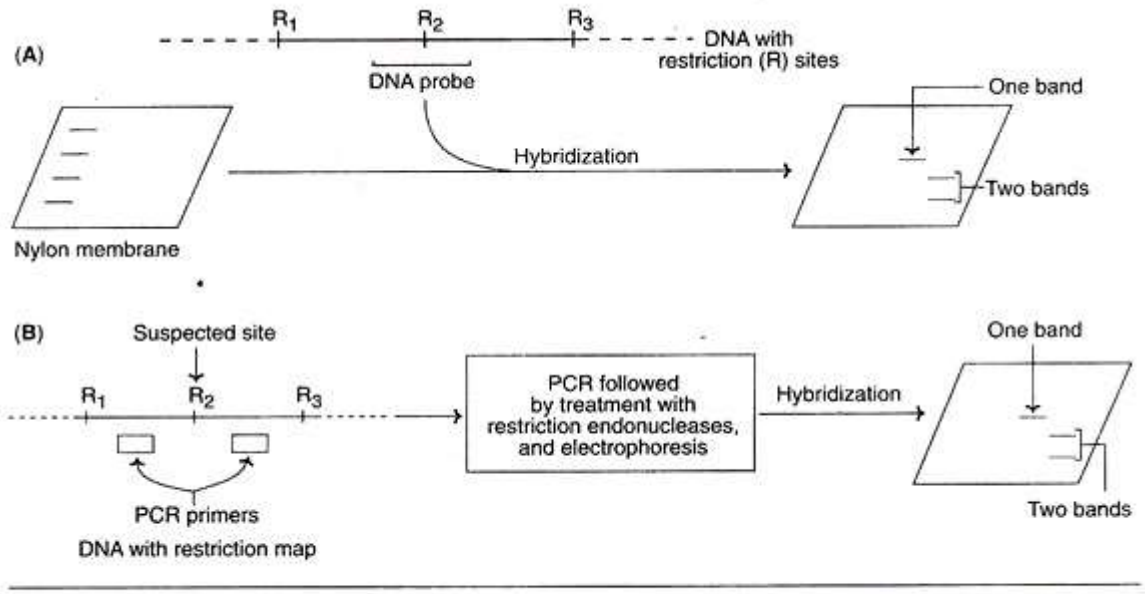


Fig. 14.6 : Two common methods used for scoring restriction fragment length polymorphism (RFLP) (A) RFLP by Southern hybridization (B) RFLP by polymerase chain reaction (PCR).

2. Polymerase chain reaction:

RFLPs can also be scored by PCR. For this purpose, PCR primers that can anneal on either side of the suspected restriction site are used. After amplification by PCR, the DNA molecules are treated with restriction enzyme and then analysed by agarose gel electrophoresis. If the restriction site is absent only one band is seen while two bands are found if the site is found (Fig. 14.6B).

Applications of RFLPs:

The approach by RFLP is very powerful and has helped many genes to be mapped on the chromosomes, e.g. sickle- cell anaemia (chromosome 11), cystic fibrosis (chromosome 7), Huntington's disease (chromosome 4), retinoblastoma (chromosome 13), Alzheimer's disease (chromosome 21).

Variable Number Tandem Repeats (VNTRs):

VNTRs, also known as mini-satellites, like RFLPs, are DNA fragments of different length. The main difference is that RFLPs develop from random mutations at the site of restriction enzyme activity while VNTRs are formed due to different number of base sequences between two points of a DNA molecule. In general, VNTRs are made up of tandem repeats of short base sequences (10-100 base pairs). The number of elements in a given region may vary, hence they are known as variable number tandem repeats.

An individual's genome has many different VNTRs and RFLPs which are unique to the individual. The pattern of VNTRs and RFLPs forms the basis of DNA fingerprinting or DNA profiling. In the Fig. 14.7, two different DNA molecules with different number of copies (bands) of VNTRs are shown. When these molecules are subjected to restriction endonuclease action (at two sites R_1 and R_2), the VNTR sequences are released, and they can be detected due to variability in repeat sequence copies. These can be used in mapping of genomes, besides their utility in DNA fingerprinting.

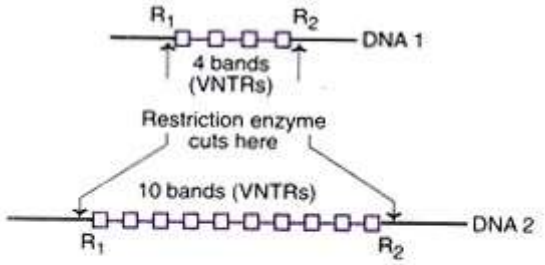


Fig. 14.7 : A diagrammatic representation of variable number tandem repeats (VNTRs). Each band (or copy) represents a repeating sequence in the DNA (e.g. 100 base pairs each). R_1 and R_2 indicate the sites cut by a restriction enzyme.

VNTRs are useful for the detection of certain genetic diseases associated with alterations in the degree of repetition of microsatellites e.g. Huntington's chorea is a disorder which is found when the VNTRs exceed 40 repeat units.

Limitations of VNTRs:

The major drawback of VNTRs is that they are not evenly distributed throughout the genome. VNTRs tend to be localized in the telomeric regions at the ends of the chromosomes.

Use of RFLPs and VNTRs in Genetic Fingerprinting:

RFLPs caused by variations in the number of VNTRs between two restriction sites can be detected (Fig. 14.8). The DNAs from three individuals with different VNTRs are cut by the specific restriction endonuclease. The DNA fragments are separated by electrophoresis, and identified after hybridization with a probe complementary to a specific sequence on the fragments.

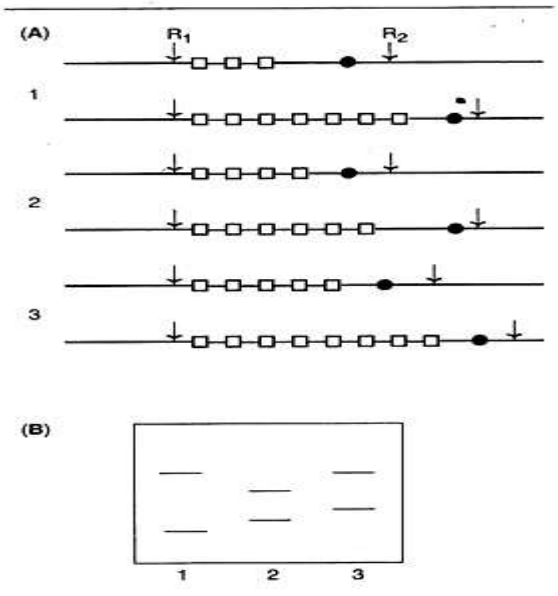
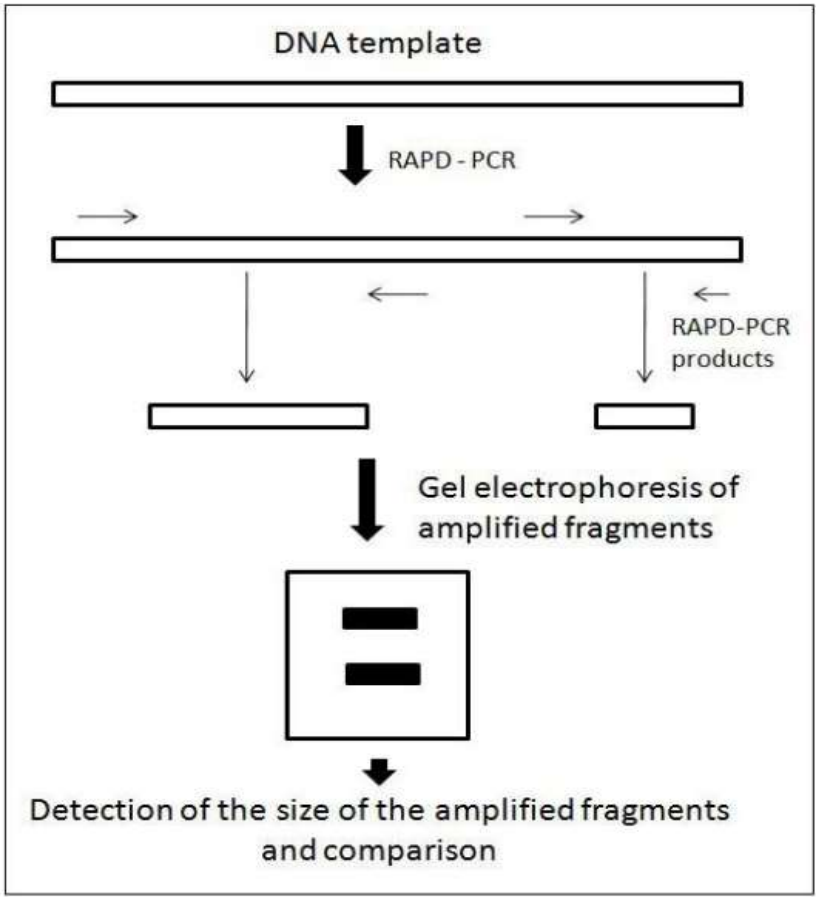


Fig. 14.8 : Use of restriction fragment length polymorphisms (RFLPs) caused by variable number tandem repeats (VNTRs) in genetic fingerprinting (A) An illustration of DNA structure from three individuals (B) Hybridized pattern of DNA fragment with a probe complementary to the sequence shown in black circles (1, 2 and 3 represent the individuals; R_1 and R_2 indicate restriction sites; coloured squares are the number of VNTRs) .

Randomly Amplified Polymorphic DNA:

RAPDs are based on random PCR amplification. The procedure is carried out by randomly designing primers for PCR which will amplify several different regions of the genome by chance. Such a primer results in amplification of only those DNA regions that have near them, inverted copies of the primer's own sequence.

The PCR products consist of DNA bands representing different sizes of the amplified DNA. The set of amplified DNA fragments is called randomly amplified polymorphic DNA (RAPD). Certain bands may be unique for an individual and can serve as DNA markers in mapping analysis.



Amplified fragment length polymorphism (AFLP):

AFLP is a novel technique involving a combination of RFLP and RAPD. AFLP is based on the principle of generation of DNA fragments using restriction enzymes and oligonucleotide adaptors (or linkers), and their amplification by PCR. Thus, this technique combines the usefulness of restriction digestion and PCR.

The DNA of the genome is extracted. It is subjected to restriction digestion by two enzymes (a rare cutter e.g. MseI; a frequent cutter e.g. EcoRI). The cut ends on both sides are then ligated to known sequences of oligonucleotides (Fig. 53.5).

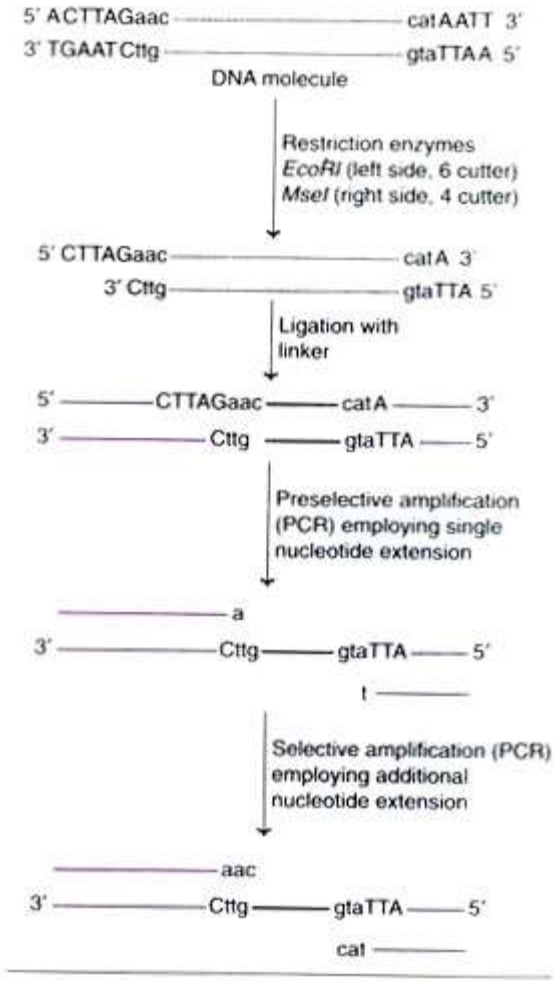


***Fig. 53.5** : A diagrammatic representation of the amplified fragment length polymorphism (AFLP)*
***(Note** : The lower case letters represent the sequences found within the amplified region; the coloured lines indicate linkers).*

PCR is now performed for the pre-selection of a fragment of DNA which has a single specific nucleotide. By this approach of pre-selective amplification, the pool of fragments can be reduced from the original mixture. In the second round of amplification by PCR, three nucleotide sequences are amplified.

This further reduces the pool of DNA fragments to a manageable level (< 100). Autoradiography can be performed for the detection of DNA fragments. Use of radiolabeled primers and fluorescently labeled fragments quickens AFLP.AFLP analysis is tedious and requires the involvement of skilled technical personnel. Hence some people are not in favour of this technique. In recent years, commercial kits are made available for AFLP analysis. AFLP is very sensitive and reproducible. It does not require prior knowledge of sequence information. By AFLP, a large number of polymorphic bands can be produced and detected.

Microsatellites (Simple Tandem Repeats):

Microsatellites are short repeat units (10-30 copies) usually composed of dinucleotide or tetra nucleotide units. These simple tandem repeats (STRs) are more popular than mini-satellites (VNTRs) as DNA markers for two reasons.

1. Microsatellites are throughout the genome.

2. PCR can be effectively and conveniently used to identify the length of polymorphism.

Two variants (alleles) of DNA molecules with 5 and 10 repeating units of a dimer nucleotides (GA) are depicted in Fig. 14.9.

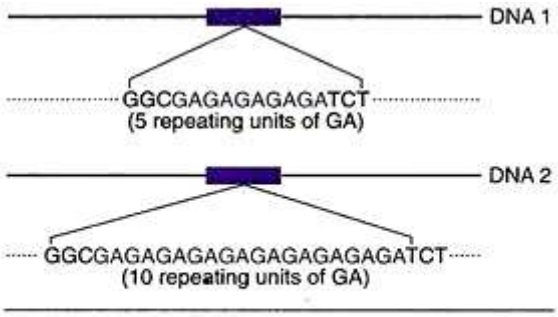


Fig. 14.9 : Two alleles of DNA molecules representing 5 and 10 dimer repeating units.

By use of PCR, the region surrounding the microsatellites is amplified, separated by agarose gel electrophoresis and identified.

Single Nucleotide Polymorphisms (SNPs):

SNPs represent the positions in the genome where some individuals have one nucleotide (e.g. G) while others have a different nucleotide (e.g. C). There are large numbers of SNPs in genomes. It is estimated that the human genome contains at least 3 million SNPs. Some of these SNPs may give rise to RFLPs. SNPs are highly useful as DNA markers since there is no need for gel electrophoresis and this saves a lot of time and labour. The detection of SNPs is based on the oligonucleotide hybridization analysis (Fig. 14.10).

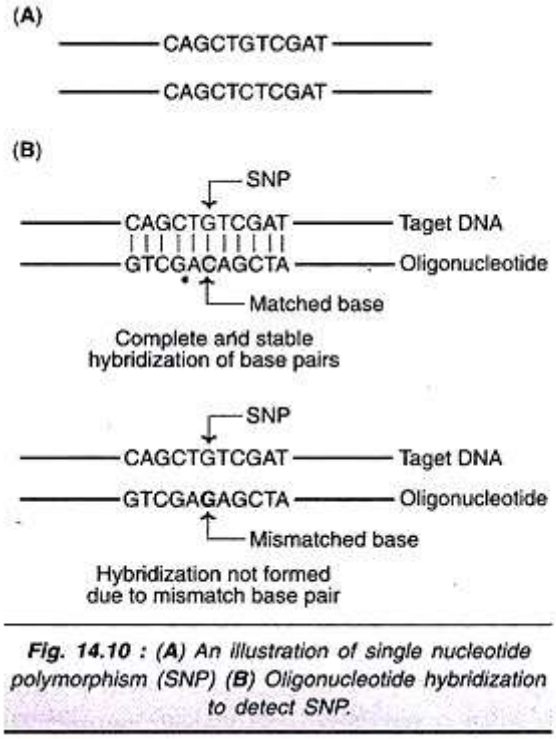


Fig. 14.10 : (A) An illustration of single nucleotide polymorphism (SNP) (B) Oligonucleotide hybridization to detect SNP.

An oligonucleotide is a short single-stranded DNA molecule synthesized in the laboratory with a length not usually exceeding 50 nucleotides. Under appropriate conditions, this nucleotide sequence

will hybridize with a target DNA strand if both have completely base paired structure. Even a single mismatch in base pair will not allow the hybridization to occur. DNA chip technology is most commonly used to screen SNPs hybridization with oligonucleotide. About one-half of missense mutations that are SNPs are estimated to cause genetic disease in humans. A non-coding SNP can also affect gene function if it is located in the promoter region or in the gene regulatory region. A small number of SNPs can create a restriction site, or eliminate an already existing restriction site. SNP-induced alterations in restriction sites are detected by using the restriction enzyme followed by Southern blot analysis or PCR.

An individual SNP locus can be analysed by using the technique of allele-specific oligonucleotide (ASO) hybridisation. The search for one particular SNP locus in humans is a challenge, because this is one base pair that is polymorphic out of the three billion base pairs in the human genome.In the ASO technique, a short oligonucleotide that is complementary to one SNP allele is synthesised and mixed with the target DNA. Hybridisation is performed under high stringency conditions that would allow only a perfect match between probe and the target DNA. That means, the oligonucleotide will not hybridize with target DNA that has any other SNP allele at that locus. Positive result of hybridisation indicates the SNP locus precisely. A more recent technique of DNA Microarrays can be used for simultaneous typing of hundreds or thousands of SNPs. Details of this technique used for SNPs and genome wide gene expression are described later in this section.

Current Technology of DNA Fingerprinting:

In the forensic analysis of DNA, the original techniques based on RFLPs and VNTRs are now largely replaced by microsatellites (short tandem repeats). The basic principle involves the amplification of microsatellites by polymerase chain reaction followed by their detection. It is now possible to generate a DNA profile by automated DNA detection system (comparable to the DNA sequencing

Genetic Profiling: DNA profiling (also called DNA fingerprinting) is the process of determining an individual's DNA characteristics, which are as unique as fingerprints. DNA analysis intended to identify a species, rather than an individual, is called DNA barcoding.

DNA profiling is a forensic technique in criminal investigations, comparing criminal suspects' profiles to DNA evidence so as to assess the likelihood of their involvement in the crime. It is also used in parentage testing, to establish immigration eligibility, and in genealogical and medical research. DNA profiling has also been used in the study of animal and plant populations in the fields of zoology, botany, and agriculture. Starting in the 1980s scientific advances allowed for the use of DNA as a mechanism for the identification of an individual. The first patent covering the modern process of DNA profiling was filed by Dr. Jeffrey Glassberg in 1983, based upon work he had done while at Rockefeller University in 1981. Glassberg, along with two medical doctors, founded Lifecodes Corporation to bring this invention to market. The Glassberg patent was issued in Belgium BE899027A1, Canada FR2541774A1, Germany DE3407196 A1, Great Britain GB8405107D0, Japan JPS59199000A, United States as US5593832A. In the United Kingdom, Geneticist Sir Alec Jeffreys independently developed a DNA profiling process in beginning in late 1984 while working in the Department of Genetics at the University of Leicester.

The process, developed by Jeffreys in conjunction with Peter Gill and Dave Werrett of the Forensic Science Service (FSS), was first used forensically in the solving of the murder of two teenagers who had been raped and murdered in Narborough, Leicestershire in 1983 and 1986. In the murder inquiry, led by Detective David Baker, the DNA contained within blood samples obtained voluntarily from around 5,000 local men who willingly assisted Leicestershire Constabulary with the investigation, resulted in the exoneration of Richard Buckland, an initial suspect who had confessed to one of the crimes, and the subsequent conviction of Colin Pitchfork on January 2, 1988. Pitchfork, a local bakery employee, had coerced his co-worker Ian Kelly to stand in for him when providing a blood sample—

Kelly then used a forged passport to impersonate Pitchfork. Another co-worker reported the deception to the police. Pitchfork was arrested, and his blood was sent to Jeffrey's lab for processing and profile development. Pitchfork's profile matched that of DNA left by the murderer which confirmed Pitchfork's presence at both crime scenes; he pleaded guilty to both murders.

Although 99.9% of human DNA sequences are the same in every person, enough of the DNA is different that it is possible to distinguish one individual from another, unless they are monozygotic (identical) twins. DNA profiling uses repetitive sequences that are highly variable, called variable number tandem repeats (VNTRs), in particular short tandem repeats (STRs), also known as microsatellites, and minisatellites. VNTR loci are similar between closely related individuals, but are so variable that unrelated individuals are unlikely to have the same VNTRs.

In India DNA fingerprinting was started by Dr. VK Kashyap and Dr. Lalji Singh. Singh was an Indian scientist who worked in the field of DNA fingerprinting technology in India, where he was popularly known as the "Father of Indian DNA fingerprinting". In 2004, he received the Padma Shri in recognition of his contribution to Indian science and technology.

Proteomics:

The term proteomics was coined in mid 1990s at the back drop of successful genomics. In bioinformatics point of view proteomics is the databases of protein sequence, databases of predicted protein structures and more recently, databases of protein expression analysis. As more protein structures are identified, the relationship between structure and functions became easier to predict.

In addition, databases of protein structure and corporating tools facilitating the identification of common protein structure and their predicted functions. In this technique individually purified ligands such as proteins, peptides, antibodies, antigens, and carbohydrates are spotted on to a derivatized surface and are generally used for examining protein expression levels for protein profiling. A major challenge facing plant biotechnology and other bioinformatics research community is the translation of complete genome DNA sequence data into protein structure and predicted functions. Such a steps will provide the key link between the genotypes of an organism and its expressed phenotype.

The growth of proteomics is a direct result of advances made in large scale nucleotide sequencing of expressed sequence tags (EST). Although mass spectrometry or more popularly MS technology has been considered as versatile tool for examining simultaneous expression of more than 1000 proteins and identification, mapping of post-translational modifications (Table 25.5).These methods performed in a latest array of technology resulted in large-scale characterization of protein location, protein-protein interaction and protein functions.

Table 25.5 Proteomics tools

Method	Description	Applications
1. Mass spectrophoto-meter	Digest protein and fragment peptide to identification proteins,	Protein identification, sequence post translational modification
2. Chip	Synthesise proteins, peptides, antigens, antibody into a avery format and spot onto slides	Protein interaction with protein, lipid and small molecules, drug discover, post translational modi-fications.
3. Bioinformatics	Insilico proteomics	Mining database predicting protein interaction.

In-silico methodologies are being developed to identify protein interaction from genome sequence. For example, 6809 putative protein-protein interaction has been identified in Escherichia coli and more than 45,000 have been identified in yeast and large number of these interactions is functionally related.

Types of Proteomics:

i. Structural Proteomics:

One of the main targets of proteomics investigation is to map the structure of protein complexes or the proteins present in a specific cellular organelle known as cell map or structural proteins. Structural proteomics attempt to identify all the proteins within a protein complex and characterization all protein-protein interactions. Isolation of specific protein complex by purification can simplify the proteomic analysis.

ii. Functional Proteomics:

It mainly includes isolation of protein complexes or the use of protein ligands to isolate specific types of proteins. It allows selected groups of proteins to be studied its chracteristics which can provide important information about protein signalling and disease mechanism etc.

Significance of Proteomics:

i. Protein profiling:

Bioinformatics has been widely employed in protein-profiling, where question of protein structural information for the purpose of protein identification, characterization and database is carried out. The spectrum of protein expressed in a cell type provides the cell with its unique identity. It explores how the protein complement changes in a cell type during development in response to environmental stress.

ii. Protein arrays:

Protein microarrays facilitate the detection of protein protein interaction and protein expression profiling. Several protein microarray examples indicate that protein arrays hold great promise for the global analysis of protein-protein and protein-ligand interaction.

iii. Proteomics to a phosphorylation:

In post-translational modification of protein, mass spectrometer (MS) can be used to identify novel phosphorylation. Measure changes in phosphorylation state of protein takes place in response to an effective and determining phosphorylation sites in proteins.

Identification of phosphorylation sites can provide information about the mechanism of enzyme regulation and protein kinase and phosphotases involved. A proteomics approach for this process has an advantage that one can study all the phosphorylating proteins in a cell at the same time.

iv. Proteome mining:

Proteome mining is a functional proteomic approach used to extract information from the analysis of specific sub-proteomics. In principle, it is based on the assumption. In principle, it is based oil the assumption that all drug like molecule selectively compete with a natural cellular ligand for a binding site on a protein target.

Basic Concepts of Proteomics:

The gene transcripts that an individual can make in a lifetime—termed as transcriptome (by analogy with the term genome)—refers to the haploid set of chromosomes carrying all the functional genes.

Similarly, all the proteins made by an organism are now grouped under the shade of proteomics. Proteomics involves the systematic study of proteins in order to provide a comprehensive view of the structure, function and role in the regulation of a biological system.

These include protein-protein interaction, protein modification, protein function and its localization studies. The aim of proteomics is not only to identify all the proteins in a cell but also to create a complete three-dimensional map of the cell indicating where proteins are located. Coupled with advances in bioinformatics, this approach to comprehensively describing biological systems will undoubtedly have a major impact on our understanding of the phenotype of both normal and diseased cells. The proteome (term coined by Mark Wilkins in 1995) of a given cell is the total number of proteins at any given instant and it is highly dynamic in response to internal and external cues. Proteins can be modified by post-translational modifications, undergo translocations within the cell or be synthesized or degraded.

Therefore, the examination of proteins of a cell at a particular time reflects the immediate protein environment in which it is studied. A cellular proteome is the collection of proteins found in a particular cell type under the influence of a particular set of environmental conditions like exposure to hormone stimulation. A complete set of proteins from all of the various cellular proteomes will form an organism's complete proteome. An interesting finding of the Human Genome Project is that there are far more proteins in the human proteome (~ 400,000 proteins) than there are protein-coding genes in the human genome (~ 22,000 genes). The large increase in protein diversity is thought to be due to alternative splicing and post-translational modification of proteins. This indicates that protein diversity cannot be fully characterized by gene expression analysis alone. Proteomics, thus is a useful tool for characterizing cells and tissues of interest.

The first protein studies that can be called proteomics began with the introduction of two dimensional gel electrophoresis of E. coli proteins (O'Ferrall, 1975) followed by mouse and guinea pig protein studies (Ksole, 1975). Although 2-dimensional electrophoresis (2-DE) was a major step forward and many proteins could be separated and visualized by this technique but it was not enough for the protein identification through any sensitive protein sequencing technology. After certain efforts the first major technology for the identification of protein was protein sequencing by Edman degradation (Edman, 1949). This technology was used for the identification of proteins from 2-D gels to create first 2D database (Celis et al. 1987). Another most important development in protein identification was Mass Spectrometry (MS) technology (Andersen et al. 2000). Protein sequencing by MS technology has been increased due to its sensitivity of analysis, tolerate protein complexes and amenable to high throughput operations.

Although several advancements have been made in protein identification (by MS or Edman sequencing) without having the database of large scale DNA sequencing of expressed sequences and genomic DNA, proteins could not be characterized because different protein isoforms can be generated from a single gene through several modifications (Fig. 18.1). And the majority of DNA and protein sequences have been accumulated within a short period of time.

In 1995, the sequencing of the genome of an organism was done for the first time in Haemophilus influenzae (Fleischmann et al. 1995). Till date, sequencing of several other eukaryotic genomes have been completed viz. Arabidopsis thaliana (Tabata, 2000), *Sachcharomyces cerevisiae* (Goffeau, 1996), Caenorhabditis elegans (Abbott, 1998), Oryza (Matsumoto, 2001) and human (Venter, 2001).

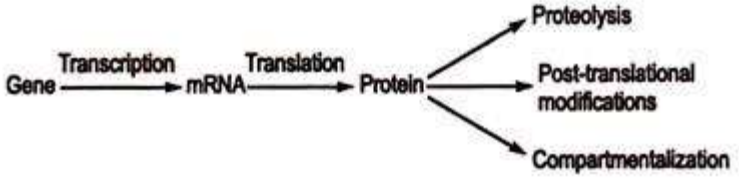


Fig. 18·1 : *Diagrammatic representation of a gene expression showing formation of many protein isoforms from a single gene. After transcription of the gene, mRNA is alternatively spliced or edited to form a mature mRNA that is translated to the protein. Proteins can be regulated by additional mechanism of proteolysis, compartmentalization and certain other modifications*

For protein expression profiling, a common procedure is the analysis of mRNA by different methods including serial analysis of gene expression (SAGE) (Velculescu et al. 1995) and DNA microarray technology (Shalon, 1996). However, the level of transcription of a gene gives only a rough idea of the real level of expression of that gene.

An mRNA may be produced in abundance, but at the same time degraded rapidly, or translated inefficiently keeping the amount of protein minimum. Proteins having been formed are subjected to post-translational modifications also. Different post-translational modifications or proteolysis and compartmentalization regulate the protein functions in the cell (Fig. 18.1).The average number of proteins formed per gene was predicted to be one or two in bacterium, three in yeast and three or more in humans (Wilkins et al. 1996). In response to extra-cellular responses, a number of proteins undergo post-translational modifications. Protein phosphorylation is an important signalling mechanism and dis-regulation of protein kinase and phosphatase can result oncogenesis (Hunter, 1995).

Through proteome analysis, changes in the modifications of many proteins expressed by a cell can be analyzed after translation. Another important feature of a protein is its localization in the cell. The mis-localization of proteins is known to have an adverse effect on cellular function (cystic fibrosis) (Drumm and Collins, 1993). The cell growth, programmed cell death and the decision to proceed through the cell cycle are all regulated by signal transduction through protein complexes (Pippin et al. 1993). The protein interaction can be detected by using yeast two-hybrid system (Rain et al. 2001).

To Understand a Proteome, Three Distinct Type of Analysis must be Carried Out:

(1) Protein-expression proteomics is the quantitative study of the protein expression of the entire proteome or sub-proteome of two samples that differ by some variable. Identification of novel proteins in signal transduction and disease specific proteins are major outcome of this approach.
(2) Structural proteomics attempts to identify all the proteins within a complex or organelle, determine their localization, and characterize all protein-protein interactions. The major goal of these studies is to map out the structure of protein complexes or cellular organelle proteins (Blackstock and Weir, 1999).
(3) Functional proteomics allows the study of a selected group of proteins responsible in signalling pathways, diseases and protein-protein interactions. This may be possible by isolating the specific sub-proteomes by affinity-chromatography for further analysis (Fig. 18.2):

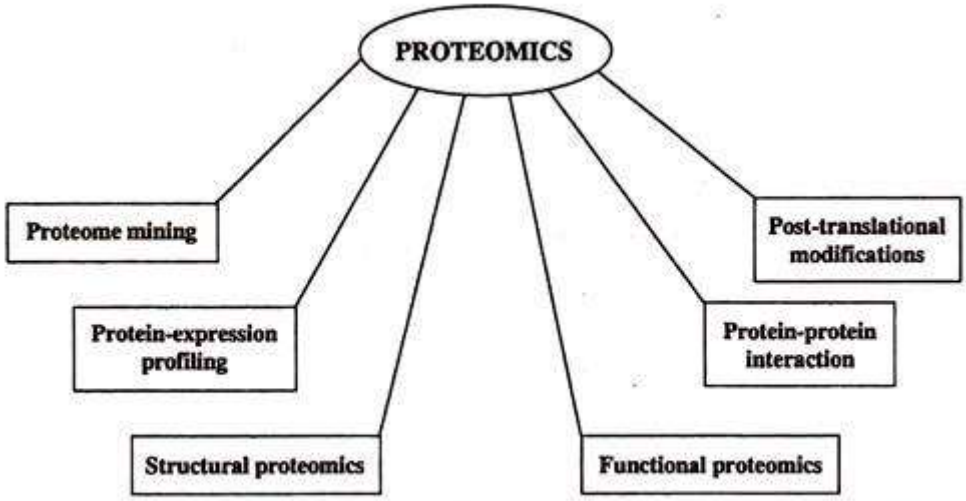


Fig. 18·2 : *Understanding the applications of proteomics (Graves and Haystead, 2002)*

Technology of Proteomics:

Measurement of the level of a gene transcript does not necessarily give clear picture of protein products formed. Therefore, for the measurement of real gene expression, the proteins should be analyzed. Before the identification and measurement of the activity, all the proteins in a proteome for any instant should be separated from each other.

A Typical Proteomics Experiment (e. g. Protein Expression Profiling) can be Divided into the following Categories:

(i) Separation and isolation of protein

(ii) The acquisition of protein structural information for protein identification and characterization

(iii) Database utilization.

(i) Protein Separation and Isolation:

An essential component of proteomics is the protein electrophoresis, the most effective way to resolve a complex mixture of proteins. Two types of electrophoresis are available as one and two-dimensional electrophoresis. In one dimensional gel electrophoresis (1-DE), proteins are resolved on the basis of their molecular masses. Proteins are stable enough during 1-DE due to their solubility in sodium dodycyl sulphate (SDS). Proteins with molecular mass of 10-300 kDa can be easily separated through 1-DE.

But with complex protein mixtures, results with 1-DE are limited, so for more complex protein mixture such as crude cell lysate, the best separation tool available is two dimensional gel electrophoresis (2-DE) (O'Ferrall, 1975). Here, proteins are separated according to their net charges in first dimension and according to their molecular masses in second dimension.

As a single 2-DE gel can resolve thousands of proteins, it remains a powerful tool for the cataloguing of proteins. Two-dimensional electrophoresis has the ability to resolve proteins that have gone under some post-translational modifications as well as protein expression of any two samples can be

compared quantitatively and qualitatively. Recently pH gradients have been introduced to 2-DE which greatly improved the reproducibility of this technique (Bjellqvist et al. 1993).However, few problems with 2-DE still remain to be solved. Despite efforts to automate protein analysis by 2-DE, it is still a labour-intensive and time-consuming process. Another major limitation of 2-DE is the inability to detect low copy number proteins when a total cell lysate is analysed (Link et al. 1997; Shevchenko et al. 1996) as well as inefficiency to speed up the in-gel digestion process also.

Therefore, alternatives have been searched to bypass protein gel electrophoresis. One approach is proteolytic digestion of protein mixture to convert them into peptides and then purify the peptides before subjecting them to analysis by mass spectrometry (MS). Peptide purification has been simplified through liquid chromatography (Link et al. 1999; McCormack et al. 1997), capillary electrophoresis (Figeys et al. 1999; Tong et al. 1999) and reverse phase chromatography (Opiteck et al. 1997).

Recently, Juan et al. (2005) have developed a new approach to speed up the protein identification process utilizing 'microwave' technology. Proteins excised from the gels are subjected to trypsin digestion by microwave irradiation, which rapidly produces peptides fragments. These fragments could be analysed by MALDI (Matrix Assisted Laser Desorption/Ionization). Despite much downstream research on certain alternatives to 2-DE, this is the most widely utilized technique for proteome studies.

(ii) Acquisition of Protein Structures: Protein Identification:

Edman Sequencing (ES):

One of the earliest methods used for protein identification was micro sequencing by Edman chemistry to obtain N-terminal amino acid sequences. This technique was introduced by Edman in 1949. In Edman sequencing, N-terminal of a protein is sequenced to determine its true start site. Edman sequencing is more applicable sequencing method for the identification of proteins separated by SDS-Polyacrylamide gel electrophoresis.

This method has been used extensively in the starting years of proteomics but certain limitations have emerged in recent time. One of the major limitations is the N-terminal modification of proteins. If any protein is blocked on N-terminal before sequencing, then it is very difficult to identify the protein.

To overcome this problem a novel approach of mixed peptide sequencing (Damer et al. 1998) has been employed recently. In this approach, a protein is converted into peptides by cleavage with cyanogen bromide (CNBr) or skatole followed by the Edman sequencing of peptides.

Mass Spectrometry (MS):

The most significant breakthrough in proteomics has been the mass spectrometric identification of gel-separated proteins. Due to its high sensitivity levels, identification of proteins in protein complexes/mixtures and high throughput, this technique has been proved far better than ES.

In mass spectrometry, proteins are digested into peptides in the gel itself by suitable protease such as trypsin, because proteins, as such, are difficult to elute out from the gels. Moreover, molecular weight of proteins is not usually suitable for database identification. In contrast, peptides can be eluted from the gels easily and matching of even a small set of peptides to the database is quite sufficient to identify a protein.

There are Two Main Approaches to Mass Spectrometric Protein Identification:

(i) "Electrospray ionization" (ESI) involves the fragmentation of individual peptides followed by direct ionization through electrospray in a tandem mass spectrometer. In ESI, a liquid sample flows from a microcapillary tube into the orifice of the mass spectrometer, where a potential difference between the capillary and the inlet to the mass spectrometer results in the generation of a fine mist of charged droplets (Fenn et al. 1989; Hunt et al. 1981).

It has the ability to resolve peptides in a mixture, isolate one species at a time and dissociate it into amino or carboxy-terminal containing fragments designated 'b' and 'y', respectively.

(ii) In "Peptide mass mapping" approach (Henzel et al. 1993) the mass spectrum of the eluted peptide mixture is acquired, which result in a peptide mass fingerprint of the protein being studied. The mass spectrum is obtained by a relatively simple 'mass spectrometric method-matrix assisted laser desorption/ ionization' (MALDI).

In this approach, tryptic peptide mixture is analysed because trypsin cleaves proteins at the amino acid arginine and lysine. As the tryptic peptides can be predicted theoretically for any protein, the predicted peptide masses can be compared with those obtained experimentally by MALDI analysis. If the sufficient number of peptide matches with the existing protein sequence in database, the accuracy for protein identification is high.

After the protease cleavages of the proteins, they are analysed by mass analysis also. Mass analysis follows the conversion of proteins or peptides into molecular ions. These ions got separated in a mass spectrometer based on their mass/charge (m/z) ratio. It is determined by the time it takes for the ions to reach the detector. Hence the instrument is called a time of flight (TOF) instrument. The relationship that allows the m/z ratio to be determined is $E = 1/2\ (m/z)v^2$. In this equation. E is the energy imparted on the charged ions as a result of the voltage that is applied by the instrument and V is the velocity of the ions down the flight path. As peptide ions are introduced into the collision chamber, they interact with collision gas and undergo fragmentation along the peptide backbone (Fig. 18.4).

Because all the ions are exposed to the same electric field, all similarly charged ions will have similar energies. Therefore, based on the above equation, ions that have larger mass must have lower velocities and hence will require longer times to reach the detector. Different steps involved in mass spectrometry are described in a flow chart in Fig. 18.3.

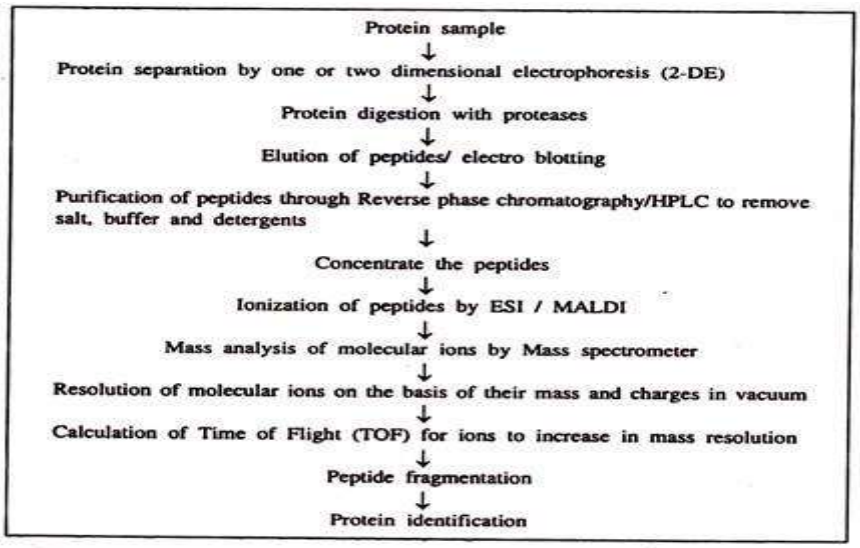


Fig. 18·3 : *A schematic representation of protein identification through Mass spectrometry. All the proteins present in the protein muixture of a cell lysate are identified with this method.*

(iii) Database Utilization:

Initially, sequencing of some proteins or peptides followed by the submission of sequences together created an assembly of proteins called protein database. Proteolytic digestion of many proteins are also predicted theoretically and deposited in database. Hence, at present, so much information has been accumulated that we can search for a homology between a new peptide sequence and the existing sequences in the database to identify the protein.

The major goal of database searching is to identify a large number of proteins—quickly and accurately. All the information accumulated through Edman sequencing or mass spectrometry are used to identify the proteins. In peptide mass fingerprinting database searching, the mass of a unknown peptide after proteolytic digestion is compared to the perdicted mass of peptide from theoretical digestion of proteins in database. In amino acid sequence database searching, the sequence of amino acids from a peptide is identified and can be used to search databases to find the protein from which it was derived.

Collection of protein sequence databases are thus designed to represent a partial list of an organism's genome, that is, the genes and all of the proteins they encode. The protein families are usually classified according to their evolutionary history inferred from sequence homology.

These databases are excellent tools for gene discovery, comparative genomics and molecular evolution. The purpose of database similarity searching is the sensitive detection of sequence homologues, regardless of the species relationship in order to infer similarity of function from similarity of sequence.

Recently, Chromatography-based proteomics is used to measure the concentration of low molecular weight peptides in complex mixtures such as plasma or sera. These technologies use time-of-flight (TOF) spectroscopy with matrix-assisted or surface- enhanced laser desorption/ionization to produce a spectrum of mass-to-charge (m/z) ratios that can be analysed in order to identify unique signatures from its chromatography pattern.

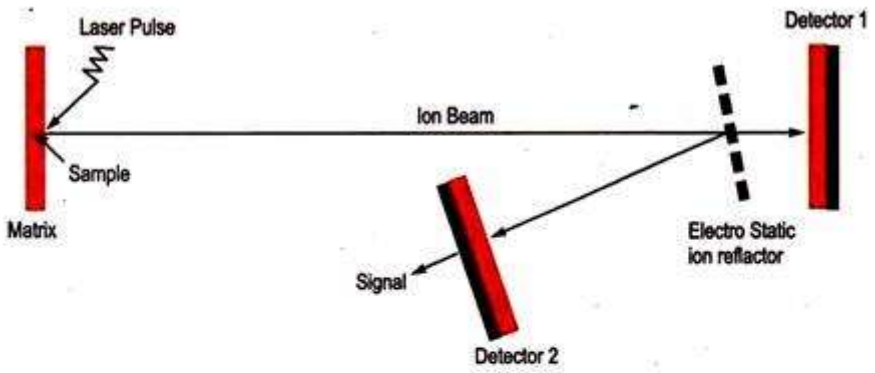


Fig. 18-4 : *Principle behind MALDI-TOF mass spectrometry. A sample is placed on the matrix and ionize by the laser beam. Due to the potential developed between the matrix and the sample, ions start moving towards the detector and get reflected by a reflector in the mid-way. Again after a flight in the tube the ions are detected by another detector. The time taken by these ions in the flight tubes depends on their masses. Therefore, we can calculate the ratio between the mass of an ion and the time of flight in the tube taken by that particular ion*

Applications of Proteomics:

1. Post-Translational Modifications:

Proteomics studies involve certain unique features as the ability to analyze post- translational modifications of proteins. These modifications can be phosphorylation, glycosylation and sulphation as well as some other modifications involved in the maintenance of the structure of a protein.

These modifications are very important for the activity, solubility and localization of proteins in the cell. Determination of protein modification is much more difficult rather than the identification of proteins. As for identification purpose, only few peptides are required for protease cleavages followed by database alignment of a known sequence of a peptide. But for determination of modification in a protein, much more material is needed as all the peptides do not have the expected molecular mass need to be analysed further.

For example, during protein phosphorylation events, phosphopeptides are 80 Da heavier than their unmodified counterparts. Therefore, it gives, rise to a specific fragment (PO^{3-} mass 79) bind to metal resins, get recognized by specific antibodies and later phosphate group can be removed by phosphatases (Clauser et al. 1999; Colledge and Scott, 1999). So protein of interest (post-translationally modified protein) can be detected by Western blotting with the help of antibodies or ^{32}P-labelling that recognize only the active state of molecules. Later, these spots can be identified by mass spectrometry.

2. Protein-Protein Interactions:

The major attribution of proteomics towards the development of protein interactions map of a cell is of immense value to understand the biology of a cell. The knowledge about the time of expression of a particular protein, its level of expression, and, finally, its interaction with another protein to form an intermediate for the performance of a specific biological function is currently available.

These intermediates can be exploited for therapeutic purposes also. An attractive way to study the protein-protein interactions is to purify the entire multi-protein complex by affinity based methods using GST-fusion proteins, antibodies, peptides etc.

The yeast two-hybrid system has emerged as a powerful tool to study protein-protein interactions (Haynes and Yates, 2000). According to Pandey and Mann (2000) it is a genetic method based on the modular structure of transcription factors in the close proximity of DNA binding domain to the activation domain induces increased transcription of a set of genes.

The yeast hybrid system uses ORFs fused to the DNA binding or activation domain of GAL4 such that increased transcription of a reporter gene results when the proteins encoded by two ORFs interact in the nucleus of the yeast cell. One of the main consequences of this is that once a positive interaction is detected, simply sequencing the relevant clones identifies the ORF. For this reason it is a generic method that is simple and amenable to high throughput screening of protein-protein interactions.Phage display is a method where bacteriophage particles are made to express either a peptide or protein of interest fused to a capsid or coat protein. It can be used to screen for peptide epitopes, peptide ligands, enzyme substrate or single chain antibody fragments.

Another important method to detect protein-protein interactions involves the use of fluorescence resonance energy transfer (FRET) between fluorescent tags on interacting proteins. FRET is a non-radioactive process whereby energy from an excited donor fluorophore is transferred to an acceptor fluorophore. After excitation of the first fluorophore, FRET is detected either by emission from the second fluorophore using appropriate filters or by alteration of the fluorescence lifetime of the donor.

A proteomics strategy of increasing importance involves the localization of proteins in cells as a necessary first step towards understanding protein function in complex cellular networks. The discovery of GFP (green fluorescent protein) and the development of its spectral variants has opened the door to analysis of proteins in living cells by use of the light microscope.

Large-scale approaches of localizing GFP-tagged proteins in cells have been performed in the genetically amenable yeast S. pombe (Ding et al. 2000) and in Drosophila (Morin et al. 2001). To localize proteins in mammalian cells, a strategy was developed that enables the systematic GFP tagging of ORFs from novel full-length cDNAs that are identified in genome projects.

3. Protein Expression Profiling:

The largest application of proteomics continues to be protein expression profiling. The expression levels of a protein sample could be measured by 2-DE or other novel technique such as isotope coded affinity tag (ICAT). Using these approaches the varying levels of expression of two different protein samples can also be analysed.

This application of proteomics would be helpful in identifying the signalling mechanisms as well as disease specific proteins. With the help of 2-DE several proteins have been identified that are responsible for heart diseases and cancer (Celis et al. 1999). Proteomics helps in identifying the cancer cells from the non-cancerous cells due to the presence of differentially expressed proteins. The technique of Isotope Coded Affinity Tag has developed new horizons in the field of proteomics. This involves the labelling of two different proteins from two different sources with two chemically identical reagents that differ in their masses due to isotope composition (Gygi et al. 1999). The biggest advantage of this technique is the elimination of protein quantitation by 2-DE. Therefore, high amount of protein sample can be used to enrich low abundance proteins.

Different methods have been used to probe genomic sets of proteins for biochemical activity. One method is called a biochemical genomics approach, which uses parallel biochemical analysis of a proteome comprised of pools of purified proteins in order to identify proteins and the corresponding ORFs responsible for a biochemical activity. The second approach for analyzing genomic sets of proteins is the use of functional protein microarrays, in which individually purified proteins are separately spotted on a surface such as a glass slide and then analysed for activity. This approach has huge potential for rapid high-throughput analysis of proteomes and other large collections of proteins, and promises to transform the field of biochemical analysis.

4. Molecular Medicine:

With the help of the information available through clinical proteomics, several drugs have been designed. This aims to discover the proteins with medical relevance to identify a potential target for pharmaceutical development, a marker(s) for disease diagnosis or staging, and risk assessment—both for medical and environmental studies. Proteomic technologies will play an important role in drug discovery, diagnostics and molecular medicine because of the link between genes, proteins and disease.

As researchers study defective proteins that cause particular diseases, their findings will help develop new drugs that either alter the shape of a defective protein or mimic a missing one. Already, many of the best-selling drugs today either act by targeting proteins or are proteins themselves. Advances in proteomics may help scientists eventually create medications that are “personalized” for different individuals to be more effective and have fewer side effects. Current research is looking at protein families linked to disease including cancer, diabetes and heart disease.

Probable Questions:

1. Define DNA Fingerprinting. What are its applications.
2. How DNA Markers are used in disease diagnosis and fingerprinting?
3. Write a note on RFLP with diagram.
4. Write a note on AFLP with diagram.
5. Write a note on RAPD with diagram.
6. Write a note on SNP with diagram.
7. Write a note on VNTR with diagram.
8. Write a note on STR with diagram.
9. What are the types of Proteomics.
10. Write down the significance of proteomics.
11. What you know about Edman Degradation technique?
12. How Mass spectrometry is used in protein study?
13. What are the applications of Proteomics?

Suggested Readings:

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7th Edition.
6. Gene cloning and DNA Analysis by T. A. Brown, Sixth Edition.
7. Genetics . Verma and Agarwal.

Unit-III

Sociobiology, Altruism, Kin selection and inclusive fitness, Haplodiploidy, Imprinting phenomena

Objective: In this unit you will learn about altruism, kin selection and inclusive fitness. You will also learn about Haplodiploidy and imprinting phenomena.

Sociobiology:

Sociobiology is based on the premise that some behaviours (social and individual) are at least partly inherited and can be affected by natural selection. It begins with the idea that behaviours have evolved over time, similar to the way that physical traits are thought to have evolved. It predicts that animals will act in ways that have proven to be evolutionarily successful over time. This can, among other things, result in the formation of complex social processes conducive to evolutionary fitness.
The discipline seeks to explain behaviour as a product of natural selection. Behaviour is therefore seen as an effort to preserve one's genes in the population. Inherent in sociobiological reasoning is the idea that certain genes or gene combinations that influence particular behavioural traits can be inherited from generation to generation
For example, newly dominant male lions often kill cubs in the pride that they did not sire. This behaviour is adaptive because killing the cubs eliminates competition for their own offspring and causes the nursing females to come into heat faster, thus allowing more of his genes to enter into the population. Sociobiologists would view this instinctual cub-killing behaviour as being inherited through the genes of successfully reproducing male lions, whereas non-killing behaviour may have died out as those lions were less successful in reproducing. Sociobiologists believe that human behaviour, as well as nonhuman animal behaviour, can be partly explained as the outcome of natural selection. They contend that in order to fully understand behaviour, it must be analysed in terms of evolutionary considerations.
Natural selection is fundamental to evolutionary theory. Variants of hereditary traits which increase an organism's ability to survive and reproduce will be more greatly represented in subsequent generations, i.e., they will be "selected for". Thus, inherited behavioural mechanisms that allowed an organism a greater chance of surviving and/or reproducing in the past are more likely to survive in present organisms. That inherited adaptive behaviours are present in nonhuman animal species has been multiply demonstrated by biologists, and it has become a foundation of evolutionary biology. However, there is continued resistance by some researchers over the application of evolutionary models to humans, particularly from within the social sciences, where culture has long been assumed to be the predominant driver of behaviour.

Sociobiology is based upon two fundamental premises:

- Certain behavioural traits are inherited,
- Inherited behavioural traits have been honed by natural selection. Therefore, these traits were probably "adaptive" in the environment in which the species evolved.

Sociobiology uses Nikolaas Tinbergen's four categories of questions and explanations of animal behaviour. Two categories are at the species level; two, at the individual level. The species-level categories (often called "ultimate explanations") are

- the function (i.e., adaptation) that a behaviour serves and
- the evolutionary process (i.e., phylogeny) that resulted in this functionality. The individual-level categories (often called "proximate explanations") are

- the development of the individual (i.e., ontogeny) and
- the proximate mechanism (e.g., brain anatomy and hormones).

Sociobiologists are interested in how behaviour can be explained logically as a result of selective pressures in the history of a species. Thus, they are often interested in instinctive, or intuitive behaviour, and in explaining the similarities, rather than the differences, between cultures. For example, mothers within many species of mammals – including humans – are very protective of their offspring. Sociobiologists reason that this protective behaviour likely evolved over time because it helped the offspring of the individuals which had the characteristic to survive. This parental protection would increase in frequency in the population. The social behaviour is believed to have evolved in a fashion similar to other types of nonbehavioural adaptations, such as a coat of fur, or the sense of smell.

Individual genetic advantage fails to explain certain social behaviours as a result of gene-centred selection. E.O. Wilson argued that evolution may also act upon groups The mechanisms responsible for group selection employ paradigms and population statistics borrowed from evolutionary game theory. Altruism is defined as "a concern for the welfare of others". If altruism is genetically determined, then altruistic individuals must reproduce their own altruistic genetic traits for altruism to survive, but when altruists lavish their resources on non-altruists at the expense of their own kind, the altruists tend to die out and the others tend to increase. An extreme example is a soldier losing his life trying to help a fellow soldier. This example raises the question of how altruistic genes can be passed on if this soldier dies without having any children.

Within sociobiology, a social behaviour is first explained as a sociobiological hypothesis by finding an evolutionarily stable strategy that matches the observed behaviour. Stability of a strategy can be difficult to prove, but usually, it will predict gene frequencies. The hypothesis can be supported by establishing a correlation between the gene frequencies predicted by the strategy, and those expressed in a population. Altruism between social insects and littermates has been explained in such a way. Altruistic behaviour, behaviour that increases the reproductive fitness of others at the apparent expense of the altruist, in some animals has been correlated to the degree of genome shared between altruistic individuals. A quantitative description of infanticide by male harem-mating animals when the alpha male is displaced as well as rodent female infanticide and fetal resorption are active areas of study. In general, females with more bearing opportunities may value offspring less, and may also arrange bearing opportunities to maximize the food and protection from mates. An important concept in sociobiology is that temperament traits exist in an ecological balance. Just as an expansion of a sheep population might encourage the expansion of a wolf population, an expansion of altruistic traits within a gene pool may also encourage increasing numbers of individuals with dependent traits.

Studies of human behaviour genetics have generally found behavioural traits such as creativity, extroversion, aggressiveness, and IQ have high heritability. The researchers who carry out those studies are careful to point out that heritability does not constrain the influence that environmental or cultural factors may have on those traits.

Altruism:

Altruism is the principle and moral practice of concern for happiness of other human beings, resulting in a quality of life both material and spiritual. It is a traditional virtue in many cultures and a core aspect of various religious traditions and secular worldviews, though the concept of "others" toward whom concern should be directed can vary among cultures and religions. In an extreme case, altruism may become a synonym of selflessness which is the opposite of selfishness.
In a common way of living, it doesn't deny the singular nature of the subject, but realizes the traits of the individual personality in relation to the others, with a true, direct and personal interaction with each of them. It is focusing both on the single people and the whole community. In an (not only)

Christian practice, it is the law of love direct to the ego and his neighbour. The word "altruism" was coined by the French philosopher Auguste Comte in French, as *altruisme*, for an antonym of egoism. He derived it from the Italian *altrui*, which in turn was derived from Latin *alteri*, meaning "other people" or "somebody else".
Altruism in biological observations in field populations of the day organisms can be defined as an individual performing an action which is at a cost to themselves (e.g., pleasure and quality of life, time, probability of survival or reproduction), but benefits, either directly or indirectly, another third-party individual, without the expectation of reciprocity or compensation for that action. Steinberg suggests a definition for altruism in the clinical setting, that is "intentional and voluntary actions that aim to enhance the welfare of another person in the absence of any quid pro quo external rewards". Altruism can be distinguished from feelings of loyalty, in that whilst the latter is predicated upon social relationships, altruism does not consider relationships. Much debate exists as to whether *"true"* altruism is possible in human psychology. The theory of psychological egoism suggests that no act of sharing, helping or sacrificing can be described as truly altruistic, as the actor may receive an intrinsic reward in the form of personal gratification. The validity of this argument depends on whether intrinsic rewards qualify as "benefits".
The term *altruism* may also refer to an ethical doctrine that claims that individuals are morally obliged to benefit others. Used in this sense, it is usually contrasted with egoism, which claims individuals are morally obligated to serve themselves first. The concept has a long history in philosophical and ethical thought. The term was originally coined in the 19th century by the founding sociologist and philosopher of science, Auguste Comte, and has become a major topic for psychologists (especially evolutionary psychology researchers), evolutionary biologists, and ethologists. Whilst ideas about altruism from one field can affect the other fields, the different methods and focuses of these fields always lead to different perspectives on altruism. In simple terms, altruism is caring about the welfare of other people and acting to help them.

Kin Selection:

Kin selection is the evolutionary strategy that favours the reproductive success of an organism's relatives, even at a cost to the organism's own survival and reproduction. Kin altruism can look like altruistic behaviour whose evolution is driven by kin selection. Kin selection is an instance of inclusive fitness, which combines the number of offspring produced with the number an individual can ensure the production of by supporting others, such as siblings. John Maynard Smith may have coined the actual term "kin selection" in 1964.
Charles Darwin discussed the concept of kin selection in his 1859 book, *The Origin of Species*, where he reflected on the puzzle of sterile social insects, such as honey bees, which leave reproduction to their mothers, arguing that a selection benefit to related organisms (the same "stock") would allow the evolution of a trait that confers the benefit but destroys an individual at the same time. R.A. Fisher in 1930 and J.B.S. Haldane in 1932 set out the mathematics of kin selection, with Haldane famously joking that he would willingly die for two brothers or eight cousins. In 1964, W.D. Hamilton popularised the concept and the major advance in the mathematical treatment of the phenomenon by George R. Price which has become known as Hamilton's rule. In the same year John Maynard Smith used the actual term kin selection for the first time.

According to Hamilton's rule, kin selection causes genes to increase in frequency when the genetic relatedness of a recipient to an actor multiplied by the benefit to the recipient is greater than the reproductive cost to the actor. Hamilton proposed two mechanisms for kin selection. First, kin recognition allows individuals to be able to identify their relatives. Second, in viscous populations, populations in which the movement of organisms from their place of birth is relatively slow, local interactions tend to be among relatives by default. The viscous population mechanism makes kin selection and social cooperation possible in the absence of kin recognition. In this case, nurture kinship, the treatment of individuals as kin as a result of living together, is sufficient for kin selection, given reasonable assumptions about population dispersal rates. Note that kin selection is not the same thing as group selection, where natural selection is believed to act on the group as a

whole.
In humans, altruism is both more likely and on a larger scale with kin than with unrelated individuals; for example, humans give presents according to how closely related they are to the recipient. In other species, vervet monkeys use allomothering, where related females such as older sisters or grandmothers often care for young, according to their relatedness. The social shrimp *Synalpheusregalis* protects juveniles within highly related colonies.

The earliest mathematically formal treatments of kin selection were by R.A. Fisher in 1930 and J.B.S. Haldane in 1932and 1955.J.B.S. Haldane fully grasped the basic quantities and considerations in kin selection, famously writing "I would lay down my life for two brothers or eight cousins". Haldane's remark alluded to the fact that if an individual loses its life to save two siblings, four nephews, or eight cousins, it is a "fair deal" in evolutionary terms, as siblings are on average 50% identical by descent, nephews 25%, and cousins 12.5% (in a diploid population that is randomly mating and previously outbred). But Haldane also joked that he would truly die only to save more than a single identical twin of his or more than two full siblings. In 1955 he clarified:

Let us suppose that you carry a rare gene that affects your behaviour so that you jump into a flooded river and save a child, but you have one chance in ten of being drowned, while I do not possess the gene, and stand on the bank and watch the child drown. If the child's your own child or your brother or sister, there is an even chance that this child will also have this gene, so five genes will be saved in children for one lost in an adult. If you save a grandchild or a nephew, the advantage is only two and a half to one. If you only save a first cousin, the effect is very slight. If you try to save your first cousin once removed the population is more likely to lose this valuable gene than to gain it. … It is clear that genes making for conduct of this kind would only have a chance of spreading in rather small populations when most of the children were fairly near relatives of the man who risked his life.

Inclusive fitness:

In evolutionary biology, inclusive fitness is one of two metrics of evolutionary success as defined by W. D. Hamilton in 1964:

- **Personal fitness** is the number of offspring that an individual begets (regardless of who rescues/rears/supports them)
- **Inclusive fitness** is the number of offspring equivalents that an individual rears, rescues or otherwise supports through its behaviour (regardless of who begets them)

An individual's own child, who carries one half of the individual's genes, is defined as one offspring equivalent. A sibling's child, who will carry one-quarter of the individual's genes, is 1/2 offspring equivalent. Similarly, a cousin's child, who has 1/16 of the individual's genes, is 1/8 offspring equivalent.

From the gene's point of view, evolutionary success ultimately depends on leaving behind the maximum number of copies of itself in the population. Prior to Hamilton's work, it was generally assumed that genes only achieved this through the number of viable offspring produced by the individual organism they occupied. However, this overlooked a wider consideration of a gene's success, most clearly in the case of the social insects where the vast majority of individuals do not produce offspring.

Hamilton showed mathematically that, because other members of a population may share one's genes, a gene can also increase its evolutionary success by indirectly promoting the reproduction and survival of other individuals who also carry that gene. This is variously called "kin theory", "kin selection theory" or "inclusive fitness theory". The most obvious category of such individuals is close genetic relatives, and where these are concerned, the application of inclusive fitness theory is often

more straightforwardly treated via the narrower kin selection theory.
Hamilton's theory, alongside reciprocal altruism, is considered one of the two primary mechanisms for the evolution of social behaviours in natural species and a major contribution to the field of sociobiology, which holds that some behaviours can be dictated by genes, and therefore can be passed to future generations and may be selected for as the organism evolves.

Although described in seemingly anthropomorphic terms, these ideas apply to all living things, and can describe the evolution of innate and learned behaviours over a wide range of species including insects, small mammals or humans.

Belding's ground squirrel provides an example. The ground squirrel gives an alarm call to warn its local group of the presence of a predator. By emitting the alarm, it gives its own location away, putting itself in more danger. In the process, however, the squirrel may protect its relatives within the local group (along with the rest of the group). Therefore, if the effect of the trait influencing the alarm call typically protects the other squirrels in the immediate area, it will lead to the passing on of more copies of the alarm call trait in the next generation than the squirrel could leave by reproducing on its own. In such a case natural selection will increase the trait that influences giving the alarm call, provided that a sufficient fraction of the shared genes include the gene(s) predisposing to the alarm call. *Synalpheus regalis*, a eusocial shrimp, also is an example of an organism whose social traits meet the inclusive fitness criterion. The larger defenders protect the young juveniles in the colony from outsiders. By ensuring the young's survival, the genes will continue to be passed on to future generations. Inclusive fitness is more generalized than strict kin selection, which requires that the shared genes are *identical by descent*. Inclusive fitness is not limited to cases where "kin" ('close genetic relatives') are involved.

Haplodiploidy:

Approximately 15% of all arthropods reproduce through haplodiploidy. Yet it is unclear how this mode of reproduction affects other aspects of reproductive ecology. In this review we outline predictions on how haplodiploidy might affect mating system evolution, the evolution of traits under sexual or sexual antagonistic selection, sex allocation decisions and the evolution of parental care. We also give an overview of the phylogenetic distribution of haplodiploidy. Finally, we discuss how comparisons between different types of haplodiploidy (arrhenotoky, PGE with haploid vs somatically diploid males) might help to discriminate between the effects of virgin birth, haploid gene expression and those of haploid gene transmission.

Haplodiploidy is a sex-determination system in which males develop from unfertilized eggs and are haploid, and females develop from fertilized eggs and are diploid. Haplodiploidy is sometimes called arrhenotoky. Haplodiploidy determines the sex in all members of the insect orders Hymenoptera (bees, ants, and wasps) and Thysanoptera ('thrips'). The system also occurs sporadically in some spider mites, Hemiptera, Coleoptera (bark beetles), and rotifers.
In this system, sex is determined by the number of sets of chromosomes an individual receives. An offspring formed from the union of a sperm and an egg develops as a female, and an unfertilized egg develops as a male. This means that the males have half the number of chromosomes that a female has, and are haploid. The haplodiploid sex-determination system has a number of peculiarities. For example, a male has no father and cannot have sons, but he has a grandfather and can have grandsons. Additionally, if a eusocial-insect colony has only one queen, and she has only mated once, then the relatedness between workers (diploid females) in a hive or nest is 3⁄4. This means the workers in such monogamous single-queen colonies are significantly more closely related than in other sex determination systems where the relatedness of siblings is usually no more than 1⁄2. It is this point which drives the kin selection theory of how eusociality evolved. Whether haplodiploidy did in fact pave the way for the evolution of eusociality is still a matter of debate.

Another feature of the haplodiploidy system is that recessive lethal and deleterious alleles will be removed from the population rapidly because they will automatically be expressed in the males (dominant lethal and deleterious alleles are removed from the population every time they arise, as they kill any individual they arise in). Haplodiploidy is not the same thing as an X0 sex-determination system. In haplodiploidy, males receive one half of the chromosomes that females receive, including autosomes. In an X0 sex-determination system, males and females receive an equal number of autosomes, but when it comes to sex chromosomes, females will receive two X chromosomes while males will receive only a single X chromosome.

Several models have been proposed for the genetic mechanisms of haplodiploid sex-determination. The model most commonly referred to is the complementary allele model. According to this model, if an individual is heterozygous for a certain locus, it develops into a female, whereas hemizygous and homozygous individuals develop into males. In other words, diploid offspring develop from fertilized eggs, and are normally female, while haploid offspring develop into males from unfertilized eggs. Diploid males would be infertile, as their cells would not undergo meiosis to form sperm. Therefore, the sperm would be diploid, which means that their offspring would be triploid. Since hymenopteran mother and sons share the same genes, they may be especially sensitive to inbreeding: Inbreeding reduces the number of different sex alleles present in a population, hence increasing the occurrence of diploid males.
After mating, each fertile hymenopteran female stores sperm in an internal sac called the spermatheca. The mated female controls the release of stored sperm from within the organ: If she releases sperm as an egg passes down her oviduct, the egg is fertilized. Social bees, wasps, and ants can modify sex ratios within colonies which maximizes relatedness among members and generates a workforce appropriate to surrounding conditions. In other solitary hymenopterans, the females lay unfertilized male eggs on poorer food sources while laying the fertilized female eggs on better food sources, possibly because the fitness of females will be more adversely affected by shortages in their early life. Sex ratio manipulation is also practiced by haplodiploid ambrosia beetles, who lay more male eggs when the chances for males to disperse and mate with females in different sites are greater.

Imprinting Phenomena:

Genomic imprinting is an epigenetic phenomenon that causes genes to be expressed in a parent- of-origin-specific manner. Forms of genomic imprinting have been demonstrated in fungi, plants and animals. As of 2014, there are about 150 imprinted genes known in the mouse and about half that in humans.
Genomic imprinting is an inheritance process independent of the classical Mendelian inheritance. It is an epigenetic process that involves DNA methylation and histone methylation without altering the genetic sequence. These epigenetic marks are established ("imprinted") in the germline (sperm or egg cells) of the parents and are maintained through mitotic cell divisions in the somatic cells of an organism. Appropriate imprinting of certain genes is important for normal development. Human diseases involving genomic imprinting include Angelman syndrome and Prader–Willi syndrome.

Imprinting mechanisms:

Imprinting is a dynamic process. It must be possible to erase and re-establish imprints through each generation so that genes that are imprinted in an adult may still be expressed in that adult's offspring. (For example, the maternal genes that control insulin production will be imprinted in a male but will be expressed in any of the male's offspring that inherit these genes.) The nature of imprinting must therefore be epigenetic rather than DNA sequence dependent. In germline cells the imprint is erased and then re-established according to the sex of the individual, i.e. in the developing sperm (during spermatogenesis), a paternal imprint is established, whereas in developing oocytes (oogenesis), a maternal imprint is established. This process of erasure and reprogramming is

necessary such that the germ cell imprinting status is relevant to the sex of the individual. In both plants and mammals there are two major mechanisms that are involved in establishing the imprint; these are DNA methylation and histone modifications.
Recently, a new study has suggested a novel inheritable imprinting mechanism in humans that would be specific of placental tissue and that is independent of DNA methylation (the main and classical mechanism for genomic imprinting). Among the hypothetical explanations for this exclusively human phenomenon, two possible mechanisms have been proposed: either a histone modification that confers imprinting at novel placental-specific imprinted *loci* or, alternatively, a recruitment of DNMTs to these loci by a specific and unknown transcription factor that would be expressed during early trophoblast differentiation.

Hypotheses on the origins of imprinting

A widely accepted hypothesis for the evolution of genomic imprinting is the "parental conflict hypothesis". Also known as the kinship theory of genomic imprinting, this hypothesis states that the inequality between parental genomes due to imprinting is a result of the differing interests of each parent in terms of the evolutionary fitness of their genes The father's genes that encode for imprinting gain greater fitness through the success of the offspring, at the expense of the mother. The mother's evolutionary imperative is often to conserve resources for her own survival while providing sufficient nourishment to current and subsequent litters. Accordingly, paternally expressed genes tend to be growth-promoting whereas maternally expressed genes tend to be growth-limiting. In support of this hypothesis, genomic imprinting has been found in all placental mammals, where post-fertilisation offspring resource consumption at the expense of the mother is high; although it has also been found in oviparous birds where there is relatively little post-fertilisation resource transfer and therefore less parental conflict.

However, our understanding of the molecular mechanisms behind genomic imprinting show that it is the maternal genome that controls much of the imprinting of both its own and the paternally- derived genes in the zygote, making it difficult to explain why the maternal genes would willingly relinquish their dominance to that of the paternally-derived genes in light of the conflict hypothesis.

Another hypothesis proposed is that some imprinted genes act co-adaptively to improve both foetal development and maternal provisioning for nutrition and care. In it a subset of paternally expressed genes are co-expressed in both the placenta and the mother's hypothalamus. This would come about through selective pressure from parent-infant coadaptation to improve infant survival. Paternally expressed 3 (Peg3) is a gene for which this hypothesis may apply.

Others have approached their study of the origins of genomic imprinting from a different side, arguing that natural selection is operating on the role of epigenetic marks as machinery for homologous chromosome recognition during meiosis, rather than on their role in differential expression. This argument centers on the existence of epigenetic effects on chromosomes that do not directly affect gene expression, but do depend on which parent the chromosome originated from. This group of epigenetic changes that depend on the chromosome's parent of origin (including both those that affect gene expression and those that do not) are called parental origin effects, and include phenomena such as paternal X inactivation in the marsupials, nonrandom parental chromatid distribution in the ferns, and even mating type switching in yeast. This diversity in organisms that show parental origin effects has prompted theorists to place the evolutionary origin of genomic imprinting before the last common ancestor of plants and animals, over a billion years ago.

Natural selection for genomic imprinting requires genetic variation in a population. A hypothesis for the origin of this genetic variation states that the host-defense system responsible for silencing foreign DNA elements, such as genes of viral origin, mistakenly silenced genes whose silencing turned

out to be beneficial for the organism. There appears to be an over-representation of retrotransposed genes, that is to say genes that are inserted into the genome by viruses, among imprinted genes. It has also been postulated that if the retrotransposed gene is inserted close to another imprinted gene, it may just acquire this imprint

Imprinted genes in Mammals :

That imprinting might be a feature of mammalian development was suggested in breeding experiments in mice carrying reciprocal chromosomal translocations. Nucleus transplantation experiments in mouse zygotes in the early 1980s confirmed that normal development requires the contribution of both the maternal and paternal genomes. The vast majority of mouse embryos derived from parthenogenesis (called parthenogenones, with two maternal or egg genomes) and androgenesis (called androgenones, with two paternal or sperm genomes) die at or before the blastocyst/implantation stage. In the rare instances that they develop to post-implantation stages, gynogenetic embryos show better embryonic development relative to placental development, while for androgenones, the reverse is true. Nevertheless, for the latter, only a few have been described.

No naturally occurring cases of parthenogenesis exist in mammals because of imprinted genes. However, in 2004, experimental manipulation by Japanese researchers of a paternal methylation imprint controlling the *Igf2* gene led to the birth of a mouse (named Kaguya) with two maternal sets of chromosomes, though it is not a true parthenogenone since cells from two different female mice were used. The researchers were able to succeed by using one egg from an immature parent, thus reducing maternal imprinting, and modifying it to express the gene Igf2, which is normally only expressed by the paternal copy of the gene.Parthenogenetic/gynogenetic embryos have twice the normal expression level of maternally derived genes, and lack expression of paternally expressed genes, while the reverse is true for androgenetic embryos. It is now known that there are at least 80 imprinted genes in humans and mice, many of which are involved in embryonic and placental growth and development. Hybrid offspring of two species may exhibit unusual growth due to the novel combination of imprinted genes.

Various methods have been used to identify imprinted genes. In swine, Bischoff *et al.* 2009 compared transcriptional profiles using short-oligonucleotide microarrays to survey differentially expressed genes between parthenotes (2 maternal genomes) and control fetuses (1 maternal, 1 paternal genome). An intriguing study surveying the transcriptome of murine brain tissues revealed over 1300 imprinted gene loci (approximately 10-fold more than previously reported) by RNA-sequencing from F1 hybrids resulting from reciprocal crosses. The result however has been challenged by others who claimed that this is an overestimation by an order of magnitude due to flawed statistical analysis. In domesticated livestock, single-nucleotide polymorphisms in imprinted genes influencing foetal growth and development have been shown to be associated with economically important production traits in cattle, sheep and pigs.

Probable Questions:

1. Write a note about altruism. What is its significance.
2. What do you mean by kin selection? what is its significance.
3. What is Haplodiploidy?
4. What is Genetic imprinting? How it affects human behaviour?
5. Write about hypothesis on genetic imprinting.
6. Define and explain sociobiology.
7. Write a note on inclusive fitness.

Suggested readings:

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7^{th} Edition.
6. Gene cloning and DNA Analysis by T. A. Brown, Sixth Edition.
7. Genetics . Verma and Agarwal.

Unit-IV

Human genome project and the age of genomics

Objective: In this unit you will learn about different aspects of human genome project and about genomics.

Meaning of Genomics:

The term genomics was first used by Thomas Roderick in 1986. It refers to the study of structure and function of entire genome of a living organism. Genome refers to the basic set of chromosomes. In a genome, each type of chromosome is represented only once. Now genomics is being developed as a sub discipline of genetics which is devoted to the mapping, sequencing and functional analysis of genomes.

Main points related to genomics are listed below:

i. It is a computer aided study of structure and function of entire genome of an organism.

ii. It deals with mapping and sequencing of genes on the chromosomes.

iii. It is a rapid and accurate method of gene mapping. It is more accurate than recombination mapping and deletion mapping techniques.

iv. The genomic techniques are highly powerful, efficient and effective in solving complex genetic problems.

v. Now use of genomic techniques has become indispensable in plant breeding and genetics.

Types of Genomics:

The discipline of genomics consists of two parts, viz. structural genomics and functional genomics.

These are defined as under:

i. Structural Genomics:

It deals with the study of the structure of entire genome of a living organism. In other words, it deals with the study of the genetic structure of each chromosome of the genome. It determines the size of the genome of a species in mega-bases [Mb] and also the genes present in the entire genome of a species.

ii. Functional Genomics:

The study of function of all genes present in the entire genome is known as functional genomics. It deals with transcriptome and proteome. The transcriptome refers to complete set of RNAs transcribed from a genome and proteome refers to complete set of proteins encoded by a genome.

3. Classification of Genomics:

The genomics can be classified as plant genomics, animal genomics, eukaryotic genomics and prokaryotic genomics.

These are defined as follows:

(i) Plant Genomics:

It deals with the study of structure and function of entire genome of plant species.

(ii) Animal Genomics:

It deals with the study of structure and function of entire genome of animal species.

(iii) Eukaryotic Genomics:

It deals with the study of structure and function of entire genome of higher [multi-cellular] organisms.

(iv) Prokaryotic Genomics:

It deals with the study of structure and function of entire genome of unicellular organisms.

Whole Genome Sequence Data:

Complete nucleotide sequences of nuclear, mitochondrial and chloroplast genomes have already been worked out in large number of prokaryotes and several eukaryotes. By the year 2005, among prokaryotes, approx. 1400 viral genomes, 250 bacterial genomes (230 eubacteria and 20 archaea), 500 mitochondrial genomes, 35 chloroplast genomes have been fully sequenced.

Among the eukaryotes namely the whole genome of Saccharomyces cerevisiae (yeast), Coenorhabditis elegans (nematode), fruitfly (Drosophila melanogaster), Human (Homo sapiens), Crucifer weed (Arabidopsis thaliana) and rice (Oryza sativa) have been sequenced already and data available for annotation studies.

The sequence data of eukaryotic nuclear genome is an important source of identification, discovery and isolation of important genes. This data is very much helpful in variety of application relevant to animal, plant and microbial biotechnology.

Functional Genomics:

Functional genomics is to place all of the genes in the genome of an organism within a functional frame work. Actually, in every organism about 12-15% genes are structural genes which are expressed for certain characters. These are transcribed in a given cell. This is helpful in overall functioning of the cell and organism.

Functional genomics brings together genetics with gene transcripts, proteins and metabolites by analyzing genome sequencing. Functional genomics is driving a shift from vertical analysis of single genes, proteins or metabolites towards horizontal analysis of full suites of genes, proteins and metabolites. This may help in molecular participation of a given biological process. This offers the prospect of determining a truly holistic picture of life.

Functional Genomics Toolbox:

The functional genomics emerged in response to the challenges posed by complete genome sequences. To understand this process it is necessary to know the biochemical and physiological function of every gene product and their complexes.

The activity of genes manifests at a number of different levels, including RNA, protein and metabolite levels and analyses at these levels can provide insight not only into the possible function of individual gene but also the cooperation that occurs between genes and gene products to produce a defined biological outcome.

The technology, involved in defining functional genomics are DNA or oligonucleotide microarray technology for determining mRNA, 2D gels and mass spectroscopy and other methods for analyzing different proteins and GC-MS or LC-MS for identifying and quantifying different metabolites in a cell. High throughput methods for forward and reverse genetics are also integral to functional genomics (Fig. 27.25).

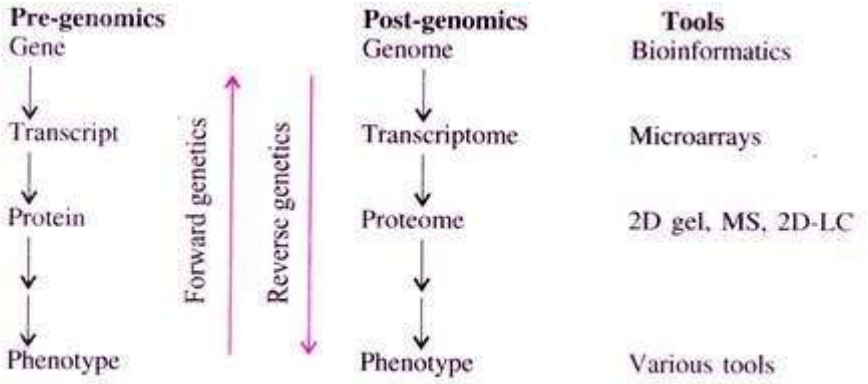


Fig. 27.25 : Tools of functional genomics.

Methods in Functional Genomics:

Functional genomics lies on gene expression, profiling (mRNA) in protein expression, reverse genetics, the generation of targeted mutations in genes of interest besides forward mutation rate, the generation of random mutations in the genome for desirable mutants and bioinformatics.

These criteria help in providing maximum information of a particular organism. This helps in understanding the biological process at the molecular level and also useful to identify novel genes regulating this process. To understand the gene function, it is desirable to identify genes and to understand its expression at the whole genome level.

There are many prokaryotic and eukaryotic organisms whose genomes are fully sequenced. The current discovery is mapping of whole sequences of genes present in human genome. It is possible to assign functions to novel genes and proteins, and to understand biological processes at the molecular level. The integrated understanding of the control of gene expression and knowledge of signal transduction, cell signalling and overall cell function are dynamic tools to study regulation of gene expression in any given cell type. In yeast cells, transcripts associated with different parts of the cell cycle form discrete clusters.

These studies allowed sequence tags encoding proteins of unknown function to be assigned to putative classes based on their clustering with genes of known function. Here, role of functional genomics will be to test those repetitive functions and apply to resolve complex biological processes.

Future Prospects:

It has good future as briefly given below:

(a) In Human Pathology:

Application of gene expression profiling in understanding the human cells and tissues to disease is under way. It would be possible in future to study the modifications in gene sequence during infection. Such studies will yield fundamental insights into the etiology and pathogenesis.

(b) In Parallel Sequencing:

In a number of laboratories, most of the sequences are generated using different approaches. Integrating very different datasets is not as simple as assembling a sequence itself which serves as an absolute standard. Although transcriptional profiling can be used to construct the standardized databases based on absolute RNA and protein levels, yet this is clearly not the case for relative gene expression data.

Significance of Genomics:

All the information's require input in probability theory, database management arid manipulation, and computer science.

This will help in:

(a) Identification of open reading frame sequences,

(b) Gene splicing sites (introns),

(c) Gene annotation (inter-genomic comparisons) and

(d) Determination of sequence patterns of regulatory sites and gene regulations.

Human Genome Project:

Introduction:

The term genome (introduced by H. Winkter in 1920) refers to one complete copy of the genetic information (DNA) or one complete set of chromosome (monoploid or haploid) of an organism. The term genomics (term coined by T.H. Roderick in 1987) denotes mapping, sequencing and functional analysis of genomes.

The Human Genome Project (HGP) was launched on 1st October, 1990 for sequencing entire genome of 2.75 billion nucleotide pairs. This megaproject was a 13 year project coordinated by the U.S. Department of Energy and the National Institute of Health. During the early years of the HGP, the Wellcom Trust (U.K.) becomes a major partner; additional contributions come from Japan, France, Germany, China and others.

The project was completed in 2003. James Watson was the first directors of human genome project and after a period of two years he was replaced by Francis Collins in 1993. Two important scientists associated with HGP were Francis Collins, director of the HGP and J. Craig Venter, founding president of Celera Genomics. HGP was closely associated with rapid development of a new era in biology called as Bioinformatics.

Meaning of Human Genome Project:

The Human Genome Project (HGP) is an International collaborative research programme which started in 1990 and completed in 2003, whose goal was the complete mapping and understanding of the three billion DNA subunits (bases), and to identify all human genes, making them accessible for further biological study.

History of Human Genome Project:

In U.S., the HGP was carried out by the Department of Energy (Human Genome Program) directed by Ari Patrinos, and National Institute of Health (Human Genome Research Institute) directed by Francis Collins. In 2001, Craig Venter, CEO of Celera Genomics, co-announced the completion (90%) of sequencing of the human genome (draft sequence).

The full sequence was completed and published in 2003 (finished sequence). More refined sequence is available in 2006 and correction of minor errors (1 less in 10000 DNA subunits) requires some time to come.

The Birth and Activity of Human Genome Project:

The human genome project (HGP) was conceived in 1984, and officially begun in earnest in October 1990. The primary objective of HGP was to determine the nucleotide sequence of the entire human nuclear genome. In addition, HGP was also entrusted to elucidate the genomes of several other model organisms e.g. Escherichia coli, Saccharomyces cerevisiae (yeast), Caenorhabditis elegans (roundworm), Mus musculus (mouse). James Watson (who elucidated DNA structure) was the first Director of HGP.

In 1997, United States established the National Human Genome Research Institute (NHGRI). The HGP was an international venture involving research groups from six countries—USA, UK, France, Germany, Japan and China, and several individual laboratories and a large number of scientists and technicians from various disciplines. This collaborative venture was named as International Human Genome Sequencing Consortium (IHGSQ and was headed by Francis Collins.

A total expenditure of $3 billion, and a time period of 10-15 years for the completion of HGP was expected. A second human genome project was set up by a private company — Celera Genomics, of Maryland USA in 1998. This team was led by Craig Venter. Very rapid and unexpected progress occurred in HGP with good cooperation between the two teams of workers and improved methods in sequencing.

Goals of Human Genome Project:

Human Genome Project had many goals some of the important goals were outlined below:

1. To identify all the approximately 20,000-25,000 genes in human DNA.

2. To determine the sequences of the 3 billion base pairs that makes up human DNA.

3. To store this information in data base.

4. To develop improvised tools for data analysis.

5. To transfer related technologies to other sectors, such as industries.

6. To address the ethical, legal and social issues (ELSI) that may arise from the project.

The methodologies involved two major approaches identifying all genes of the genome and their sequencing. For sequencing, the total DNA from a cell is isolated and converted into fragments of relatively small sizes and cloned in suitable host, this generates a genomic library of the organism.

The complete sequencing of the first human chromosome, small chromosome 22, was published in December 1999. Then chromosome 21 was completely sequenced in May 2000. The first draft sequence of entire human genome was published in the famous scientific journal "Nature" on 16th February, 2001.

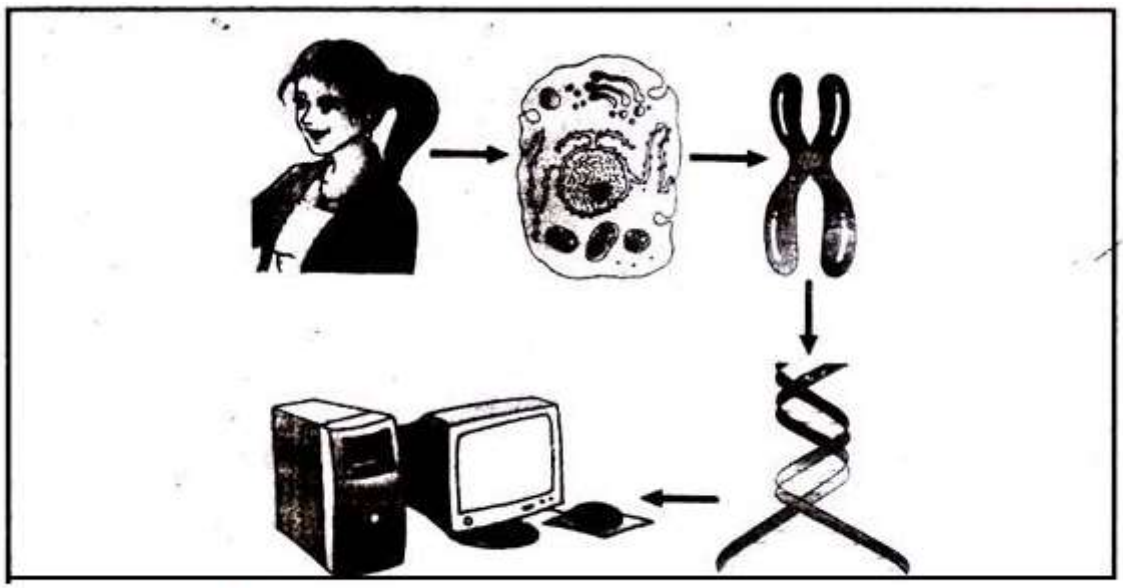


Fig 5.23 A Representative Diagram of Human Genome Project

Important Features of Human Genome:

1. The human genome contains 3164.7 million nucleotide bases.

2. The average gene consists of 3000 bases, but gene size vary greatly (the largest human gene is dystrophin containing 2.4 million bases).

3. The total number of genes in the genome is estimated at 30,000 and all (99.9 percent) nucleotide bases are exactly the same in all people

4. Functions of about 50% of the discovered genes are still unknown.

5. Less than 2% of the genes of the genome codes for proteins.

6. Chromosome 1 has most genes (2968) and the Y has the fewest (231).

7. Repeated sequences (AT-AT-AT or GC-GC-GC…….) make up very large portion of the human genome.

8. Scientists have identified about 1.4 million locations where single base DNA differences (SNPs-single nucleotide polymorphism, pronounced as 'snips') occur on humans.

Human Genome Project was an undertaking by many countries to acquire complete knowledge of the organisation, structure and functions of the human genome. Such a multinational undertaking was called as International Human Genome Sequencing. HGP was regarded as the most ambitious project ever undertaken by humans.

The project had the benefits to identify all human genes and also mutated genes causing diseases. Complete knowledge on the genome sequence will enable the scientists in future to gain knowledge on the types of proteins encoded by these genes. The cloning and sequencing of the disease causing alleles (mutated genes) will largely help in the diagnosis and treatment of diseases.

Human Genome Size:

A genome is an organism's complete set of deoxyribonucleic acid (DNA), a chemical compound that contains the genetic instructions needed to develop and direct the activities of an organism. The human genome contains approx. Three billion base pairs which reside in 23 pairs of chromosomes.

Each chromosome contains hundreds and thousands of genes, and ranges in size from about 50000000 to 300000000 base pairs. The total number of genes is 30000 (approx.) and accounts for only 25% of the DNA; the rest is extra-genic DNA.

Human Genome Project Mapping:

Before beginning a sequencing project of the human genome, it was first necessary to produce a good framework map. Two general methods were developed for mapping human genome — standard method and whole genome short-gun method.

The standard method involves finding a segment of the genome and locating where it belongs. Genetic maps based on recombination frequencies between markers are useful in ordering genes. Molecular markers like RFLP, VNTRs (Microsatellites), STSs, SNPs have been used in mapping human genome. The whole genome shotgun sequencing method involves shearing of genomic DNA followed by cloning, to produce a genomic library.

This is followed by sequencing of cloned DNA fragments at random, followed by shotgun assembly, i.e., the assembly of the fragment sequences into larger units on the basis of their overlaps. Groups of cloned DNA segments that can be aligned in an overlapping fashion to cover a region of the human genome are referred as contigs. Yeast Artificial Chromosomes (YACs) were initially used as cloning agents when primary task was mapping. However, as the emphasis of the project shifted to sequencing. Bacterial Artificial Chromosomes (BACs) were used.

Human Genome Project Sequence:

Sequencing means determining the exact order of the base pairs in a segment of DNA. The primary method used by the HGP to produce the finished version of the human genetic code is map-based or BAC- based sequencing. The human DNA is fragmented into pieces that are relatively large, cloned in the bacteria, stored for replication as required.

A collection of BAG clones containing the entire human genome is called a BAC-library. In this method, each BAC clone is mapped to determine the location of that fragment in human chromosome and then the DNA letters are sequenced from each clone and their spatial relation to sequenced human DNA in other BAC clones.

For sequencing, each BAC clone is cut into still smaller fragments that are about 2000 bases in length. These pieces are called **"sub-clones".** A **"sequencing reaction"** is carried out on these sub-clones. With the help of a computer then the short sequences are assembled into contiguous stretches of sequence of the clones.

In a short the whole process can be summarized:

i. Chromosomes, which range in size from 50 million to 250 million bases, must first be broken into much shorter pieces (sub-cloning step).

ii. Each short piece is used as a template to generate a set of fragments that differ in length from each other by a single base that will be identified in a later step (template preparation and sequencing step).

iii. The fragments in a set are separated by gel electrophoresis (separation step).

iv. The final base at the end of each fragment is identified (base-calling step). This process recreates the original sequence of As, Ts, Cs and Gs for each short piece generated in the first step.

v. After the bases are 'read', computers are used to assemble the short sequences (in blocks of about 500 bases each called the read length) into long continuous stretches that are analysed for errors, gene coding regions, and other characteristics.

vi. Finished sequence is submitted to major public sequence databases, making Human Genome Project sequence data thus freely available to anyone around the world (Fig. 18.18).

The human genome reference sequence do not represent any one person's genome. Rather the knowledge obtained is applicable to everyone because all humans share the same basic set of genes and genomic regulatory regions that control the development.

Researchers collected blood (female) or sperm (male) samples from different races like European, African, American (North, Central, South) and Asian ancestry and a few samples were processed as DNA resources.

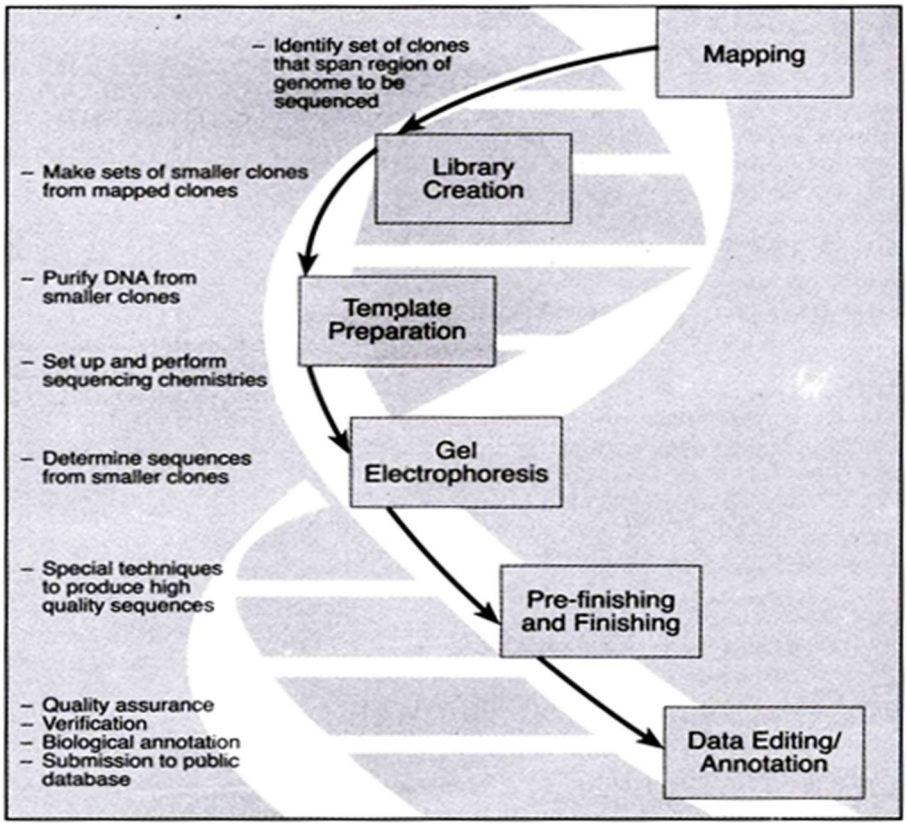


Fig. 18.18: DNA sequencing process

Outcome of Human Genome Project:

i. The human genome contains 3164.7 million chemical nucleotide bases (A, C, T and G).

ii. The average gene consists of 3000 bases, but sizes vary greatly, largest known human gene is **"dystrophin"** – 2.4 million bases.
iii. Total number of genes estimated 30000 approx.

iv. Almost all (99.9%) nucleotide bases are exactly the same in all people.

v. 50% genes are unknown for function.

vi. Less than 2% genomes code for proteins.

vii. Repeated sequences (junk DNA) is 50% of the human genome. This may contribute to create new genes, to modify and reshuffle the existing genes.

viii. A-T rich regions are gene-poor and G-C rich regions are gene-dense. Chromosome-I has the most genes (2968) and the Y chromosome has the fewest (231).

ix. Scientists have identified about 1.4 million locations where single base DNA differences (SNPs) occur in human, these findings will help to localize the disease associated sequences in the chromosomes.

x. Finding the DNA sequences underlying such common diseases as cardiovascular disease, diabetes, arthritis and cancers is being aided by human variation maps (SNPs) generated in HGP.

Announcement of the draft sequence of human genome:

The date 26th June 2000 will be remembered as one of the most important dates in the history of science or even mankind. It was on this day, Francis Collins and Craig Venter, the leaders of the two human genome projects, in the presence of the President of U.S., jointly announced the working drafts of human genome sequence. The detailed results of the teams were later published in February 2001 in scientific journals Nature (IHGSC) and Science (Celera Genomics).

Mapping of the Human Genome:

The most important objective of human genome project was to construct a series of maps for each chromosome. In Fig. 12.1, an outline of the different types of maps is given.

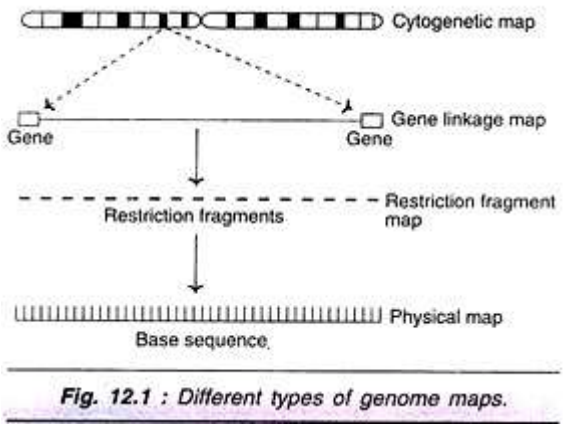


Fig. 12.1 : Different types of genome maps.

1. Cytogenetic map:

This is a map of the chromosome in which the active genes respond to a chemical dye and display themselves as bands on the chromosome.

2. Gene linkage map:

A chromosome map in which the active genes are identified by locating closely associated marker genes. The most commonly used DNA markers are restriction fragment length polymorphism (RFLP), variable number tandems repeats (NTRs) and short tandem repeats (STRs). VNTRs are also called as minisatellites while STRs are microsatellites.

3. Restriction fragment map:

This consists of the random DNA fragments that have been sequenced.

4. Physical map:

This is the ultimate map of the chromosome with highest resolution base sequence. Physical map depicts the location of the active genes and the number of bases between the active genes.

Organization of Human Genome:

An outline of the organization of the human genome is given in Fig. 12.2. Of the 3200 Mb, only a small fraction (48 Mb) represents the actual genes, while the rest is due to gene-related sequences (introns, pseudo genes) and inter-genic DNA (long interspersed nuclear elements, short inter-spread nuclear elements, microsatellites, DNA transposons etc.). Inter-genic DNA represents the parts of the genome that lie between the genes which have no known function. This is appropriately regarded as junk DNA.

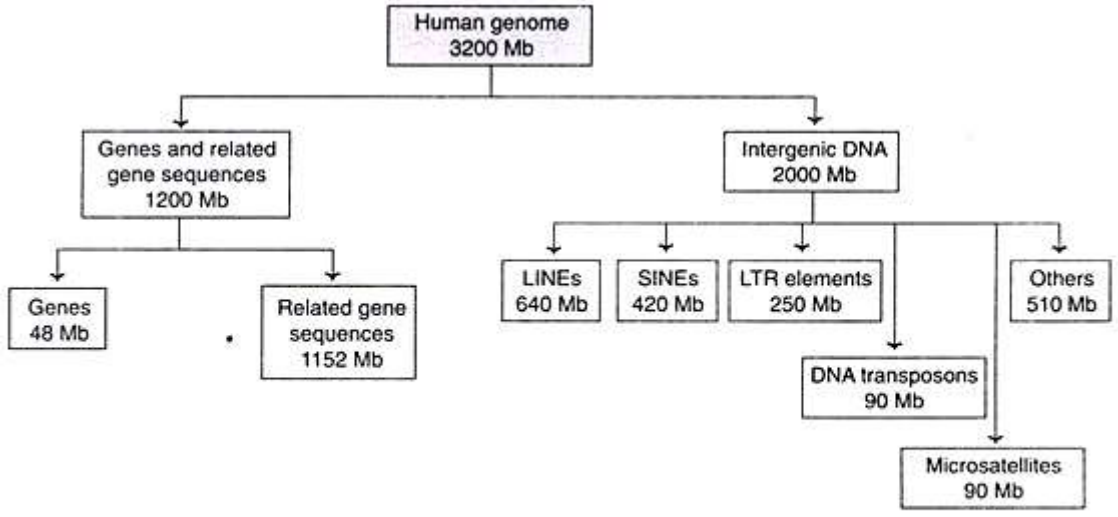


Fig. 12.2 : An overview of the organization of human genome (LINEs-Long interspersed nuclear elements; SINEs–Short interspersed nuclear elements; LTR-Long terminal repeats).

Genes Present in Human Genome:

The two genome projects differ in their estimates of the total number of genes in humans. Their figures are in the range of 30,000-40,000 genes. The main reason for this variation is that it is rather difficult to specifically recognize the DNA sequences which are genes and which are not.

Before the results of the HGP were announced, the best guess of human genes was in the range of 80,000-100,000. This estimate was based on the fact that the number of proteins in human cells are 80,000-100,000, and thus so many genes expected. The fact that the number of genes is much lower than the proteins suggests that the RNA editing (RNA processing) is widespread, so that a single mRNA may code for more than one protein.

A diagrammatic representation of a typical structure of an average human gene is given in Fig. 12.3. It has exons and introns.

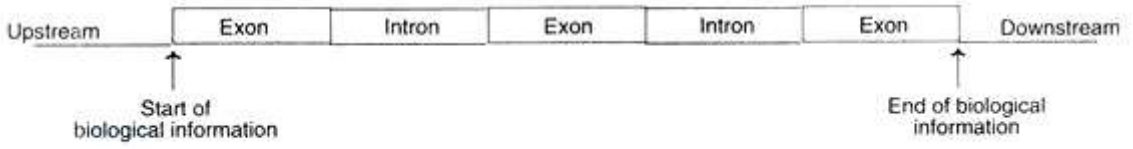


Fig. 12.3 : A diagrammatic representation of a typical structure of an average human gene.

A broad categorization of human gene catalog in the form of a pie chart is depicted in Fig. 12.4. About 17.5% of the genes participate in the general biochemical functions of the cells, 23% in the maintenance of genome, 21% in signal transduction while the remaining 38% are involved in the production of structural proteins, transport proteins, immunoglobins etc.

Human Genes Encoding Proteins:

It is now clear that only 1.1-1.5% of the human genome codes for proteins. Thus, this figure 1.1-1.5% represents exons of genome.

As already described, a huge portion of the genome is composed of introns, and inter-genic sequences (junk DNA).

The major categories of the proteins encoded by human genes are listed in Table 12.4. The function of at least 40% of these proteins are not known.

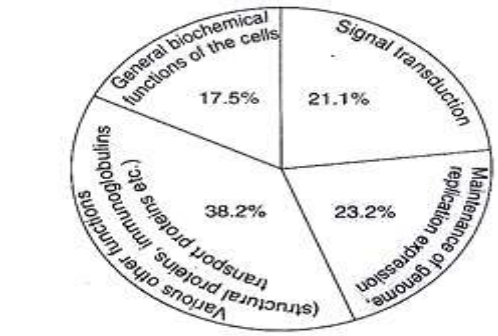


Fig. 12.4 : A pie chart showing a broad categorization of the human gene catalog (About 13000 genes whose functions are not known are not included).

Marked Differences in Individual Chromosomes:

The landscape of human chromosomes varies widely. This includes many features such as gene number per mega base, GC content, density of SNPs and number of transposable elements. For instance, chromosome 19 has the richest gene content (23 genes per mega base) while chromosome 13 and Y chromosome have the least gene content (5 genes per mega base).

Other Interesting/Important Features of Human Genome:

For more interesting features of human genome, refer Table 12.3.

i. It is surprising to note that the number of genes found in humans is only twice that present in the roundworm (19,099) and thrice that of fruit fly (13,001).

ii. Around 200 genes appear to have been derived from bacteria by lateral transfer. Surprisingly, none of these genes are present in non-vertebrate eukaryotes.

iii. The proteins encoded by human genes are more complex than that of invertebrates.

iv. The flood of the data of human genome projects will be highly useful for bioinformatics and biotechnology.

TABLE 12.4 Different categories of proteins encoded by human genes (*based on the Human Genome Project report, 2001*)

Category of proteins	*Percentage*	*Actual number of genes*
Unknown functions	41.0%	12,809
Nucleic acid enzymes	7.5%	2,308
Transcription factors	6.0%	1,850
Receptors	5.0%	1,543
Hydrolases	4.0%	1,227
Regulatory proteins (G-proteins, cell cycle regulators etc.)	3.2%	988
Protooncogenes	2.9%	902
Structural proteins of cytoskeleton	2.8%	876
Kinases	2.8%	868

*(**Note** : This table is based on the rough draft of human genome reported by Celera Genomics. The percentages are derived from a total of 26,383 genes)*

Genomes of Some Other Organisms Sequenced:

Sequencing of genomes is not confined to humans. For obvious reasons and significance, human genome sequencing attracted worldwide attention. In fact, the first genome sequence of the bacteriophage QX174 was determined in 1977. Yeast was the first eukaryotic organism to be sequenced (1986). Recently, the mouse, an animal model closest to human has been sequenced. A selected list of genomes that have been sequenced is given in Table 12.5.

TABLE 12.5 A selected list of genomes that have been sequenced

Name of the species	*Genome size (Mb/kb)*	*Comments (year)*
Bacteriophage QX174	5.38 kb	First genome sequenced (1977).
Plasmid pBR 322	4.3 kb	First plasmid sequenced (1979).
Yeast chromosome III	315 kb	First chromosome sequenced (1992).
Haemophilus influenzae	1.8 Mb	First genome of cellular organism to be sequenced (1995).
Saccharomyces cerevisiae	12 MB	First eukaryotic organism to be sequenced (1996).
Arabidopsis thaliana	125 MB	First plant genome to be sequenced (2000).
Homo sapiens (human)	3200 MB	First mammalian genome to be sequenced (2001).
Oryza sativa (rice)	430 MB	First crop plant genome to be sequenced (2002).
Mus musculis (mouse)	3300 MB	Animal model closest to human (2003).

Ethics and Human Genome:

The research on human genomes will make very sensitive data available that will affect the personal and private lives of individuals. For instance, once it is known that a person carries genes for an incurable disease, what would be the strategy of an insurance company? How will the society treat him/her?

There is a possibility that individuals with substandard genome sequences may be discriminated. Human genome results may also promote racial discrimination categorizing the people with good and bad genome sequences. Considering the gravity of ethics related to a human genome, about 3% of the HGP budget was earmarked for ethical research.

In the 1990s, there was a move by some scientists to patent the genes they discovered. This created an uproar in the public and scientific community. Fortunately, the idea of patenting genes (of human genome sequencing) was dropped. The fear still exists that genetic information will be used for commercial purposes.

Probable Questions:

1. Define genome and genomics.
2. Write down the types of genomics.
3. What do you mean by functional genomics? How functional genomics is studied?
4. How Human genome project was evolved.
5. What are the goals of HGP?
6. Write down the main features of human genome.
7. Write down the outcomes of HGP.
8. Write down different types of mapping.

Suggested Readings:

1. Molecular Cell Biology by Lodish, Fourth Edition.
2. The Cell – A Molecular Approach by Cooper and Hausman, Fourth Edition
3. Principles of Genetics by Snustad and Simmons, Sixth Edition.
4. Molecular Biology of the Cell – by Bruce Alberts
5. Cell and Molecular Biology by Gerald Karp, 7^{th} Edition.
6. Gene cloning and DNA Analysis by T. A. Brown, Sixth Edition.
7. Genetics . Verma and Agarwal.

Disclaimer:

The study materials of this book have been collected from various books, e-books, journals and other e sources.

Post-Graduate Degree Programme (CBCS)

in

ZOOLOGY

SEMESTER-III

SOFT CORE THEORY PAPER

REPRODUCTIVE BIOTECHNOLOGY

ZST-306

SELF LEARNING MATERIAL

DIRECTORATE OFOPEN AND DISTANCE LEARNING

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Director's Message

Satisfying the varied needs of distance learners, overcoming the obstacle of distance and reaching the unreached students are the threefold functions catered by Open and Distance Learning (ODL) systems. The onus lies on writers, editors, production professionals and other personnel involved in the process to overcome the challenges inherent to curriculum design and production of relevant Self Learning Materials (SLMs). At the University of Kalyani a dedicated team under the able guidance of the Hon'ble Vice-Chancellor has invested its best efforts, professionally and in keeping with the demands of Post Graduate CBCS Programmes in Distance Mode to devise a self-sufficient curriculum for each course offered by the Directorate of Open and Distance Learning (DODL), University of Kalyani.

Development of printed SLMs for students admitted to the DODL within a limited time to cater to the academic requirements of the Course as per standards set by Distance Education Bureau of the University Grants Commission, New Delhi, India under Open and Distance Mode UGC Regulations, 2017 had been our endeavour. We are happy to have achieved our goal.

Utmost care and precision have been ensured in the development of the SLMs, making them useful to the learners, besides avoiding errors as far as practicable. Further suggestions from the stakeholders in this would be welcome.

During the production-process of the SLMs, the team continuously received positive stimulations and feedback from Professor (Dr.) Sankar Kumar Ghosh, Hon'ble Vice-Chancellor, University of Kalyani, who kindly accorded directions, encouragements and suggestions, offered constructive criticism to develop it within proper requirements. We gracefully, acknowledge his inspiration and guidance.

Sincere gratitude is due to the respective chairpersons as well as each and every member of PGBOS (DODL), University of Kalyani. Heartfelt thanks is also due to the Course Writers-faculty members at the DODL, subject-experts serving at University Post Graduate departments and also to the authors and academicians whose academic contributions have enriched the SLMs. We humbly acknowledge their valuable academic contributions. I would especially like to convey gratitude to all other University dignitaries and personnel involved either at the conceptual or operational level of the DODL of University of Kalyani.

Their persistent and co-ordinated efforts have resulted in the compilation of comprehensive, learner-friendly, flexible texts that meet the curriculum requirements of the Post Graduate Programme through Distance Mode.

All the Self Learning Materials are self writing and collected from e-book, journals and websites.

Prof Manas Mohan Adhikary
Director
Directorate of Open and Distance Learning
University of Kalyani

SOFT CORE THEORY PAPER (ZST- 306)

REPRODUCTIVE BIOTECHNOLOGY

Module	Unit	Content	Credit	Class	Time (h)	Page No.
ZST - 306 (REPRODUCTIVE BIOTECHNOLOGY)	I	Cell culture laboratory design and equipment, media andreagents.	1.0	1	1	
	II	*In vitro* fertilization. cryo-preservation and frozen egg transfer,embryo transfer, Intra Cytoplasmic Sperm Injection(ICSI). *In vitro* embryoculture and Assisted Reproductivetechnology.		1	1	
	III	An overview of Cloningtechniques and Disease diagnostic markers and gene therapy		1	1	
	IV	TransgenicTechnology in animals.		1	1	

Unit-I

Cell culture laboratory design and equipment, media and reagents

Objective: In this unit you will learn aboutCell culture laboratory design and equipment, and also about media andreagents.

Introduction:

Animal cell culture basically involves the in vitro (in the laboratory) maintenance and propagation of animal cells in a suitable nutrient media. Thus, culturing is a process of growing cells artificially. Cell culture has become an indispensable technology in various branches of life sciences.

The general parameters need to be considered are: (i) Cell Quantitation (ii) Equipment and Medium (iii) pH and Buffer Systems (iv)Oxygen (v) Growth Kinetics (vi) Types of Culture Processes (vii) Other Practical Considerations.In the laboratories, small scale cultures of cells in flasks (usually 1-5 litre volume) are done for establishing the cell lines. Such cell cultures are useful for studying the morphology, growth, metabolism etc. Large-scale cultures are required for semi-industrial (100-1,000 I capacity) and large- scale industrial (5,000-20,000 I capacity) use of cells for production of wide range of biologically important compounds (e.g. enzymes, antibodies, hormones, interferon's, plasminogen activator, interleukins).

The terms fermenter and bioreactor are in common use while dealing with the industrial use of cells. A fermenter usually refers to the containment system for the cultivation of prokaryotic cells (bacteria, fungi), while a bioreactor grows the eukaryotic cells (mammalian, insect). Scale-up refers to the process of developing the culture systems in stages from a laboratory to the industry.Scale-up although tedious, labour intensive and expensive, is required for the production of commercially important products. For a better understanding of scale-up, certain basic and fundamental concepts of cell culture should be clear.

Cell Culture — General Considerations:
There are several parameters that need to be considered for appropriate growth, proliferation and maintenance of cells in culture.

A good understanding of these parameters, listed below is also necessary for scale-up:

i. Cell quantitation.

ii. Equipment and medium.

iii. pH and buffer systems.

iv. Oxygen.

v. Growth kinetics.

vi. Types of culture processes.

vii. Other practical considerations.

i. Cell Quantitation:

The total number of cells in a culture can be measured by counting in a haemocytometer. It is however, not possible to identify the viable and non-viable cells by this method.

Cell viability:

The viability of cells can be detected by use of dyes e.g. tryphan blue. The principle is based on the fact that the dye is permeable to dead cells while the viable cells do not take up dye.

Indirect measurements for cell viability:

The viability of cells can be measured by their metabolic activity. Some of the most commonly used parameters are listed:

i. Glucose utilization.

ii. Oxygen consumption.

iii. Pyruvate production.

iv. Carbon dioxide formation.

In recent years, many laboratories have started measuring the activity of lactate dehydrogenase (LDH) to detect cell viability. Dead cells release LDH and therefore, this enzyme can be used to quantitatively measure the loss of cell viability.

ii. Equipment and Medium:

The various aspects of equipment and medium used in culture laboratory.

Culture vessels:

The materials made up of glass or stainless steel are commonly used for cell cultures. Borosilicate glass (e.g. Pyrex) is preferred as it can better withstand autoclaving for suspension cultures, wherein cell attachment to the surface has to be discouraged; the culture vessels are usually treated with silicone (siliconization).

Medium and nutrients:

Appropriate selection of the medium is done based on the nutritional requirements, and the purpose for which the cultured cells are required. Eagle's basal medium and minimal essential medium are the most commonly used. The media may be supplemented with serum.

Additional feeding of certain nutrients is often required as they are quickly utilized and get exhausted. These include glucose, glutamine and cystine. For suspension cultures, media lacking calcium and magnesium are used, since their absence minimizes the surface attachment.

Non-nutrient medium supplements:

Certain non-nutrient compounds are often added to the medium for improvement of cell cultures. Sodium carboxymethyl cellulose addition to medium helps to minimize mechanical damage that may occur due to forced aeration or the forces generated by stirred impeller. Polyglycol (trade name Pluronic F-68) in the medium reduces foaming in stirred and aerated cultures.

iii. pH and Buffer Systems:

The ideal pH for animal cell cultures is around 7.4. A pH below 6.8 inhibits cell growth. The factors that can alter pH include the stability of the medium, type of buffer and its buffering capacity, concentration of glucose and headspace.

The commonly used buffer of the in vitro culture carbon dioxide-bicarbonate system (2-5% CO_2 with 10-25 mM $NaHCO_3$) is comparable to the blood buffer. The presence of phosphates in the medium improves the buffering capacity. Some laboratories use HEPES instead of bicarbonate for more efficient buffering.
As glucose is utilized by the cells, pyruvic acid and lactic acid are produced which can alter the pH. If fructose and galactose (instead of glucose) are used, the acid formation is less, but the cell growth is reduced.

iv. Oxygen:

Oxygen has to be continuously supplied to the medium throughout the life of the culture. This has to be done without causing damage to the cells. Oxygen can be supplied to the cultures in one of the following ways.

Surface aeration:

In closed system static cultures, the headspace is used for the supply of oxygen. For instance, in a 1 litre flask with 100 ml medium 900 ml of the space containing air has about 0.27 g of O_2. This O_2 is capable of supporting 10^8 cells for about 450 hours.

Sparging:

The process of bubbling gas through the culture is referred to as sparging. This is an efficient means of O_2 supply, but may often damage the cells due to effects of the bubble on the cell membrane surfaces. Use of higher air bubbles minimizes the damaging effect.

Membrane diffusion:

Adequate diffusion of oxygen into the culture can be obtained through silicone tubing which is highly permeable to gases. This approach however, is inconvenient, besides the high cost of silicone tubing.

Medium perfusion:

The medium is perfused through an oxygenation chamber before it enters the culture system. This method ensures good O_2 saturation. Medium perfusion is in fact used in glass bead system and micro carrier systems.

v. Growth Kinetics:

The standard pattern of growth of cultured cells follows a lag phase, an exponential (log) phase and a Stationary phase. Growth of cells usually means an increase in cell numbers. However, increase in cell mass may occur without replication. The following terms are in common use to represent growth of cultured cells.

Specific growth rate:
The rate of cell growth per unit amount of biomass.

Doubling time:
The time required for a population of cells to double in number or mass.

Degree of multiplication (number of doublings):
The number of times a given inoculum has replicated.

vi. Types of Culture Processes:

The different culture processes and the growth patterns of cells (represented by cell density) are depicted in Fig 37.1. They are briefly described.

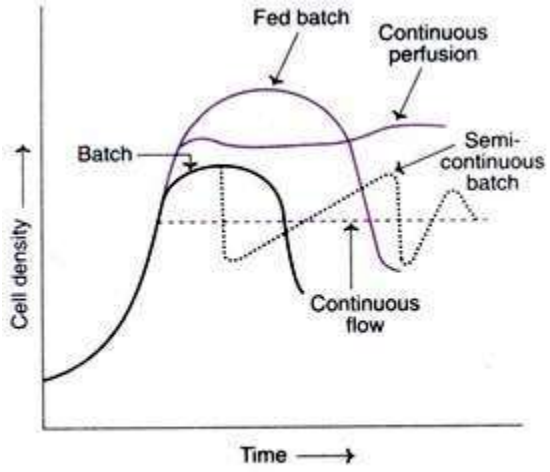


Fig. 37.1 : Comparison of different culture processes.

Batch culture:

In this technique, when the cells are inoculated into a fixed volume of the medium, they utilize the nutrients and grow, and simultaneously accumulate metabolites. As the nutrients get exhausted, toxic waste products accumulate and the cell multiplication ceases. Further, the cell density drops due to death of the cells.

Batch culture is a standard technique. Several modifications have been made to increase proliferation of cells, besides prolonging their life. The other culture processes described below are the modified batch cultures.

Fed batch culture:

There is a gradual addition of fresh medium so that the cell proliferation is much higher than the batch culture. Thus, in the fed batch culture there is an increase in the volume of culture.

Semi-continuous batch culture:

A portion of the culture medium is intermittently replaced with an equal volume of fresh medium. The growth pattern of the cells is fluctuating, with a rapid increase in cell density after each replacement of the medium.

Continuous perfusion culture:

There is a continuous addition of the medium to the culture and a withdrawal of an equal volume of used cell-free medium. The continuous perfusion process may close or open for circulation of the medium.

Continuous-flow culture:

In the continuous-flow culture, a homeostatic condition with no change in the cell numbers, nutrients and metabolites is attained. This is made possible by a balance between the addition of the medium and withdrawal of medium along with cells. This is mostly suitable for suspension cultures.

vii. Other Practical Considerations:

Besides the parameters described above, there are several other practical considerations for in vitro culture and scale-up. Some important ones are given below.

Culture surface area:
The available surface is important for the cells to grow. In general, the culture processes are planned in such a way that the surface area is not a limiting factor.

Inoculation density of cells:
As such, there is no set rule for the density of inoculation. However, inoculation with high density is preferred for better growth.

Growth phase of cells:
Cells in the late exponential (log) phase are most suitable for inoculation. The cells at the stationary phase should be avoided since they have either prolonged lag phase or no growth at all.

Stirring rate of culture:
The stirring rates of different culture lines are developed in the laboratories. It is usually in the range of 100-500 rpm for most of the cultures.

Temperature of the medium:
It is advisable to warm the medium to 37°C before adding to the culture.

Culture Media for Animal Cells:

The selection of an appropriate growth medium for the in vitro cultivation of cells is an important and essential step. The mammalian cells of an organ in the body receive nutrients from blood circulation.

For culturing these cells in vitro, it is expected that they should be provided with the components similar to those present in blood. In general, the choice of the medium mostly depends on the type of the cells to be cultured, and the purpose of the culture (growth, differentiation, and production of desired products). The culture media may be natural or artificial.

a. Natural Media:

In the early years, the natural media obtained from various biological sources were used.

Body fluids:
Plasma, serum, lymph, amniotic fluid, ascitic and pleural fluids, aqueous humour from eyes and insect hemolymph were in common use. These fluids were tested for sterility and toxicity before their utility.

Tissue extracts:
Among the tissue extracts, chick embryo extract was the most commonly employed. The extracts of liver, spleen, bone marrow and leucocytes were also used as culture media. Some workers still prefer natural media for organ culture.

b. Artificial Media:

The artificial media (containing partly defined components) have been in use for cell culture since 1950.

The minimal criteria needed for choosing a medium for animal cell cultures are listed below:

i. The medium should provide all the nutrients to the cells.

ii. Maintain the physiological pH around 7.0 with adequate buffering.

iii. The medium must be sterile, and isotonic to the cells.

The basis for the cell culture media was the balanced salt solution which was originally used to create a physiological pH and osmolarity required to maintain cells in vitro. For promoting growth and proliferation of cells, various constituents (glucose, amino acids, vitamins, growth factors, antibiotics etc.) were added, and several media developed.

Addition of serum to the various media is a common practice. However, some workers in recent years have started using serum-free media. The physicochemical properties of media required for tissue cultures are briefly described. This is followed by a brief account on balanced salt solutions, commonly used culture media and the serum-free media.

Physicochemical Properties of Culture Media:

The culture media is expected to possess certain physicochemical properties (pH, O_2, CO_2, buffering, osmolarity, viscosity, temperature etc.) to support good growth and proliferation of the cultured cells.

pH:
Most of the cells can grow at a pH in the range of 7.0-7.4, although there are slight variations depending on the type of cells (i.e. cell lines). The indicator phenol red is most commonly used for visible detection of pH of the media.

Its colouration at the different pH is shown below:
At pH 7.4 — Red

At pH 7.0 — Orange

At pH 6.5 — Yellow

At pH 7.8 — Purple

CO_2, bicarbonate and buffering:

Carbon dioxide in the medium is in a dissolved state, the concentration of which depends on the atmospheric CO_2 tension and temperature. CO_2 in the medium exists as carbonic acid (H_2CO_3), and bicarbonate (HCO^-_3) and H^+ ions as shown below.

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO^-_3$$

As is evident from the above equation, the concentrations of CO_2, HCO^-_3 and pH are interrelated. By increasing the atmospheric CO_2, the pH will be reduced making the medium acidic.
Addition of sodium bicarbonate (as a component of bicarbonate buffer) neutralizes bicarbonate ions.

$$NaHCO_3 \leftrightarrow Na^+ + HCO^-_3$$

In fact, the commercially available media contain a recommended concentration of bicarbonate, and CO_2 tension for the required pH. In recent years HEPES (hydroxyethyl piperazine 2-sulfonic acid) buffer which is more efficient than bicarbonate buffer is being used in the culture media.
However, bicarbonate buffer is preferred by most workers because of the low cost, less toxicity and nutritional benefit to the medium. This is in contrast to HEPES which is expensive, besides being toxic to the cells. The presence of pyruvate in the medium results in the increased endogenous production of CO_2 by the cells. This is advantageous since the dependence on the exogenous supply of CO_2 and HCO^-_3 will be less. In such a case, the buffering can be achieved by high concentration of amino acids.

Oxygen:

A great majority of cells in vivo are dependent on the O_2 supply for aerobic respiration. This is in fact made possible by a continuous supply of O_2 to the tissues by hemoglobin. The cultured cells mostly rely on the dissolved O_2 in the medium which may be toxic at high concentration due to the generation of free radicals. Therefore, it is absolutely necessary to supply adequate quantities of O_2 so that the cellular requirements are met, avoiding toxic effects.Some workers add free-radical scavengers (glutathione, mercaptoethanol) to nullify the toxicity. Addition of selenium to the medium is also advocated to reduce O_2 toxicity. This is because selenium is a cofactor for the synthesis of glutathione.In general, the glycolysis occurring in cultured cells is more anaerobic when compared to in vivo cells. Since the depth of the culture medium influences the rate of O_2 diffusion, it is advisable to keep the depth of the medium in the range 2-5 mm.

Temperature:

In general, the optimal temperature for a given cell culture is dependent on the body temperature of the organism, serving as the source of the cells. Accordingly, for cells obtained from humans and warm blooded animals, the optimal temperature is 37°C.In vitro cells cannot tolerate higher temperature and most of them die if the temperature goes beyond 40°C. It is therefore absolutely necessary to maintain a constant temperature (± 0.5°C) for reproducible results.

If the cells are obtained from birds, the optimal temperature is slightly higher (38.5°C) for culturing. For cold blooded animals (poikilotherms) that do not regulate their body heat (e.g. cold-water fish), the culture temperature may be in the range of 15-25°C. Besides directly influencing growth of cells, temperature also affects the solubility of CO_2 i.e. higher temperature enhances solubility.

Osmolality:

In general, the osmolality for most of the cultured cells (from different organisms) is in the range of 260-320 mosm/kg. This is comparable to the osmolality of human plasma (290 mosm/kg). Once an osmolality is selected for a culture medium, it should be maintained at that level (with an allowance of ± 10 mosm/kg). Whenever there is an addition of acids, bases, drugs etc. to the medium, the osmolality gets affected. The instrument osmometer is employed for measuring osmolalities in the laboratory.

Balanced Salt Solutions:

The balanced salt solutions (BSS) are primarily composed of inorganic salts. Sometimes, sodium bicarbonate, glucose and HEPES buffer may also be added to BSS. Phenol red serves as a pH indicator.

The important functions of balanced salt solutions are listed hereunder:

i. Supply essential inorganic ions.

ii. Provide the requisite pH.

iii. Maintain the desired osmolality.

iv. Supply energy from glucose.

In fact, balanced salt solutions form the basis for the preparation of complete media with the requisite additions. Further, BSS is also useful for a short period (up to 4 hours) incubation of cells.

The composition of two most widely used BSS namely Earle's BSS and Hank's BSS is given in Table 34.1.

Table 34.1 Composition (g/l) of balanced salt solutions (BSS)

Ingradient	*Earle's BSS*	*Hank's BSS*
NaCl	6.68	8.0
KCl	0.4	0.4
$CaCl_2$ (anhydrous)	0.02	0.14
$MgSO_4.7H_2O$	0.2	0.1
$NaHCO_3$	2.2	0.35
$NaH_3PO_4.H_2O$	0.14	—
$Na_2HPO_4.7H_2O$	—	0.09
KH_2PO_4	—	0.06
D-Glucose	1.0	1.0
Phenol red	0.01	0.01
HEPES, Na salt (buffer)	13.02	2.08

Complete Culture Media:

In the early years, balanced salt solutions were supplemented with various nutrients (amino acids, vitamins, serum etc.) to promote proliferation of cells in culture. Eagle was a pioneer in media formulation. He determined (during 1950-60) the nutrient requirements for mammalian cell cultures. Many developments in media preparation have occurred since then. There are more than a dozen media now available for different types of cultures.

Some of them are stated below:

EMEM—Eagle's minimal essential medium

DMEM—Dulbecco's modification of Eagle's medium

CMEM—Glasgow's modification of Eagle's medium

RPMI 1630 and RPMI 1640—Media from Rosewell Park Memorial Institute.

The other important culture media are Ham's F10, and F12, TC 199 and CMRL 1060. The detailed composition of three commonly used media namely Eagle's MEM, RPMI 1640 and Ham's F12 is given in Table 34.2. The complete media, in general, contains a large number of components amino acids, vitamins, salts, glucose, other organic supplements, growth factors and hormones, and antibiotics, besides serum. Depending on the medium, the quality and quantity of the ingredients vary. Some important aspects of the media ingredients are briefly described.

Table 34.2 Composition of three commonly used culture media

Component	*Eagle's MEM*	*RPMI 1640*	*Ham's F 12*
Amino acids			
L-Alanine			8.91
L-Arginine HCl	105	200	211
L-Asparagine H_2O		50	15.0
L-Aspartic acid		20	13.3
L-Cystine	24	50	24.0
L-Glutamic acid		20	14.7
L-Glutamine	292	300	146.2
Glycine		10	7.51
L-Histidine HCl H_2O	31	15	21.0
L-Isoleucine	52	50	3.94
L-Leucine	52	50	13.12
L-Lysine	58	40	36.54
L-Methionine	15	15	4.48
L-Phenylalanine	32	15	4.96
L-Proline		20	34.5
L-Serine		30	10.51
L-Threonine	48	20	11.91
L-Tryptophan	10	5	2.042
L-Tyrosine	36	20	5.43
L-Valine	46	20	11.7
Glutathione (red)		1	
L-Hydroxyproline		20	
Vitamins			
D-Biotin		0.2	0.007
Ca D-pantothenate	1	0.25	0.26
Choline chloride	1	3.0	13.96
Folic acid	1	1.0	1.32
i-Inositol	2		18.02
Nicotinamide	1	35	0.037
p-Aminobenzoic acid		1.0	
Pyridoxine HCl		1	0.062
Pyridoxal HCl	1		
Riboflavin	0.1	0.2	0.038
Thiamine HCl	1	1.0	0.34
Vitamin B_{12}		0.005	1.36

Table 34.2 contd. next column

Component	Eagle's MEM	RPMI 1640	Ham's F 12
Inorganic salts			
$CaCl_2.2H_2O$	200		44.1
$CaNO_3.4H_2O$		100	
$CuSO_4.5H_2O$			0.0025
$FeSO_4.7H_2O$			0.83
KCl	400	400	223
$MgSO_4.7H_2O$	220	100	133
NaCl	6800	6000	7599
$NaHCO_3$	2000	2000	1176
$Na_2HPO_4.7H_2O$		1512	268
$NaH_2PO_4.2H_2O$	150		
Other components			
D-Glucose	1000	2000	1801
Phenol red		5.0	1.2
Sodium pyruvate			110
Lipoic acid			0.21
Linoleic acid			0.084
Hypoxanthine			4.08
Putrescine 2HCl			0.16

Amino acids:

All the essential amino acids (which cannot be synthesized by the cells) have to be added to the medium. In addition, even the non-essential amino acids (that can be synthesized by the cells) are also usually added to avoid any limitation of their cellular synthesis. Among the non-essential amino acids, glutamine and/or glutamate are frequently added in good quantities to the media since these amino acids serve as good sources of energy and carbon.

Vitamins:

The quality and quantity of vitamins depends on the medium. For instance, Eagle's MEM contains only water soluble vitamins (e.g. B-complex, choline, inositol). The other vitamins are obtained from the serum added. The medium M 199 contains all the fat soluble vitamins (A, D, E and K) also. In general, for the media without serum, more vitamins in higher concentrations are required.

Salts:

The salts present in the various media are basically those found in balanced salt solutions (Eagle's BSS and Hank's BSS). The salts contribute to cations (Na^+, K^+, Mg^{2+}, Ca^{2+} etc.) and anions (CI^-, HCO^-_3, SO^{2-}_4, PO^{3-}_4), and are mainly responsible for the maintenance of osmolality. There are some other important functions of certain ions contributed by the salts.

i. Ca^{2+} ions are required for cell adhesion, in signal transduction, besides their involvement in cell proliferation and differentiation.

ii. Na^+, K^+ and CI^- ions regulate membrane potential.

iii. PO^{3-}_4, SO^{2-}_4 and HCO^{-}_3 ions are involved in the maintenance of intracellular charge; besides serving as precursors for the production of certain important compounds e.g. PO^{3-}_4 is required for ATP synthesis.

Glucose:

Majority of culture media contain glucose which serves as an important source of energy. Glucose is degraded in glycolysis to form pyruvate/lactate. These compounds on their further metabolism enter citric acid cycle and get oxidized to CO_2. However, experimental evidence indicates that the contribution of glucose for the operation of citric acid cycle is very low in vitro (in culture cells) compared to in vivo situation. Glutamine rather than glucose supplies carbon for the operation of citric acid cycle. And for this reason, the cultured cells require very high content of glutamine.

Hormones and growth factors:

For the media with serum, addition of hormones and growth factors is usually not required. They are frequently added to serum-free media.

Other organic supplements:

Several additional organic compounds are usually added to the media to support cultures. These include certain proteins, peptides, lipids, nucleosides and citric acid cycle intermediates. For serum-free media, supplementation with these compounds is very useful.

Antibiotics:

In the early years, culture media invariably contained antibiotics. The most commonly used antibiotics were ampicillin, penicillin, gentamycin, erythromycin, kanamycin, neomycin and tetracycline. Antibiotics were added to reduce contamination. However, with improved aseptic conditions in the present day tissue culture laboratories, the addition of antibiotics is not required. In fact, the use of antibiotics is associated with several disadvantages.

i. Possibility of developing antibiotic-resistant cells in culture.

ii. May cause anti-metabolic effects and hamper proliferation.

iii. Possibility of hiding several infections temporarily.

iv. May encourage poor aseptic conditions.

The present recommendation is that for the routine culture of cells, antibiotics should not be added. However, they may be used for the development of primary cultures.

Serum:

Serum is a natural biological fluid, and is rich in various components to support cell proliferation. The major constituents found in different types of sera are listed in Table 34.3. The most commonly used sera are calf serum (CS), fetal bovine serum (FBS), horse serum and human serum. While using human serum, it must be screened for viral diseases (hepatitis B, HIV).

Table 34.3 Major constituents of serum

Proteins	
	Albumin
	Globulins
	Fetuin
	Fibronectin
	Transferrin
	Protease inhibitors
	(α_1-antitrypsin)
Amino acids	
	Almost all the 20
Lipids	
	Cholesterol
	Phospholipids
	Fatty acids
Carbohydrates	
	Glucose
	Hexosamine
Other organic compounds	
	Lactic acid
	Pyruvic acid
	Polyamines
	Urea
Vitamins	
	Vitamin A
	Folic acid
Growth factors	
	Epidermal growth factor
	Platelet-derived growth factor
	Fibroblast growth factor
Hormones	
	Hydrocortisone
	Thyroxine
	Triiodothyronine
	Insulin
Inorganics	
	Calcium
	Sodium
	Potassium
	Chlorides
	Iron
	Phosphates
	Zinc
	Selenium

Approximately 5-20% (v/v) of serum is mostly used for supplementing several media. Some of the important features of the serum constituents are briefly described.

Proteins:

The in vitro functions of serum protein are not very clear. Some of them are involved in promoting cell attachment and growth e.g. fetuin, fibronectin. Proteins increase the viscosity of the culture medium, besides contributing to buffering action.

Nutrients and metabolites:

Serum contains several amino acids, glucose, phospholipids, fatty acids, nucleosides and metabolic intermediates (pyruvic acid, lactic acid etc.). These constituents do contribute to some extent for the nutritional requirements of cells. This may however, be insignificant in complex media with well supplemented nutrients.

Growth factors:

There are certain growth factors in the serum that stimulate the proliferation of cells in the culture:

i. Platelet-derived growth factor (PDGF).

ii. Fibroblast growth factor (FGF).

iii. Epidermal growth factor (EGF).

iv. Vascular endothelial growth factor (VEGF).

v. Insulin-like growth factors (IGF-1, IGF-2).

In fact, almost all these growth factors are commercially available for use in tissue culture.

Hormones:

Hydrocortisone promotes cell attachment, while insulin facilitates glucose uptake by cells. Growth hormone, in association with somatomedins (IGFs), promotes cell proliferation.

Inhibitors:

Serum may also contain cellular growth inhibiting factors. Majority of them are artefacts e.g. bacterial toxins, antibodies. The natural serum also contains a physiological growth inhibitor namely transforming growth factor β (TGF-β). Most of these growth inhibitory factors may be removed by heat inactivation (at 56°C for 30 minutes).

Selection of Medium and Serum:

As already stated, there are around a dozen media for the cell cultures. The selection of a particular medium is based on the cell line and the purpose of culturing. For instance, for chick embryo fibroblasts and HeLa cells, EMEM is used.The medium DMEM can be used for the cultivation of neurons. A selected list of cells and cell lines along with the media and sera used is given in Table 34.4. In fact, information on the selection of appropriate medium for a particular cell line is available from literature.

Table 34.4 A selected list of the cells or cell lines along with the media and serum used for their culture

Cells or cell line	*Medium*	*Serum*
Chick embryo fibroblasts	EMEM	CS
Chinese hamster ovary (CHO)	EMEM, Ham's F12	CS
HeLa cells	EMEM	CS
Human leukemia	RPMI 1640	FB
Mouse leukemia	Fischer's medium, RPMI 1640	FB, HoS
Neurons	DMEM	FB
Mammary epithelium	RPMI 1640, DMEM	FB
Hematopoietic cells	RPMI 1640, Fischer's medium	FB
Skeletal muscle	DMEM, F 12	FB, HoS
Glial cells	MEM, F 12, DMEM	FB
3T3 eells	MEM, DMEM	CS

The selection of serum is also based on the type of cells being cultured.

The following criteria are taken into consideration while choosing serum:
i. Batch to batch variations.

ii. Quality control.

iii. Efficiency to promote growth and preservation of cells.

iv. Sterility.

v. Heat inactivation.

In recent years, there is a tendency to discontinue the use of serum, and switch over to more clearly defined media.

Supplementation of the Medium with Tissue Extracts:

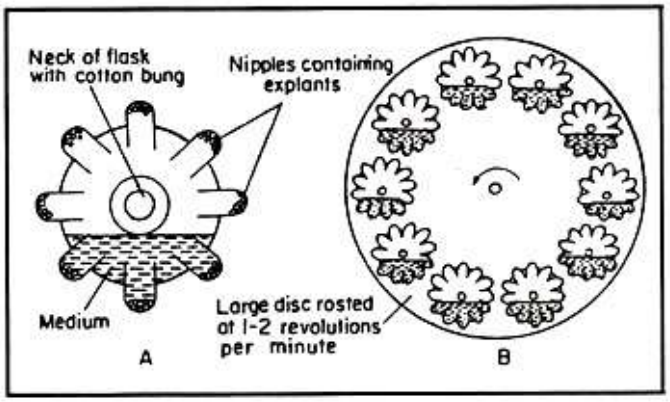
Besides serum, the culture media can also be supplemented with certain tissue extracts and microbial culture extracts. The examples are—chick embryo extract, proteolytic digests of beef heart, bactopeptone, lactalbumin hydrolysate, tryptose. The chick embryo extract was found to contain both high molecular weight and low molecular weight compounds that support growth and proliferation of cells.

A. Batch Culture:

Batch culture is a type of suspension culture where the cell material grows in a finite volume of agitated liquid medium.For instance, cell material in 20 ml or 40 ml or 60 ml liquid medium in each passage constitute a batch culture. Batch suspension cultures are most commonly maintained in conical flasks incubated on orbital platform shakers at the speed of 80-120 rpm.

Slowly Rotating Cultures:

Single cells and cell aggregates are grown in a specially designed flask, the nipple flask. Each nipple flask possesses eight nipple-like projections. The capacity of each flask is 250 ml. Ten flasks are loaded in a circular manner on the large flat disc of a vertical shaker (Fig 4.3). When the flat disc rotates at the speed of 1-2 rpm, the cell within each nipple of the flask are alternately bathed in culture medium and exposed to air.

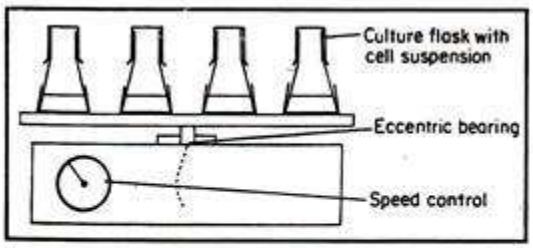


❒ Fig 4.3

A. Detail of a nipple flask. B. Large disc loaded with 10 nipple flasks used for growing cell suspension cultures

Shake Cultures:

It is a very simple and effective system of suspension culture. In this method, single cells and cell aggregates in fixed volume of liquid medium are placed in conical flasks. Conical flasks are mounted with the help of clips on a horizontal large square plate of an orbital platform shaker. The square plate moves by a circular motion at the speed of 60-180 rpm (Fig4.4).

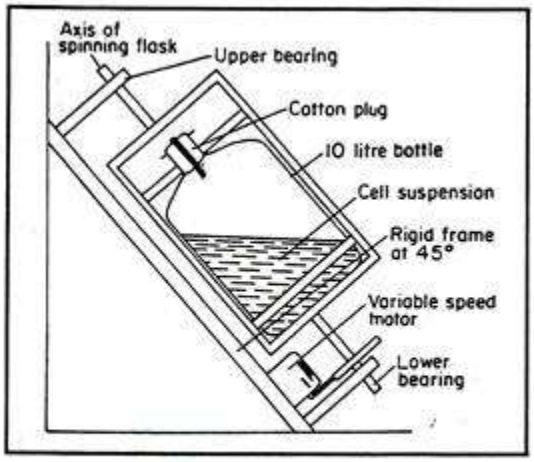


❒ Fig 4.4

Side view of a platform shaker loaded with suspension cultures contained in conical flasks

Spinning Cultures:

Large volumes of cell suspension may be cultured in 10L bottles which are rotated in a culture spinner at 120 rpm at an angle of 45° (Fig4.5).

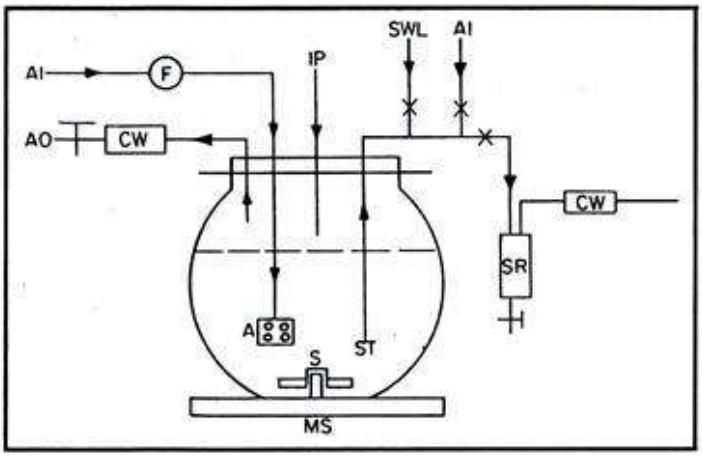


❒ Fig 4.5

Diagram of a 10 litre spinning culture apparatus

Stirred Culture:

This system is also used for large-scale batch culture (1.5 to 10 litres). In this method, the large culture vessel is not rotated but the cell suspension inside the vessel is kept dispersed continuously by bubbling sterile air through culture medium. The use of an internal magnetic stirrer is the most convenient way to agitate the culture medium safely. The magnetic stirrer revolves at 200-600 rpm. The culture vessel is a 5 to 10 litres round-bottom flask (Fig 4.6).



❒ Fig 4.6

Stirred batch culture unit. Arrow indicate direction of flow of air; AI = air input; F = sterilizing glass-fibre air filter; AO = air outlet; CW = cotton wool; IP = inoculation port; A = aerator; S = stirrer magnet; ST = sample tube; MS = magnetic stirrer; SWL = sterile water line; SR = sample receiver; (Diagram after Dr. P. King)

B. Continuous Culture System:

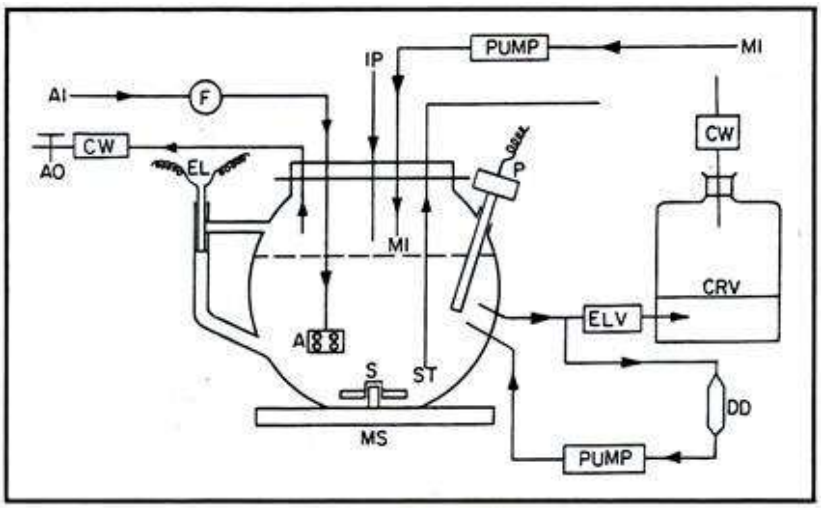
This system is very much similar to stirred culture. But in this system, the old liquid medium is continuously replaced by the fresh liquid medium to stabilize the physiological states of the growing cells. Normally, the liquid medium is not changed until the depletion of some nutrients in the medium and the cells are kept in the same medium for a certain period.

As a result active growth phase of the cell declines the depletion of nutrient. In continuous culture system, nutrient depletion does not occur due to continuous flow of nutrient medium and the cells always remain in the steady state of active growth phase.

Chemostats:

In this system, culture vessels are generally cylindrical or circular in shape and possess inlet and outlet pores for aeration and the introduction of and removal of cells and medium. The liquid medium containing the cells is stirred by a magnetic stirrer. The introduction of fresh sterile medium, which is pumped in at a constant rate into the vessel is balanced by the displacement of an equal volume of spent or old medium and cells.

Such a system can be maintained in a steady state so that new cells are produced by division at a rate which compensates the number lost in the outflow of spent medium. Thus in a steady state condition the density, growth rate, chemical composition and metabolic activity of the cells all remain constant. Such continuous cultures are ideal for studying growth kinetics and the regulation of metabolic activity in higher plants (Fig4.7).



❒ Fig 4.7

Chemostat culture. Arrows indicate direction of flow of liquid; AI = air input; F = sterilizing glass-fibre; AO = air output; CW = cottol wool; EL = volume-sensing electrodes; ELV = volume controlling outlet valve; MI = medium input; S = stirrer magnet; ST = sample tube; P = probe for oxygen tension; DD = density detector, CRV = culture receiving vessel; MS = magnetic stirrer; IP = inoculation port (Diagram after Dr. P. King)

Turbidostats:

The turbidity of a suspension culture medium changes rapidly when the cells increase in number due to their steady state growth. The changes of turbidity of the culture medium can be measured by the changes of optical density of the medium.Again, the pH of the medium changes due to increase of cell density. In turbidostats, an automatic monitoring unit is connected with the culture vessel and such unit adjusts the medium flow in such a way as to maintain the optical density or pH at a chosen, preset level.

Probable Questions:

1 What is batch culture? What is suspension culture?

2. Discuss various artificial and natural media used in animal cell culture.

3. Discuss in brief the physiochemical properties of culture media.

4. What is complete culture media. Give examples.

5. Describe Chemostats.

6. Discuss turbidostats.

7. Briefly discuss about equipment used in animal cell culture.

Suggested readings:

1. Biotechnology by P.K. Gupta

2. Gene Cloning by T. Brown.

3. Biotechnology by N. Kumarsen.

4. Biotechnology by B.D. Singh

Unit-II

In vitro fertilization. cryo-preservation and frozen egg transfer, Embryo transfer, Intra Cytoplasmic Sperm Injection(ICSI). In vitro embryo culture and Assisted Reproductive technology

Objective: In this unit you will learn about In vitro fertilization. cryo-preservation and frozen egg transfer, Embryo transfer, Intra Cytoplasmic Sperm Injection(ICSI). In vitro embryo culture and Assisted Reproductive technology.

In vitro fertilization:

In a normal pregnancy, a male sperm penetrates a woman's egg and fertilizes it inside her body after ovulation, when a mature egg has been released from the ovaries. The fertilized egg then attaches itself to the wall of the uterus, or womb, and begins developing into a baby. This is known as natural conception. However, if natural or unassisted conception is not possible, fertility treatment is an option. IVF has been used since the late 1970s. On 25 July 1978, the first "test-tube baby," Louise Brown, was born. Robert Edwards and Patrick Steptoe, who collaborated on the procedure, are considered to be the pioneers of IVF. In 2010, Robert Edwards received the 2010 Nobel Prize in Physiology or Medicine "for the development of in-vitro fertilization."

In July 2013, an American couple had the first baby to be born through IVF as a result of next-generation DNA sequencing, a new way of screening embryos that improves IVF success rates and significantly reduces the cost of treatment. DNA sequencing technology helps doctors screen embryos created by IVF to identify those most likely to lead to successful pregnancies. In vitro fertilisation (IVF) is a process by which an egg is fertilised by sperm outside the body: in vitro. IVF is a major treatment for infertility when other methods of assisted reproductive technology have failed. The process involves monitoring and stimulating a woman's ovulatory process, removing ovum or ova (egg or eggs) from the woman's ovaries and letting sperm fertilise them in a fluid medium in a laboratory.

The fertilised egg (zygote) cultured for 2-6 days in a growth medium and is then transferred to the mother's uterus with the intention of establishing a successful pregnancy. The first successful birth of a "test tube baby," Louise Brown, occurred in 1978. Louise Brown was born as a result of natural cycle IVF where no stimulation was made. Robert G. Edwards, the physiologist who developed the treatment, was awarded the Nobel Prize in Physiology or Medicine in 2010.

The term in vitro, from the Latin meaning in glass, is used, because early biological experiments involving cultivation of tissues outside the living organism from which they came, were carried out in glass containers such as beakers, test tubes, or petri dishes. Today, the term in vitro is used to refer to any biological procedure that is performed outside the organism it would normally be occurring in, to distinguish it from an in vivo procedure, where the tissue remains inside the living organism within which it is normally found. A colloquial term for babies conceived as the result of IVF, "test tube babies," refers to the tube-shaped containers of glass or plastic resin, called test tubes, that are commonly used in chemistry labs and biology labs.

However, in vitro fertilisation is usually performed in the shallower containers called Petri dishes. One IVF method, autologous endometrial coculture, is actually performed on organic material, but is still considered in vitro.

Procedure:

Techniques may differ depending on the clinic, but IVF usually involves the following steps:

1. Suppressing the natural menstrual cycle

The woman receives a drug, usually in the form of a daily injection for about 2 weeks, to suppress their natural menstrual cycle.

2 Super ovulation

Fertility drugs containing the fertility hormone follicle stimulating hormone (FSH) are given to the woman. FSH makes the ovaries produce more eggs than usual. Vaginal ultrasound scans can monitor the process in the ovaries.

3. Retrieving the eggs

The eggs are collected through a minor surgical procedure known as "follicular aspiration." A very thin needle is inserted through the vagina and into an ovary. The needle is which is connected to a suction device. This sucks the eggs out. This process is repeated for each ovary.In 2011, researchers suggested that collecting 15 eggs from the ovaries in one cycle gives the highest chance of a successful pregnancy.Frozen or donated eggs may also be used.

4. Insemination and fertilization

The eggs that have been collected are placed together with male sperm and kept in an environmentally controlled chamber. After a few hours, the sperm should enter the egg.Sometimes the sperm is directly injected into the egg. This is known as an intracytoplasmic sperm injection (ICSI).Frozen sperm, retrieved through testicular biopsy, may be used. This is believed to be as effective as fresh sperm in achieving a successful pregnancy.The fertilized egg divides and becomes an embryo.At this point, some centres offer pre-implantation genetic diagnosis (PGD) which can screen an embryo for genetic disorders. This is somewhat controversial and is not always used.One or two of the best embryos are selected for transfer.The woman is then given progesterone or human chorionic gonadotrophin (hCG) to help the lining of the womb receive the embryo.

5. Embryo transfer

Sometimes, more than one embryo is placed in the womb. It is important that the doctor and the couple wishing to have a child discuss how many embryos should be transferred. Normally, a doctor will only transfer more than one embryo if no ideal embryos are available.The transfer of the embryo is done using a thin tube, or catheter. It enters the womb through the vagina. When the embryo sticks to the lining of the womb, healthy embryo growth can begin.

The important techniques employed in assisted reproductive technology are listed below:

i. Intrauterine insemination (IUI).

ii. In vitro fertilization and embryo transfer (IVF and ET).

iii. Gamete intra-fallopian transfer (GIFT).

iv. Zygote intra-fallopian transfer (ZIPT).

v. Intra-vaginal culture (IVC).

vi. Cytoplasmic transfer (CT).

vii. Micromanipulation (Intra-cytoplasmic sperm injection (ICSI), sub-zonal insertion (SUZI).

viii. Cryopreservation.

ix. Assisted hatching (AH).

Among these techniques, the most commonly used procedure is in vitro fertilization and embryo transfer. Important features of different types of ART are briefly described.

Intrauterine Insemination (IUI):

The infertile women (due to endometriosis, idiopathic infertility) without blockage or damage to fallopian tubes can be effectively treated by intrauterine insemination. The women with adequate ovulation and below the age of 40 years are considered for IUI.

The women are usually super-ovulated by administering gonadotrophins. This results in multiple egg development. The IUI is timed to coincide with ovulation. The semen is washed and the highly motile sperms are separated. By using a thin and soft catheter, the sperms are placed either in the cervix or in uterine cavity. The women subjects are advised to remain lying down for about 15-30 minutes following IUI.Insemination should be carefully timed for good success. If it is done, a little before the expected time of ovulation, the chances for fertilization are much higher. IUI is usually successful in the first 3-4 attempts. In any case, this approach is not recommended for more than a maximum of 6 ovulation cycles. The success rates of IUI vary considerably and are in the range of 15-30%.

In Vitro Fertilization and Embryo Transfer (IVF and ET):

In vitro fertilization broadly deals with the removal of eggs from a women, fertilizing them in the laboratory, and then transferring the fertilized eggs (zygotes) into the uterus a few days later.

Indications for IVF:

Infertility due to the following causes may be considered for IVF.

i. Failed ovulation induction

ii. Tubal diseases

iii. Cervical hostility

iv. Endometriosis

v. Idiopathic infertility (in men and women).

Ideal Subjects for IVF:

Although it is not always possible to have a choice in the selection of subjects, the following criteria are preferred.

i. Woman below 35 years.

ii. Presence of at least one functional ovary.

iii. Husband with normal motile sperm count (i.e. normal seminogram).

iv. The couple must be negative for HIV and hepatitis.

Methodology of IVF:

The in vitro fertilization broadly involves the following steps.

1. Induction of superovulation.

2. Monitoring of ovarian response.

3. Oocyte retrieval.

4. Fertilization in vitro.

5. Embryo transfer.

Induction of Superovulation:

It is well known that the success rate IVF is much higher when more embryos (3-5) are transferred. This is possible only with controlled ovarian hyper-stimulation (COH). The other advantages of COH include improvement in the quality of oocyte, control of ovulation timing, besides overcoming the ovulatory dysfunction. The following drug regimens are in use to induce superovulation.

i. Clomiphene citrate (CC).

ii. CC + human menopausal gonadotrophin (hMG).

iii. CC + follicle stimulating hormone (FSH).

iv. Human menopausal gonadotrophin.

v. Follicle stimulating hormone.

vi. Gonadotrophin releasing hormone agonists (GnRHa) + hMG (or FSH).

It is now common to use GnRH agonists to induce ovulation. These compounds act through a process called down regulation of the physiologic hypothalamic- pituitary-ovarian feedback mechanism to effectively suppress spontaneous ovulation.

Monitoring of Ovarian Response:
The follicular growth or ovarian response can be monitored by increase in serum estradiol level, increase in follicular diameter and thickening of endometrial bed.

Oocyte Retrieval:

The most common method for oocyte retrieval is carried out through vaginal route under ultrasound guidance. This method is simple and less invasive, and can be performed with analgesics only. It is easy to recognize the oocyte as a single cell surrounded by a mass of cumulus cells. The recovered oocytes are maintained in vitro culture for 4-6 hours.

Fertilization in Vitro:

The semen specimens are collected (just prior to oocyte retrival) via masturbation, processed, and incubated in protein-supplemented media for 3-4 hours prior to fertilization. The incubation results in sperm capacitation.

The retrieved oocytes are also cultured in protein-supplemented media for about 6-8 hours. For the purpose of IVF, 50,000-1, 00,000 capacitated sperms are placed in culture with a single oocyte. The signs of fertilization may be demonstrated 16-20 hours later by the presence of two pronuclei within the developing embryo. There is no need to change the regime for a single failure of IVF. Many a times, success occurs in the subsequent cycles. The two most important criteria for the success of IVF are sperm density and motility.

Embryo Transfer:

Embryo at a stage between pronuclei and blastocyst stage are transferred. Conventionally, 4- 8 cell stage embryos are transferred between 48-60 hours following insemination. The transfer procedure is carried out by use of a catheter.

Not more than three embryos are transferred (per cycle) to minimize multiple pregnancies. However, in the women above the age of 40 years, higher number of embryo may be transferred. (Note: Excess oocytes and embryos are cryopreserved for further use. This will reduce the cost, besides the risk of ovarian hyper stimulation).

Luteal phase support is given by administration of progesterone for about two weeks. By this time, the diagnosis of pregnancy can be assessed by estimating human chorionic gonadotrophin (hCG).

Success Rates of IVF:

Success of IVF varies from programme to programme and within the same programme, the success rate is dependent on the correct diagnosis of the patient, and age. The overall pregnancy rate in IVF is in the range of 25-35% per oocyte retrieval. The take home baby rate is about 15-20% per procedure.

The success rate of IVF is rather low due to the following reasons:

i. Increased risk of abortion

ii. Multiple pregnancy

iii. Ectopic pregnancy

iv. Low birth weight baby

v. Premature delivery.

The World's Picture of Test Tube Babies:

By employing in vitro fertilization and embryo transfer, the world's first test tube baby (Louise Brown) was born in UK on 28th July 1978. The world's second test tube baby (Kanupriya alias Durga) was born in Kolkata on 3rd October 1978. A team led by Subhash Mukherjee carried IVF and ET in India. Scientists responsible for the "birth of test tube babies were severely criticized then.
In fact, IVF turned out to be one of the major achievements of medical sciences in the last century. It has become a novel way of treating infertility. Today, there are more than a million test tube babies born all over the world. In 2003, the world celebrated the silver jubilee of IVF with much fanfare.

Gamete Intra-Fallopian Transfer (GIFT):

Gamete intra-fallopian transfer involves the transfer of both sperm and unfertilized oocyte into the fallopian tube. This allows the fertilization to naturally occur in vivo. The prerequisite for GIFT procedure is that the woman should have at least one normal fallopian tube.

The induction of ovulation and the monitoring procedures for GIFT are almost the same as described for IVF. A couple of hours prior to oocyte retrieval, semen specimens are collected. Two oocytes along with 2-5 lakhs motile sperms for each fallopian tube are placed in a plastic tube container. It is then inserted (by laparoscopy) 4 cm into the distal end of the fallopian tube, and the oocyte sperm combination is injected.The overall pregnancy rate is as high as 30- 40%. The take home baby rate is about 25%. This is much higher when compared to IVF. But the major limitation is the requirement of laparoscopy (a major surgical procedure) to transfer oocytes and sperms into the fallopian tubes.

Zygote Intra-Fallopian Transfer (ZIFT):

ZIFT is suitable when the infertility lies in men, or in case of failure of GIFT.The wife's oocytes are exposed to her husband's sperms in the laboratory. The fertilized eggs (zygotes) within 24 hours are transferred to the fallopian tube by using laparoscopy.ZIPT has an advantage over GIFT with male factor infertility. Further, it can be known whether the wife's oocytes have been fertilized by her husbands' sperms.

Intra Vaginal Culture (IVC):

The body's own environment is appropriately utilized in intra-vaginal culture. The retrieved oocytes and sperms are placed in a culture medium inside a sealed container. This is inserted into the vagina. The container is held by a vaginal diaphragm. Thus, the oocytes and sperms are maintained at the normal body temperature (in contrast to any incubator in the laboratory). Two to 3 three days later, the container is opened, and the fertilized and dividing zygotes are transferred into the uterus. This procedure appears simple, but the success rate is very low. Only a few centers practice this.

Cytoplasmic Transfer (CT):

Cytoplasm includes many things, the most important being mitochondria which provide energy to the cell. It is possible that deficiency in the mitochondria may leave the oocyte without the necessary power for cell division, after fertilization. This may result in abnormal cell division and poor development of embryo.

It is therefore logical to think of the transfer of cytoplasm from a donor (with active mitochondria) into the oocyte of a woman. The advantage with cytoplasmic transfer is that the mother's own genetic material is passed on to the offspring.

Two methods of cytoplasmic transfer have been developed:

1. Transfer of a small amount of cytoplasm by a tiny needle from a donor to a recipient oocyte.

2. Transfer of a large amount of cytoplasm which is fused with the recipient's cytoplasm by applying electricity.

The procedure of cytoplasmic transfer is tedious and technically difficult, besides the cost factor. At least two viable pregnancies have been so far reported in literature by this approach.

Micromanipulation:

Micromanipulation involves in vitro micro-surgically assisted fertilization procedures. This is required when the sperms are unable to penetrate the zona pellucida of oocyte and fertilize. Micromanipulations are usually done in severe cases of male factor infertility.

A diagrammatic representation of micromanipulation is depicted in Fig 18.3.

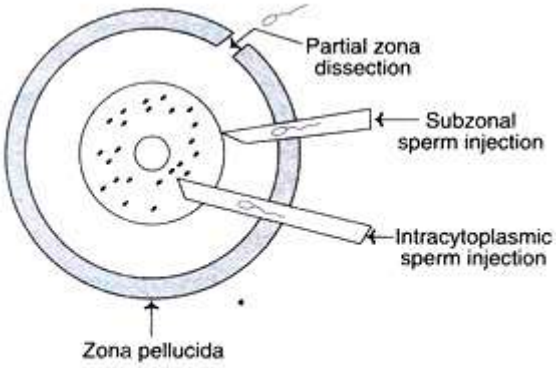


Fig. 18.3 : Micromanipulation for fertilization of an egg (microsurgically assisted fertilization).

Intra-Cytoplasmic Sperm Injection (ICSI):

Intra-cytoplasmic sperm injection is a new and novel infertility treatment utilizing the micromanipulation technology. Many of the previous treatment processes for male infertility have been abandoned in favour of ICSI. The male factor infertility could be due to low sperm counts, poor sperm motility, and poor quality of sperm to penetrate oocyte.

By partial zona dissection (PZD), the zona pellucida is opened using either chemical dissolution or a sharp instrument. A single spermatozoon can be directly injected into the cytoplasm of the oocyte through the micro-puncture of zona pellucida. A micropipette is used to hold the oocyte while the spermatozoon is deposited inside the ooplasm of the oocyte. Besides using normal sperms, round-headed sperms, sperms collected directly from the epididymis and previously cryopreserved sperms can be used in ICSI.

Among the micromanipulation techniques ICSI is the most successful one with a fertilization rate of about 65%. Attempts are on to improve this further. In fact, ICSI has revolutionized assistant reproductive technology by utilizing the sperms of husbands who were once considered to be unsuitable for fertilization process.

Sub-zonal Insertion (SUZI):

In sub-zonal insertion, the zona pellucida is punctured and sperms (1-30 in number) are injected into an area between the zona and the egg. It is expected that one of the sperms will fertilize the egg. The major limitation of SUZI is polyspermy since it is not possible to control the number of sperms that enter the egg.

Round Spermid nucleus Injection (ROSNI):

There are a few men who cannot manufacture sperms, and therefore they have a zero sperm count. For these men, it is possible to take out the round spermatids (immature cells) directly from the testicle, isolate the nucleus (containing the genetic material) and inject it into the partner's eggs. ROSNI is a recent exciting breakthrough to solve the problem of male infertility through micromanipulation.

Cryopreservation:

Preservation in a frozen state is regarded as cryopreservation. Cryopreservation is very useful in assisted reproductive technology.

i. Semen can be cryopreserved. This may be from the donors, cancer patients (before the commencement of treatment).

ii. Fertilized eggs after IVP or ICSI can be preserved.

iii. Embryos can also be preserved for transfer at a later stage.

Human embryos have been successfully preserved in the presence of cryoprotectants (1, 2-propanediol/dimethyl sulfoxide/glycerol) and stored at -196°C under liquid nitrogen. At appropriate time, the embryos are thawed, cryoprotectants removed and then transferred. Many test tube babies in fact have been born as a result of application of freezing technology.

Cryopreservation of semen:

Semen cryopreservation (commonly called **sperm banking or sperm freezing**) is a procedure to preserve sperm cells. Semen can be used successfully indefinitely after cryopreservation. For human sperm, the longest reported successful storage is 24 years. It can be used for sperm donation where the recipient wants the treatment in a different time or place, or as a means of preserving fertility for men undergoing vasectomy or treatments that may compromise their fertility, such as chemotherapy, radiation therapy or surgery.

Freezing

The most common cryoprotectant used for semen is glycerol (10% in culture medium). Often sucrose or other di-, trisaccharides are added to glycerol solution. Cryoprotectant media may be supplemented with either egg yolk or soy lecithin, with the two having no statistically significant differences compared to each other regarding motility, morphology, ability to bind to hyaluronate in vitro, or DNA integrity after thawing.

Additional cryoprotectants can be used to increase sperm viability and fertility rates post-freezing. Treatment of sperm with heparin binding proteins prior to cryopreservation showed decreased cryoinjury and generation of ROS. The addition of nerve growth factor as a cryoprotectant decreases sperm cell death rates and increased motility after thawing. Incorporation of cholesterol into sperm cell membranes with the use of cyclodextrins prior to freezing also increases sperm viability.

Semen is frozen using either a controlled-rate, slow-cooling method (slow programmable freezing or SPF) or a newer flash-freezing process known as vitrification. Vitrification gives superior post-thaw motility and cryosurvival than *slow programmable freezing*.

Thawing :

Thawing at 40 °C seems to result in optimal sperm motility. On the other hand, the exact thawing temperature seems to have only minor effect on sperm viability, acrosomal status, ATP content, and DNA. As with freezing, various techniques have been developed for the thawing process, both discussed by Di Santo et al. (2012)

Refreezing :

In terms of the level of sperm DNA fragmentation, up to three cycles of freezing and thawing can be performed without causing a level of risk significantly higher than following a single cycle of freezing and thawing. This is provided that samples are refrozen in their original cryoprotectant and are not going through sperm washing or other alteration in between, and provided that they are separated by density gradient centrifugation or swim-up before use in assisted reproduction technology.

Effect on quality :

Some evidence suggests an increase in single-strand breaks, condensation and fragmentation of DNA in sperm after cryopreservation. This can potentially increase the risk of mutations in offspring DNA. Antioxidants and the use of well-controlled cooling regimes could potentially improve outcomes. In long-term follow-up studies, no evidence has been found either of an increase in birth

defects or chromosomal abnormalities in people conceived from cryopreserved sperm compared with the general population.

Cryopreservation of embryo:

Cryopreservation of embryos is the process of preserving an embryo at sub-zero temperatures, generally at an embryogenesis stage corresponding to pre-implantation, that is, from fertilisation to the blastocyst stage.

Indications:

Embryo cryopreservation is useful for leftover embryos after a cycle of in vitro fertilisation, as patients who fail to conceive may become pregnant using such embryos without having to go through a full IVF cycle. Or, if pregnancy occurred, they could return later for another pregnancy. Spare oocytes or embryos resulting from fertility treatments may be used for oocyte donation or embryo donation to another woman or couple, and embryos may be created, frozen and stored specifically for transfer and donation by using donor eggs and sperm.

Method:

Embryo cryopreservation is generally performed as a component of in vitro fertilization (which generally also includes ovarian hyperstimulation, egg retrieval and embryo transfer). The ovarian hyperstimulation is preferably done by using a GnRH agonist rather than human chorionic gonadotrophin (hCG) for final oocyte maturation, since it decreases the risk of ovarian hyperstimulation syndrome with no evidence of a difference in live birth rate (in contrast to fresh cycles where usage of GnRH agonist has a lower live birth rate).

The main techniques used for embryo cryopreservation are vitrification versus slow programmable freezing (SPF). Studies indicate that vitrification is superior or equal to SPF in terms of survival and implantation rates. Vitrification appears to result in decreased risk of DNA damage than slow freezing.

Direct Frozen Embryo Transfer: Embryos can be frozen by SPF in ethylene glycol freeze media and transfer directly to recipients immediately after water thawing without laboratory thawing process. The world's first crossbred bovine embryo transfer calf under tropical conditions was produced by such technique on 23 June 1996 by Dr.Binoy S Vettical of Kerala Livestock Development Board, Mattupatti

Prevalence

World usage data is hard to come by but it was reported in a study of 23 countries that almost 42,000 frozen human embryo transfers were performed during 2001 in Europe.

Pregnancy outcome and determinants

In current state of the art, early embryos having undergone cryopreservation implant at the same rate as equivalent fresh counterparts. The outcome from using cryopreserved embryos has uniformly been positive with no increase in birth defects or development abnormalities, also between fresh versus frozen eggs used for intracytoplasmic sperm injection (ICSI). In fact, pregnancy rates are increased following frozen embryo transfer, and perinatal outcomes are less affected, compared to embryo transfer in the same cycle as ovarian hyperstimulation was performed. The endometrium is believed to not be optimally prepared for implantation following ovarian hyperstimulation, and therefore frozen embryo transfer avails for a separate cycle to focus on optimizing the chances of successful implantation. Children born from vitrified blastocysts have significantly higher birthweight than those born from non-frozen blastocysts. For early cleavage embryos, frozen ones appear to have at least as good obstetric outcome, measured as preterm birth and low birthweight for children born after

cryopreservation as compared with children born after fresh cycles. Oocyte age, survival proportion, and number of transferred embryos are predictors of pregnancy outcome.

Pregnancies have been reported from embryos stored for 16 years. A study of more than 11,000 cryopreserved human embryos showed no significant effect of storage time on post-thaw survival for IVF or oocyte donation cycles, or for embryos frozen at the pronuclear or cleavage stages.[11] In addition, the duration of storage had no significant effect on clinical pregnancy, miscarriage, implantation, or live birth rate, whether from IVF or oocyte donation cycles. A study in France between 1999 and 2011 came to the result that embryo freezing before administration of gonadotoxic chemotherapy agents to females caused a delay of treatment in 34% of cases, and a live birth in 27% of surviving cases who wanted to become pregnant, with the follow-up time varying between 1 and 13 years.

Assisted Hatching (AH):

Improper implantation of the embryo in the uterus is one of the limiting factors in the success of ART in humans. Assisted hatching is a novel approach for the proper implantation of the embryo in the endrometrium. The embryos in the uterus possess an outer coating namely zona pellucida (the shell). These embryos must be hatched to remove the shell, a step necessary for implantation. In certain women, particularly above 40 years age, natural hatching does not occur, and requires outside assistance.

Assisted hatching is carried out by using a Laser to make a small hole in the shell of the embryo. These embryos when transferred into the uterus hatch and get implanted.During the course of AH for 3-4 days, the women are kept on steroids (to suppress mother's immunity) and antibiotics (to counter infections). Better results are reported with this approach.

Pre-implantation Genetic Diagnosis (PGD):

The genetic defects in ovum before fertilization or in the embryo before implantation can be identified by a new medical tool namely pre-implantation genetic diagnosis. It is estimated that about 60% of the ART driven pregnancies are lost due to chromosomal abnormalities. This can be minimized or prevented by using PGD.

A direct determination of chromosomal abnormalities prior to implantation ensures a successful pregnancy and ultimate delivery of a healthy baby. One group of workers has reported an increase in the pregnancy rate from 15 to 30% by employing pre-implantation genetic diagnosis.DNA Amplification and Analysis:

The latest in PGD is the direct DNA analysis. This can be carried out by removing a single cell from 6-8-cell embryo. The DNA is removed and amplified by employing polymerase chain reaction.

Direct DNA analysis is useful for the diagnosis of several genetic diseases e.g. cystic fibrosis, sickle-cell anaemia, haemophilia, Duchene's muscular dystrophy, Tay-Sachs disease.

Ethical Advantages of PGD:

PGD is highly advantageous from the ethical point of view, since the embryos with genetic disorders can be discarded in the very stages without the formation of offspring's with undesirable characteristics

The Negative Aspects of Art:
There are certain limitations/disadvantages associated with assisted reproductive technology in humans. Some highlights are given. It must however, be noted that the advantages of ART outweigh the disadvantages.

Ovarian Hyper-stimulation Syndrome (OHSS):
Due to administration of hormones and drugs, ovarian hyper-stimulation is frequently associated with complications, sometimes even life- threatening. OHSS is more severe in women who conceived in the same cycle, and received hCG as luteal support (following embryo transfer).

Risks Associated with Pregnancy:
ART is associated with multiple pregnancy, increased risk for anemia, gestational diabetes and premature labour. Low birth weight and prematurity are closed linked with mortality and morbidity.

Premature Menopause:
Controlled ovarian hyper-stimulation (COH) causes multiple follicular utility. There is a risk of premature menopause as COH may reduce the ovarian follicles, besides faster aging. Sometimes, a single COH may use ovarian follicles, which in the normal course are equivalent to two years of ovulation during the natural menstrual cycle.

Ovarian Cancer:
The use of fertility drugs and injuries to epithelium increase the risk of ovarian cancer at least by three times when compared to normal women.

Probable Questions:

1. Define in vitro fertilization. Why it is necessary?
2. Describe different steps of IVF?
3. What is cryopreservation? What is cryoprotectant? Give examples.
4. Describe intrauterine insemination.
5. When IVF is chosen?
6. Describe in brief Gamete Intra-Fallopian Transfer (GIFT).
7. Describe in brief Zygote Intra-Fallopian Transfer (ZIFT).
8. What are the two methods of Cytoplasmic transfer. Describe any one process.
9. Describe Intra-Cytoplasmic Sperm Injection (ICSI).
10. How cryopreservation of embryo is done?
11. What is the importance of Pre-implantation Genetic Diagnosis (PGD).

Suggested readings:

1. Biotechnology by P.K. Gupta
2. Gene Cloning by T. Brown.
3. Biotechnology by N. Kumarsen.
4. Biotechnology by B.D. Singh.

Unit-III

An overview of Cloningtechniques and Disease diagnostic markers and gene therapy

Objective: In this unit you will learn about cloning techniques and markers used for disease diagnosis. Yu will also know about gene therapy techniques.

Introduction:

Gene cloning. Gene cloning involves separation of specific gene or DNA fragments from a donor cell, attaching it to small carrier molecule called vector and then replicating this recombinant vector into a host cell.

The procedure consists of following steps:

a. Isolation of DNA to be Cloned:

The DNA of interest, i.e., target DNA may be genomic DNA or complementary DNA or synthetic DNA. The genomic DNA of interest if contained in a particular restriction fragment, that can be isolated from gel after electrophoresis.

Otherwise, a complementary DNA (cDNA) fragment is prepared directly by using mRNA as template. The polyadenylated mRNAs are separated from other types of RNAs through affinity column chromatography. These mRNAs are then copied to cDNAs with the help of reverse transcriptase. In these cases as the cDNA is obtained from mRNA, so it must contain the uninterrupted coding sequence of gene and the recombinant DNA molecule will synthesize the eukaryotic gene product in prokaryotic cell. One can also synthesize the desired DNA fragment by machine.

b. Insertion of Foreign DNA Fragment into a Vector:

The cDNA thus isolated above or obtained from gene bank is fragmented by using the specific restriction enzyme to develop specific cohesive ends. The cloning vector is also treated with the same restriction enzyme, so that the cohesive ends are generated (Fig. 18.5).

For insertion of double stranded cDNA into a cloning vector, it is necessary to add to both termini single stranded DNA sequence which should be complementary to a tract of DNA at the termini of linearized vector. In order to get efficient formation of recombinant DNA molecules, addition of sticky ends on both termini is necessary.

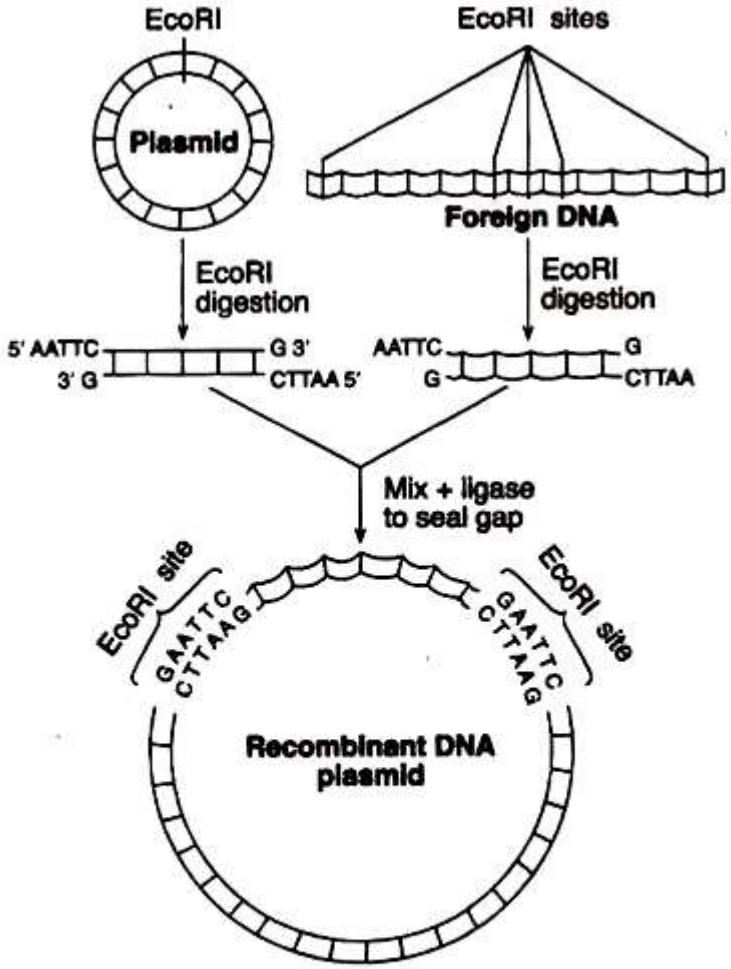


Fig. 18.5: Construction of a recombinant DNA plasmid through the use of the restriction enzyme Eco RI. The Eco RI cuts both the plasmid and the foreign DNA and by mixing, we get recombinant DNA plasmid (after Russel, 1987)

There are two methods for generation of cohesive ends on the double stranded cDNA:

(i) Use of linkers

(ii) Homopolymer tails.

Linkers are the chemically synthesized double stranded DNA oligonucleotides containing on it one or more restriction sites for cleavage by restriction enzymes (Fig. 18.6).

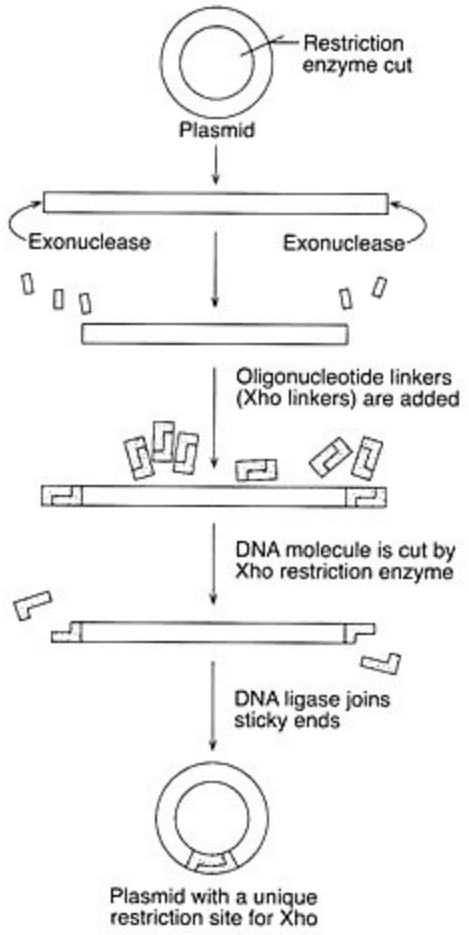


Fig. 18.6: Addition of a "linker" (carrying a restriction site) to a vector molecule

Linkers are ligated to blunt end by T4-DNA ligase. Using terminal transferase the synthesis of homopolymer tails of the defined length at both 3′ termini of double stranded DNA and vector is possible. If the poly- T tail is added at the termini of foreign DNA, then poly-A tail is added at the restriction site of the vector, so that the complementary sticky ends are formed and they get annealed by T4-DNA ligase (Fig. 18.7).

c. Transfer of Recombinant DNA into Bacterial Cell:

Before the recombinant DNA can be bulked up by cloning, it must be taken up by a suitable bacterial host cell, which is then said to be transformed, i.e., a host bacterial cell must accept the plasmid with the foreign gene, get it incorporated into its genome and start transcribing that gene.

The event of entering the plasmid with foreign DNA into the cell is known as **"transformation"**. A mild heat shock is given to the mixture which results in the uptake at higher frequency of the DNA. The selection of transformed cells is done by allowing the bacteria to grow in antibiotic selection medium.

Cloning in Eukaryotes:

In eukaryotes the nucleus is separated from the rest of cell through nuclear membrane, many of the genes are split genes with exons and introns. As such genetic engineering with eukaryotes needs special methods.

When eukaryotic genes are cloned in prokaryotes, the split genes cannot be correctly expressed, because prokaryotes do not have the machinery for splicing out the RNA transcribed from the introns of a gene. So the eukaryotic cells are needed for cloning and expression of cloned eukaryotic genes.

Among eukaryotes, DNA cloning has been done in yeast, mouse and in higher plant species. In yeast, a 2μ plasmid DNA is an appropriate cloning vehicle, which can be transferred through efficient transformation method. This involves protoplast production followed by PEG directed introduction of DNA into protoplasts.

d. Detection of Recombinant Clone:

From the large number of colonies produced by transformation to select or screen out the few colonies which contain the recombinant plasmid — the use of antibiotics is one of the most easy and useful methods for this purpose. The transformed cells can be plated on selection medium containing different antibiotics.The colonies which grow, can be said to have a plasmid, as the antibiotic resistance gene of plasmid enables the bacteria to grow. For example, the plasmid pBR 322 contains genes for ampicillin resistance (amp^r) and tetracycline resistance (tet^r). Thus the trans-formants can be detected by their plating potential on medium containing either (or both) of these antibiotics**.**

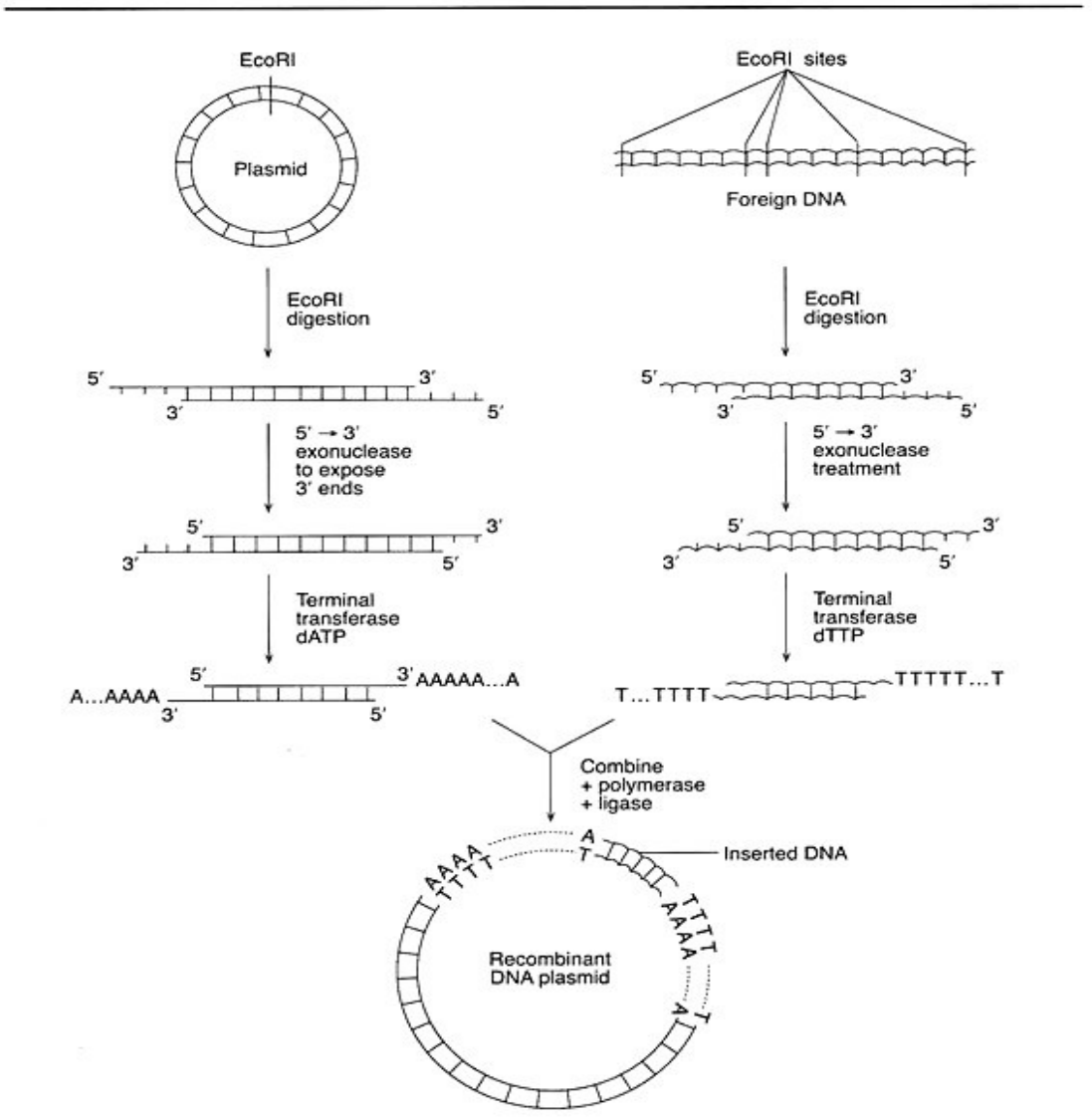


Fig. 18.7: Construction of a recombinant DNA plasmid using the enzyme terminal transferase to synthesize complementary ends on the linearized plasmid and a restriction enzyme generated fragment of foreign DNA (after Russel, 1987)

The presence of cloned DNA fragments can be detected by insertional inactivation of suitable genetic system. For example, DNA fragment of interest can be inserted into one of the antibiotic- resistance genes (tet^r) of pBR322, inactivating that gene (tet^s) and other remains active (amp^r).
To selectively kill cells with antibiotics, the original master plate with ampicillin in medium is subjected to replica plating method with both ampicillin and tetracycline. Bacteria with recombinant plasmid do not grow on replica plate, only with non-recombinant plasmid will grow. Recombinant colonies are thus identified and selected from master plate (Fig. 18.8).

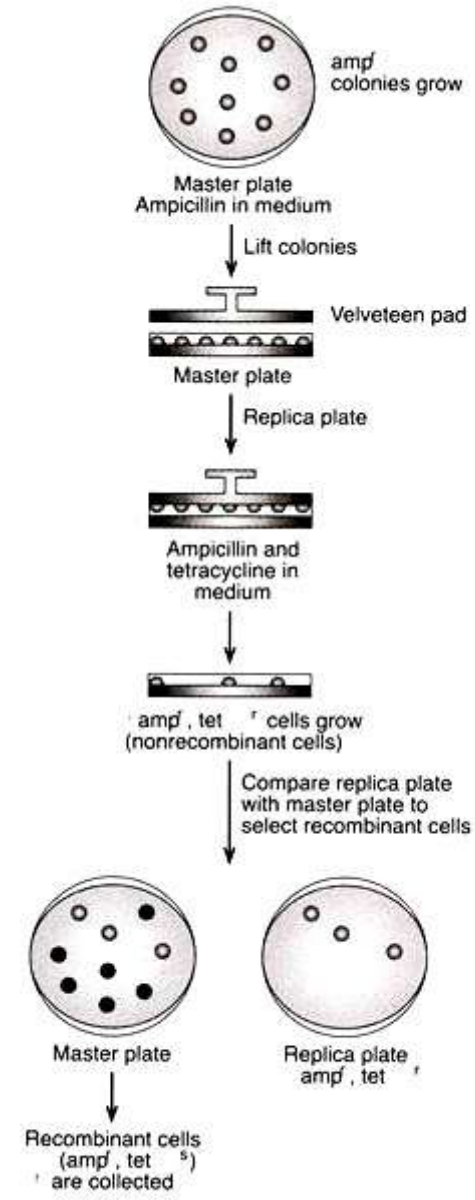


Fig. 18.8: Selecting for recombinant cells after transformation (after Barnum)

The detection of recombinant clones can also be done by using chromogenic substrates. The most popular system uses X-gal, a colourless substrate on cleavage by β-galactosidase, a blue coloured product is formed, then the expression of lac Z gene can be detected easily.

Host cells that are Lac^- are used, so that the Lac^+ phenotype will only arise when the vector is present. Furthermore, if a DNA fragment is cloned into lac Z gene (Eco RI site of Charon 16A), any

recombinants will be lac Z and therefore will not produce β-galactosidase and plaques will remain colourless in presence of X-gal.

The Vectors which are used in Gene cloning are discussed below:

1. Plasmid Vectors:

Plasmids are the extra chromosomal genetic elements commonly found in bacteria and are mostly made of double-stranded circular DNA. They are used as vectors in gene cloning, because they have a replication origin in their DNA making them competent of autonomous replication, and also because they generally have one or two restriction sites for several restriction enzymes.

Many plasmids have been genetically engineered to add useful properties. The number of copies of plasmids may vary from one to several per host cell. Some plasmids under relaxed replication control can have larger number of copies which may be sometimes as high as 1,000. Such high copy-number plasmids are specially suitable for cloning.A commonly used small cloning vector is the plasmid pBR 322 which has a circular double- stranded DNA having 4,363 base pairs, compared to the E. coli chromosome having about 4,700 x 10^3 base pairs. It carries single restriction sites for the restriction enzymes EcoR1, Hind III, Bam HI and Sal I. Thus, each of these enzymes can make single cleavages at their respective sites of pBR 322 DNA, where the foreign DNA can be inserted.

Many plasmids also have genes for antibiotic resistance which can be profitably used as selectable markers. For example, pBR322 has two genes specifying resistance to ampicillin (amp^r) and tetracycline (tet^r). Presence of two such marker genes is more advantageous than a single marker, provided one of the marker genes has a restriction site within it. When a foreign DNA fragment is inserted within the resistance gene, it results in the inactivation of the resistance gene, just as a transposon causes inactivation of a gene.

Such inactivation of an antibiotic resistance gene makes the host ceil in which the plasmid is present susceptible to the particular antibiotic. This property can be utilized for identification and selection of the host cells in which the foreign gene has been cloned. For example, pBR 322 possesses a restriction site for Hind III in the tet^r gene. When a foreign gene is cloned using Hind III in pBR 322, tet^r gene is inactivated and the host cells containing the recombinant plasmid show resistance to ampicillin because the amp' gene is intact, but not to tetracycline. This makes possible the identification of the host cells containing the cloned foreign gene, because they cannot grow on a medium in which tetracycline has been incorporated at an inhibitory concentration. Other host cells which take up only the vector DNA without the cloned gene will grow in such a medium, because their tet^r gene is intact.

Naturally occurring plasmids do not usually possess all the desirable properties which are useful for their use as cloning vectors. So they must be suitably altered by molecular biological techniques. For example, the plasmid pBR 322 was developed by several alternations. Other plasmids which are used as cloning vectors include pSC 101, pUC 8, pHC 79 etc. (p stands for plasmid).A very useful plasmid used as a vector for introducing foreign genes into plants is the Ti-plasmid of a plant pathogenic bacterium, Agrobacterium tumefaciens which infects many dicotyledonous plants causing a disease, known as crown-gall. When this organism infects a host plant, it transfers naturally a portion of the Ti-plasmid into the plant. This portion is known T-DNA. The T-DNA segment has been extensively altered by molecular biological methods to make the Ti-plasmid suitable as a vector for inserting foreign DNA into many plants to produce transgenic plants.

2. Bacteriophage Vectors:

The most commonly used bacteriophage vector is the λ-(lambda) phage of *E. coli*.The λ-DNA after it infects a host cell can either enter into a lysogenic state by integration into the host DNA, or can lead to a lytic cycle producing progeny phages. In the phage head, the DNA is present as a linear double-stranded helix.

After entering into the host cell, the molecule forms a covalently closed circular DNA with the help of single-stranded ends having complimentary base sequences (cos-sites). The λ-genome is 48.5 x 10^3 base-pair long and has a single restriction site for Eco R1. To be used as a cloning vector, the λ-DNA requires to be processed by genetical engineering techniques, so that it retains the genes necessary for carrying out the lytic cycle only and the Eco R1 recognition sequence.
The central portion of the genome which is not essential for the lytic cycle is removed to accommodate the inserted foreign DNA into λ-DNA, so that the recombinant DNA has the appropriate length to be packaged into the phage head.

One problem with the phage vectors is the length of the foreign DNA that is to be inserted. If the length of the insert is too long or too short, the recombinant DNA cannot be packaged into the phage head. The insert in case of λ-DNA should be about 10 to 20 kb, preferably 15 kb long. After the λ-DNA is processed, its length becomes too short to be packaged into the phage head. The insertion of a foreign DNA having an appropriate length (-15 kb) brings back the length to an appropriate size which can be packaged into a λ -head. The recombinant phage can then be used for propagation in E. coli yielding large number of phage particles which carry the recombinant DNA.

The insertion of a foreign DNA fragment of appropriate length which carries a gene of choice is carried out by treatment of both insert and processed λ-DNA with Eco R1. The foreign DNA, with its sticky-ends, forms base pairs with those of λ-DNA fragments to yield a recombinant DNA. The recombinant molecule is then packaged into a phage head. The phage on infection produces a circular DNA and multiplies producing a lytic cycle. From the progeny phage particles, recombinant DNA can be isolated (Fig. 9.131).For cloning larger DNA molecules, i.e. larger than 20 kb, the bacteriophage PI of E. coli can be used. This phage is also a temperate one like λ-phage, but it has a DNA genome of about 100 kb, i.e. double of λ-phage. Of its 100 kb genome, only about 15 kb is essential for replication in the host. So, a processed vector prepared from phage PI can be used for cloning much larger DNA fragments of 80 to 85 kb. The phage PI DNA is a circular molecule. The general procedure of inserting a foreign DNA into P1 vector is similar to that of λ -DNA.

3. Cosmid Vectors:

Cosmids are hybrids of plasmids and λ -phage. The cos-sites of λ-DNA are joined to a plasmid, like Col El, to produce a typical cosmid. A cosmid is generally a circular DNA molecule containing its own replication origin sequence, a selectable marker gene — like one coding for antibiotic resistance — one or two restriction sites and the cos-site of X-DNA. The cos-site helps in circularization of the cosmid DNA. The cosmid derived from Col El has a rifampicin-resistance marker (rif^r), two restriction sites for Hind III and the cos-site of λ-phage (Fig. 9.132).
Cosmids are suitable for cloning larger molecules of DNA. On the average, DNA fragments having a size of 35 to 45 kb can be inserted into a cosmid, whereas, in plasmids, the insert size is between 5 kb to 10 kb, and, in λ-vectors, the insert size is usually about 15 kb.

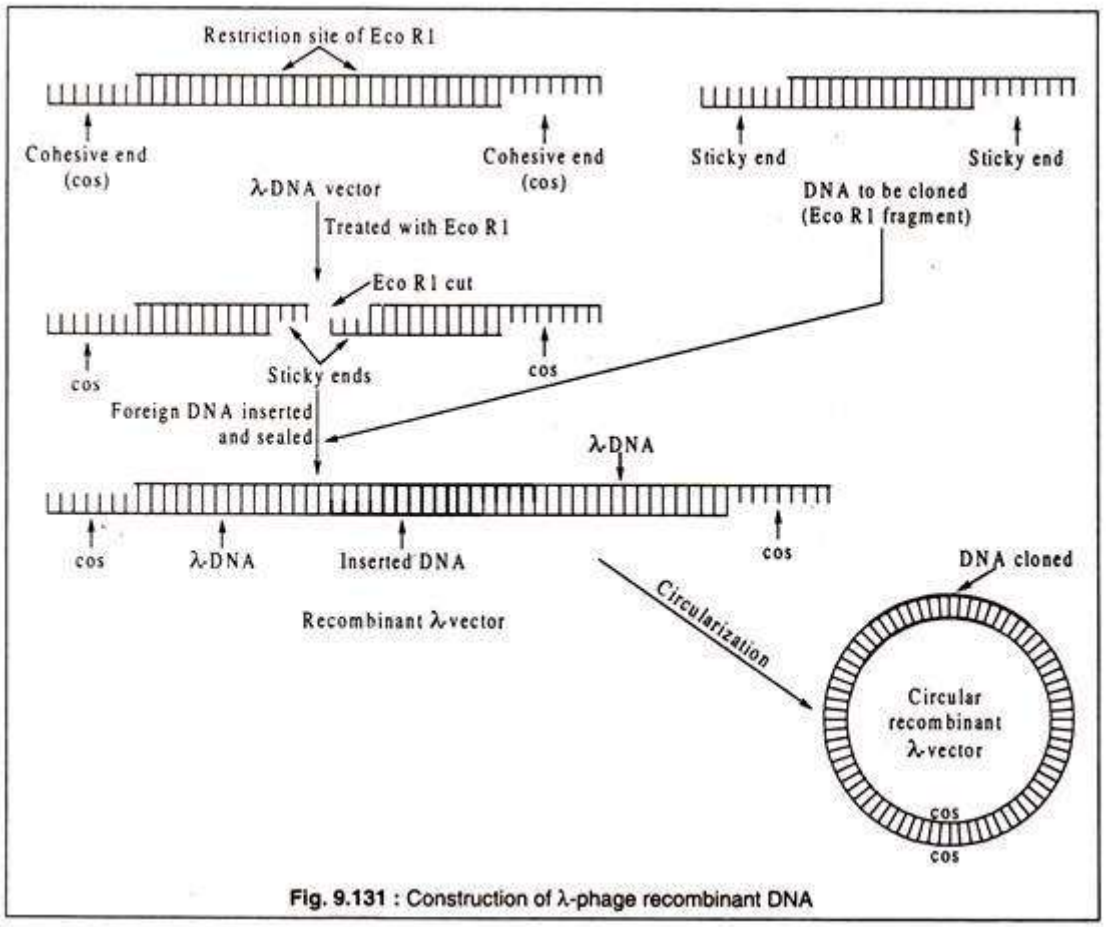


Fig. 9.131 : Construction of λ-phage recombinant DNA

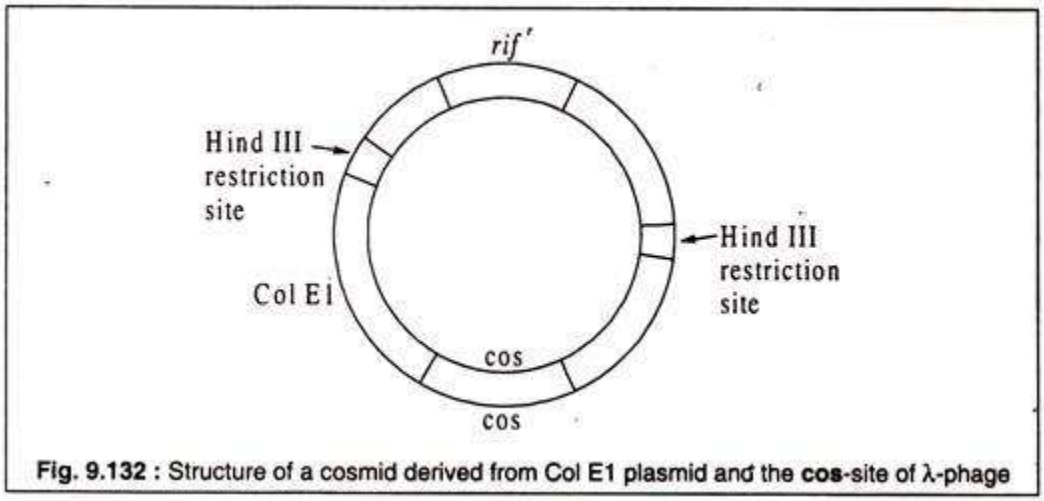


Fig. 9.132 : Structure of a cosmid derived from Col E1 plasmid and the **cos**-site of λ-phage

Procedure for Cloning Recombinant DNA

The first step in a gene cloning programme is to construct a recombinant DNA molecule containing a donor DNA segment in which a selected gene is located and a vector DNA. The donor may belong to any taxonomic group. The DNA of the donor is cleaved into fragments using any of the many restriction enzymes.The vector DNA has also to be cleaved by the same enzyme, so that both DNAs have similar sticky ends. When the donor fragments are mixed with vector fragments, the single-stranded sticky-ends form base-pairs. The free ends are then joined by DNA ligase to obtain a recombinant DNA molecule as shown in Fig. 9.130 and Fig. 9.131 using two different types of vectors.

Although joining of two DNA fragments obtained by digesting them with the same restriction enzyme is not difficult, identification of a recombinant DNA containing a specific donor gene is by no means so simple. Because, the donor DNA is often a very large molecule containing many restriction sites of a single restriction enzyme.

As a result, restriction digest consists of many fragments, only one or a few of these fragments include the gene or parts of the gene to be cloned. The insertion of these fragments into vector yields recombinant DNA, of many sorts, only a few of which contain the desired gene.In a cloning experiment it becomes essential to identify the particular recombinant molecules in which the gene is present. A straight-forward way of doing this is to identify the gene in a clone by its product. Other methods involve use of a purified DNA containing the gene of choice for insertion into the vector to prepare the recombinant molecule.The next step is the introduction of the recombinant DNA into a suitable host. This depends on the nature of vector chosen. For insertion of comparatively small DNA fragments, the vector of choice is one of the plasmids which has been suitably tailored for the purpose.

The recombinant circular plasmid vector is introduced into its bacterial host, mostly E. coli, by forced transformation, because E. coli is not normally transformable. The bacteria which have taken up the recombinant plasmid can be identified with suitable markers.For example, if the plasmid contains a gene conferring resistance to say ampicillin, only those transformed bacteria containing the plasmid can grow in an ampicillin-containing agar and the non-transformed bacteria are eliminated. This is only a preliminary selection. Because of reasons, most of the recombinant plasmids do not have the desired donor gene. For further screening, the technique of colony hybridization is often adopted to identify the bacterial clones with the desired gene.

The cloning procedure using a plasmid vector is shown in a simplified diagrammatic manner in Fig. 9.133:

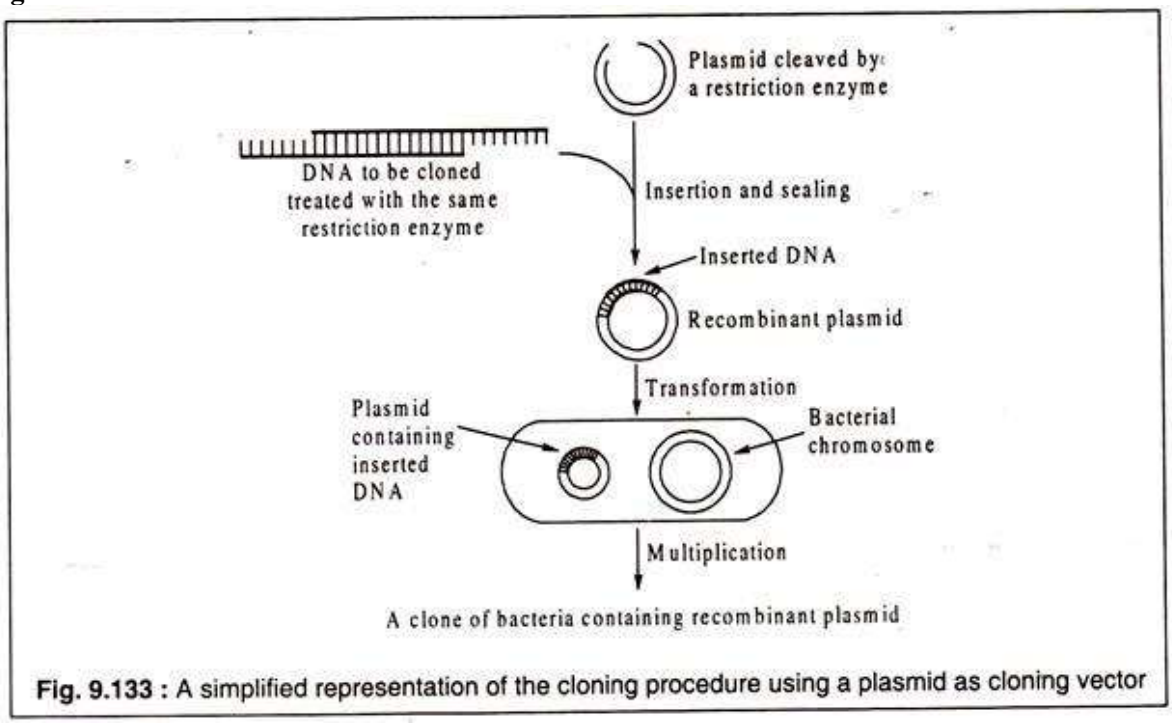


Fig. 9.133 : A simplified representation of the cloning procedure using a plasmid as cloning vector

When bacteriophage vectors are used for cloning, the recombinant DNA obtained by joining the DNA segment of the donor and the processed phage DNA is packaged into the phage head. The recombinant phage particles are allowed to infect appropriate host bacteria by the natural infection process.

The recombinant DNA of the phage multiplies producing large number of progeny phage particles which are released by lysis of the host cells. Recombinant DNA molecules can be isolated from the progeny phage particles for experimental purpose.

The procedure is shown in Fig. 9.134:

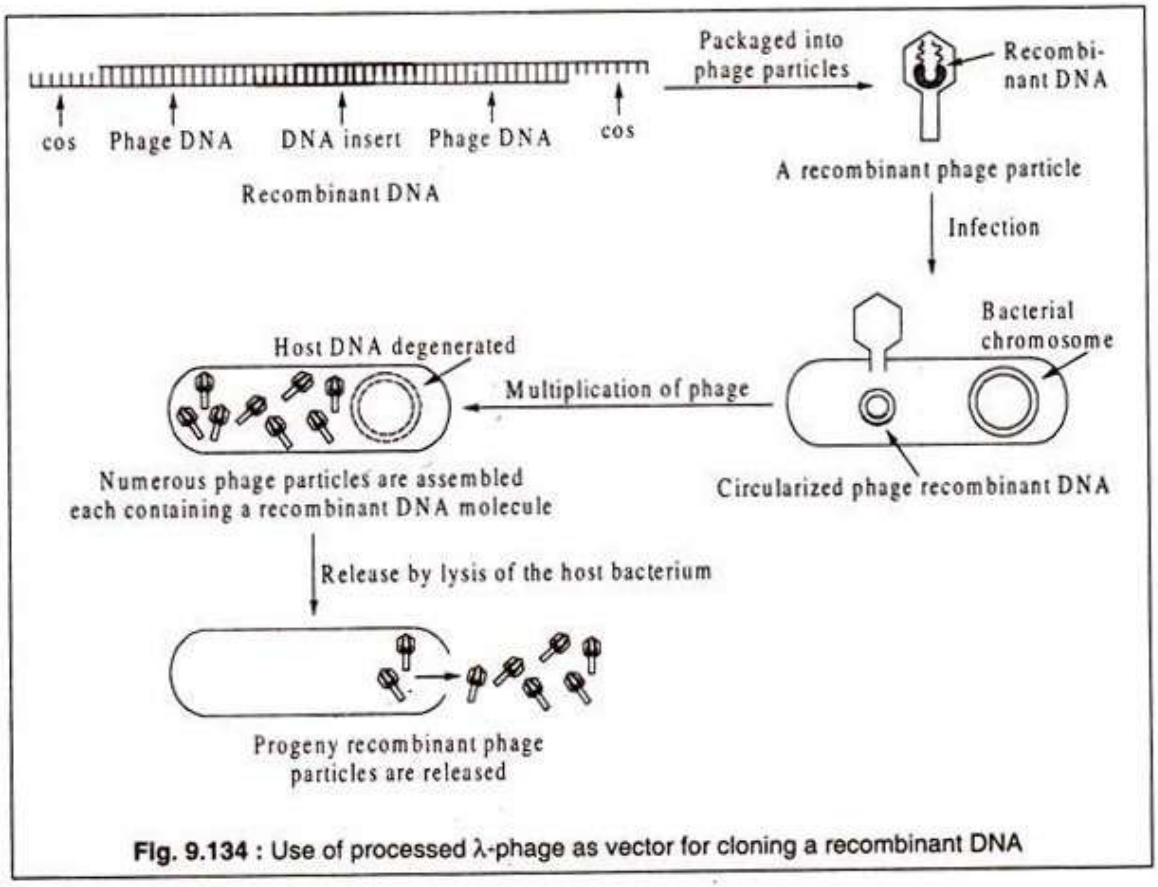


Fig. 9.134 : Use of processed λ-phage as vector for cloning a recombinant DNA

The cloning procedure using cosmid vectors is more or less similar to that of phage vectors, except that they are capable of inserting larger DNA fragments of about 40 kb size. Another important distinguishing feature of cosmid vectors is that they multiply in infected hosts as plasmids and do not form phage progeny. Therefore, the question of lysis does not arise.

All the three types of vectors — plasmids, phage and cosmids — are used for cloning recombinant DNA in bacterial hosts, mainly E. coli.

Some- vectors have been developed by genetic engineering techniques which can exist in two different hosts. These are called shuttle vectors. A vector of this type is YEp 24 which can replicate in both yeast and E. coli. These vectors contain sequences of an E. coli plasmid and a part of the yeast genome. Each part has its own replication origin and some other genes as well as restriction sites. A structure of a shuttle vector of yeast and E. coli is shown in Fig. 9.135.

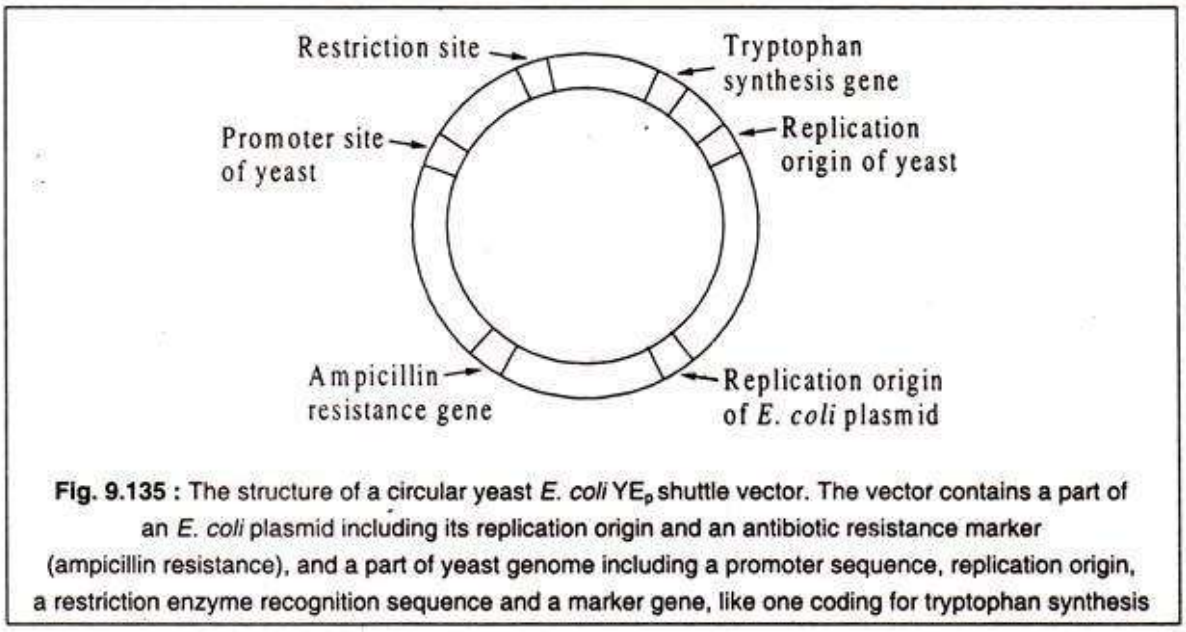


Fig. 9.135 : The structure of a circular yeast *E. coli* YE_p shuttle vector. The vector contains a part of an *E. coli* plasmid including its replication origin and an antibiotic resistance marker (ampicillin resistance), and a part of yeast genome including a promoter sequence, replication origin, a restriction enzyme recognition sequence and a marker gene, like one coding for tryptophan synthesis

Such shuttle vectors have proved useful in cloning eukaryotic genes like mammalian genes. One problem of cloning eukaryotic genes in prokaryotes is that the bacterial RNA polymerase may fail to initiate transcription of the eukaryotic gene transferred into a bacterial host, because of its inability to interact with the eukaryotic promoter.

Another problem is due to the presence of introns in the eukaryotic primary transcripts (hn-RNA) which the bacteria are unable to remove for they do not have the biochemical machinery. The first problem can be effectively solved by coupling the eukaryotic gene next to a bacterial promoter.As E. coli is selected generally as the host, its lac-promoter is often used for this purpose. The second problem of removal of introns can be solved, if the eukaryotic gene to be cloned is made free of the intron segments before its joining to the vector DNA.This can be achieved indirectly by preparing an eukaryotic gene from its m-RNA using reverse transcriptase. The processed form of m-RNA which is translated to yield the gene product is devoid of introns. A complimentary copy of m-RNA produced by reverse transcription gives a DNA without introns. Such a DNA, known as complementary DNA (c-DNA) can then be used for obtaining a recombinant DNA by joining with a suitable vector.

Use of DNA in the Diagnosis of Infectious and Genetic Diseases

I. DNA in the Diagnosis of Infectious Diseases :

The use of DNA analysis (by employing DNA probes) is a novel and revolutionary approach for specifically identifying the disease-causing pathogenic organisms. This is in contrast to the traditional methods of disease diagnosis by detection of enzymes, antibodies etc., besides the microscopic examination of pathogens.Although at present not in widespread use, DNA analysis may soon take over the traditional diagnostic tests in the years to come. Diagnosis of selected diseases by genetically engineered techniques or DNA probes or direct DNA analysis is briefly described.

Tuberculosis:

Tuberculosis is caused by the bacterium Mycobacterium tuberculosis. The commonly used diagnostic tests for this disease are very slow and sometimes may take several weeks. This is because M.

tuberculosis multiplies very slowly (takes about 24 hrs. to double; E. coli takes just 20 minutes to double).

A novel diagnostic test for tuberculosis was developed by genetic engineering, and is illustrated in Fig. 14.3. A gene from firefly, encoding the enzyme luciferase is introduced into the bacteriophage specific for M. tuberculosis. The bacteriophage is a bacterial virus, frequently referred to as luciferase reporter phage or mycophage.

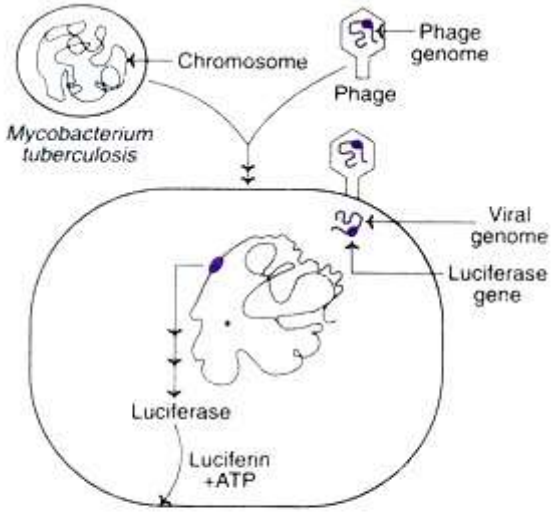


***Fig. 14.3** : Diagnosis of tuberculosis by using a genetically engineered bacteriophage (phage).*

The genetically engineered phage is added to the culture of M. tuberculosis. The phage attaches to the bacterial cell wall, penetrates inside, and inserts its gene (along with luciferase gene) into the M. tuberculosis chromosome. The enzyme luciferase is produced by the bacterium.

When luciferin and ATP are added to the culture medium, luciferase cleaves luciferin. This reaction is accompanied by a flash of light which can be detected by a luminometer. This diagnostic test is quite sensitive for the confirmation of tuberculosis. The flash of light is specific for the identification of M. tuberculosis in the culture. For other bacteria, the genetically engineered phage cannot attach and enter in, hence no flash of light would be detected.

Malaria:

Malaria, mainly caused by Plasmodium falciparum and *P. vivax*, affects about one-third of the world's population. The commonly used laboratory tests for the diagnosis of malaria include microscopic examination of blood smears, and detection of antibodies in the circulation. While the former is time consuming and frequently gives false-negative tests, the latter cannot distinguish between the past and present infections.

A specific DNA diagnostic test for identification of the current infection of *P. falciparum* has been developed. This is carried out by using a DNA probe that can bind and hybridize with a DNA fragment of *P. falciparum* genome and not with other species of Plasmodium. It is reported that this DNA probe can detect as little as 1mg of P. falciparum in blood or 10 pg of its purified DNA.

Chagas' Disease:

The protozoan parasite *Trypanosoma cruzi* causes Chagas' disease. This disease is characterized by destruction of several tissues (liver, spleen, brain, lymph nodes) by the invading parasite. Chagas' disease is diagnosed by the microscopic examination of the fresh blood samples. Immunological tests, although available, are not commonly used, since they frequently give false-positive results.

Scientists have identified a DNA fragment with 188-base pair length present in *T. cruzi* genome. This is however, not found in any other related parasite. A PCR technique is employed to amplify the 188 bp DNA fragment. This can be detected by using polyacrylamide gel electrophoresis. Thus, PCR-based amplification can be effectively used for the diagnosis of Chagas' disease.

Acquired Immunodeficiency Syndrome (AIDS):

AIDS is caused by the virus, human immunodeficiency virus (HIV). The commonly used laboratory test for detection of AIDS is the detection of HIV antibodies. However, it might take several weeks for the body to respond and produce sufficient HIV antibodies. Consequently, the antibodies test may be negative (i.e., false-negative), although HIV is present in the body. During this period, being a carrier, he/she can transmit HIV to others.

DNA probes, with radioisotope label, for HIV DNA are now available. By using PCR and DNA probes, AIDS can be specifically diagnosed in the laboratory. During the course of infection cycle, HIV exists as a segment of DNA integrated into the T-lymphocytes of the host. The T-lymphocytes of a suspected AIDS patient are isolated and disrupted to release DNA.

The so obtained DNA is amplified by PCR, and to this DNA probes are added. If the HIV DNA is present, it hybridizes with the complementary sequence of the labelled DNA probe which can be detected by its radioactivity. The advantage of DNA probe is that it can detect the virus when there are no detectable antibodies in the circulation.

HIV diagnosis in the newborn:

Detection of antibodies is of no use in the newborn to ascertain whether AIDS has been transmitted from the mother. This is because the antibodies might have come from the mother but not from the virus. This problem can be solved by using DNA probes to detect HIV DNA in the newborn.

Human Papilloma Virus:

Human papilloma virus (HPV) causes genital warts. HPV is also associated with the cervical cancer in women. The DNA probe (trade name Virapap detection kit) that specifically detects HPV has been developed. The tissue samples obtained from woman's cervix are used. HPV DNA, when present hybridizes with DNA probe by complementary base pairing, and this is the positive test.

Lyme Disease:

Lyme disease is caused by the bacterium, Borrelia burgdorferi. This disease is characterized by fever, skin rash, arthritis and neurological manifestations. The diagnosis of Lyme disease is rather difficult, since it is not possible to see B. burgdorferi under microscope and the antibody detection tests are not very reliable. Some workers have used PCR to amplify the DNA of B. burgdorferi. By using appropriate DNA probes, the bacterium causing Lyme disease can be specifically detected.

Periodontal Disease:

Periodontal disease is characterized by the degenerative infection of gums that may ultimately lead to tooth decay and loss. This disease is caused by certain bacteria. At least three distinct species of bacteria have been identified and DNA probes developed for their detection. Early diagnosis of periodontal disease will help the treatment modalities to prevent the tooth decay.

DNA Probes for Other Diseases:

In principle, almost all the pathogenic organisms can be detected by DNA probes. Several DNA probes (more than 100) have been developed and many more are in the experimental stages. The ultimate aim of the researchers is to have a stock of probes for the detection of various pathogenic organisms—bacteria, viruses, parasites. The other important DNA probes in recent years include for the detection of bacterial infections caused by E. coli (gastroenteritis) Salmonella typhi (food poisoning), *Campylobacter hyoitestinalis* (gastritis).

Diagnosis of tropical diseases:

Malaria, filariasis, tuberculosis, leprosy, schistosomiasis, leishmaniasis and trypanosomiasis are the tropical diseases affecting millions of people throughout the world. As already described for the diagnosis of malaria caused by P. falciparum, a DNA probe has been developed. A novel diagnostic test, by genetic manipulations, has been devised for the diagnosis of tuberculosis. Scientists are continuously working to develop better diagnostic techniques for other tropical diseases.

B. DNA in the Diagnosis of Genetic Diseases:

Traditional laboratory tests for the diagnosis of genetic diseases are mostly based on the estimation of metabolites and/or enzymes. This is usually done after the onset of symptoms. The laboratory tests based on DNA analysis can specifically diagnose the inherited diseases at the genetic level.DNA-based tests are useful to discover, well in advance, whether the individuals or their offspring's are at risk for any genetic disease; Further, such tests can also be employed for the prenatal diagnosis of hereditary disorders, besides identifying the carriers of genetic diseases.

By knowing the genetic basis of the diseases, the individuals can be advised on how to limit the transmission of the disease to their offspring's. It may also be possible, in due course of time, to treat genetic diseases by appropriate gene therapies. Theoretically, it is possible to develop screening tests for all single-gene diseases. Some of the important genetic diseases for which DNA analysis is used for diagnosis are briefly described.

Cystic Fibrosis:

Cystic fibrosis (CF) is a common and fatal hereditary disease. The patients produce thick and sticky mucus that clogs lungs and respiratory tract. Cystic fibrosis is due to a defect in cftr gene that encodes cystic fibrosis trans membrane regulator protein, CFTR gene is located on chromosome 7 in humans, and a DNA probe has been developed to identify this gene.

The genetic disease cystic fibrosis is inherited by a recessive pattern, i.e., the disease develops when two recessive genes are present. It is now possible to detect CF genes in duplicate in the fetal cells obtained from samples of amniotic fluid. As the test can be done months before birth, it is possible to know whether the offspring will be a victim of CF. One group of researchers have reported that CF gene can be detected in the eight-celled embryo obtained through in vitro fertilization.

Sickle-Cell Anaemia:

Sickle-cell anaemia is a genetic disease characterized by the irregular sickle (crescent like) shape of the erythrocytes. Biochemically, this disease results in severe anaemia and progressive damage to major organs in the body (heart, brain, lungs, and joints).Sickle-cell anaemia occurs due to a single amino acid change in the β-chain of haemoglobin. Specifically, the amino acid glutamate at the 6th position of β-chain is replaced by valine. At the molecular level, sickle-cell anaemia is due to a single-nucleotide change (A → T) in the β-globin gene of coding (or antisense) strand.

In the normal β-globin gene the DNA sequence is CCTGAGGAG, while in sickle-cell anaemia, the sequence is CCTGTGGAG. This single-base mutation can be detected by using restriction enzyme Mstll to cut DNA fragments in and around β-globin gene, followed by the electrophoretic pattern of the DNA fragments formed.

The change in the base from A to T in the β-globin gene destroys the recognition site (CCTGAGG) for Mstll (Fig. 14.4). Consequently, the DNA fragments formed from a sickle-cell anemia patient for β-globin gene differ from that of a normal person. Thus, sickle-cell anaemia can be detected by digesting mutant and normal β-globin gene by restriction enzyme and performing a hybridization with a cloned β-globin DNA probe.

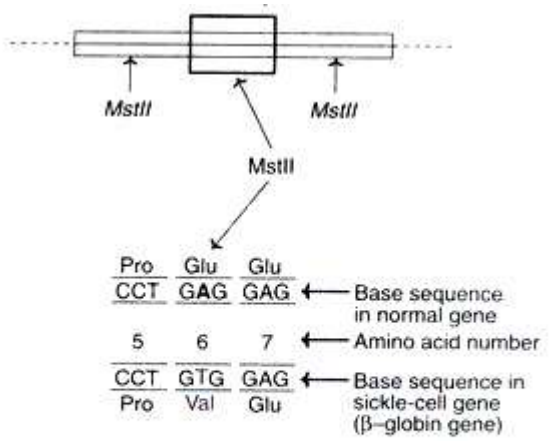


*Fig. 14.4 : Single-base change resulting in sickle-cell anemia. (**Note** : A small and relevant DNA fragment of β-globin gene magnified and shown with encoded amino acids).*

Single-nucleotide polymorphisms:
The single base changes that occur in some of the genetic diseases (e.g., sickle-cell anaemia) are collectively referred to as single-nucleotide polymorphisms (SNPs, pronounced snips). It is estimated that the frequency of SNPs is about one in every 1000 bases. Sometimes SNPs are associated with amino acid change in the protein that is encoded. A point mutation in α_1-antitrypsin gene is also a good example of SNPs, besides sickle-cell anaemia.

Duchenne's Muscular Dystrophy:
Duchenne's muscular dystrophy (DMD) is a genetic abnormality characterized by progressive wasting of leg and pelvic muscles. It is a sex-linked recessive disease that appears between 3 and 5 years of age. The affected children are unsteady on their feet as they lose the strength and control of their muscles. By the age of ten, the victims of DMD are confined to wheel chair and often die before reaching 20 years age.

The patients of DMD lack the muscle protein, namely dystrophin which gives strength to the muscles. Thus, DMD is due to the absence of a gene encoding dystrophin. For specific diagnosis of Duchenne's muscular dystrophy, a DNA probe to identify a segment of DNA that lies close to defective gene (for dystrophin) is used. This DNA segment, referred to as restriction fragment length polymorphism (RFLP), serves as a marker and can detect DMD with 95% certainty.

In the DNA diagnostic test using RFLP for DMD, DNA samples must be obtained from as many blood relatives (parents, grand-parents, uncles, aunts etc.) as possible. The RFLP patterns, constructed for the entire family are thoroughly checked for the affected and unaffected relatives. This is required since there is a wide variation in RFLPs from family to family. Thus, there is no single identifying test for the diagnosis of genetic diseases based on RFLPs analysis.

Huntington's Disease:
Huntington's disease is a genetic disease (caused by a dominant gene) characterized by progressive deterioration of the nervous system, particularly the destruction of brain cells. The victims of this

disease (usually above 50 years of age) exhibit thrashing (jerky) movements and then insanity [older name was Huntington's chorea; chorea (Creek) means to dance]. Huntington's disease is invariably fatal.

The molecular basis of Huntington's disease has been identified. The gene responsible for this disease lies on chromosome number 4, and is characterized by excessive repetition of the base triplet CAG. The victims of Huntington's disease have CAG triplet repeated 42-66 times, against the normal 11-34 times.The triplet CAG encodes for the amino acid glutamine. It is believed that the abnormal protein (with very high content of glutamine) causes the death of cells in the basal ganglia (the part of the brain responsible for motor function).

Huntington's disease can be detected by the analysis of RFLPs in blood related individuals. The clinical manifestations of this disease are observed after middle age, and by then the person might have already passed on the defective gene to his/ her offspring's.

Fragile X Syndrome:

Fragile X syndrome, as the name indicates, is due to a genetic defect in X chromosome (a sex chromosome) and affects both males and females. The victims of this disease are characterized by mental retardation. Researchers have found that sufferers of fragile X syndrome have the three nucleotide bases (CGG) repeated again and again.

It is believed that these tri-nucleotide repeats block the transcription process resulting in a protein deficiency. This protein is involved in the normal function of the nerve cells, and its deficiency results in mental retardation. A DNA probe has been developed for the detection of fragile X syndrome in the laboratory.

Other Triple Repeat Diseases:

Excessive repetition of triplet bases in DNA are now known to result in several diseases which are collectively referred to as triple repeat diseases. Besides Huntington's disease and fragile X syndrome, some more triple repeats are given below.

Friedreich's ataxia:

The tri-nucleotide GAA repeats 200 to 900 times on chromosome 9 in Friedreich's ataxia. This disease is associated with degradation of spinal cord. Spinocerebellar ataxia is another triplet disease, characterized by neuromuscular disorder, and is due to tri-nucleotide repeats of CAG by 40 to 80 times on chromosome 6.

There are a few triple repeat diseases in which the repeats tend to increase with each generation and the diseases become more severe. This also results in the onset of clinical manifestations at early ages. Kennedy's disease, also called spinobulbar muscular atropy (CAG repeat) and myotonic dystrophy (CTG) are good examples.

Are triple repeat diseases confined to humans?

Triple repeat diseases have so far not been detected in any other organisms (bacteria, fruit flies, other mammals) except in humans. More studies however, may be needed to confirm this. The occurrence of triple repeat diseases indicates that the structure of DNA may be rather unstable and dynamic. This is in contrast to what molecular biologists have been thinking all along.

Alzheimer's Disease:

Alzheimer's disease is characterized by loss of memory and impaired intellectual function (dementia). The victims of this disease cannot properly attend to their basic needs, besides being unable to speak and walk. The patients of Alzheimer's disease were found to have a specific protein, namely amyloid

in the plaques (or clumps) of dead nerve fibers in their brains. A group of researchers have identified a specific gene on chromosome 21 that is believed to be responsible for familial Alzheimer's disease.

A DNA probe has been developed to locate the genetic marker for Alzheimer's disease. The present belief is that many environmental factors and a virus may also be responsible for the development of this disease. It may be possible that in the individuals with genetic predisposition, the outside factors may be stimulatory for the onset of the disease.

Amyotrophic Lateral Sclerosis:

Amyotrophic lateral sclerosis (ALS) is characterized by degenerative changes in the motor neurons of brain and spinal cord. A gene to explain the inherited pattern of ALS was discovered. The gene, known as sodl, encoding for the enzyme superoxide dismutase is located on chromosome 21. This gene was found to be defective in families suffering from amyotrophic lateral sclerosis. In fact, certain point mutations in the sodl resulting in single amino acid changes in superoxide dismutase have been identified.

Superoxide dismutase is a key enzyme in eliminating the highly toxic free radicals that damage the cells (free radicals have been implicated in aging and several disease e.g. cancer, cataract, Parkinson's disease, Alzheimer's disease). On the basis of the function of superoxide dismutase, it is presumed that ALS occurs as a result of free radical accumulation due to a defective enzyme (as a consequence of mutated gene sodl). The deleterious effects of free radicals can be reduced by administering certain compounds such as vitamins C and E.

Another group of workers have reported that the defective superoxide dismutase cannot control a transporter protein responsible for the removal of the amino acid glutamate from the nerve cells. As a result, large quantities of glutamate accumulate in the nervous tissue leading to degenerative changes.

Cancers:

It is now agreed that there is some degree of genetic predisposition for the occurrence of cancers, although the influence of environmental factors cannot be underestimated. In fact, cancer susceptible genes have been identified in some families e.g., genes for melanoma susceptibility in humans are located on chromosomes 1 and 9.

p^{53} Gene:

The gene p^{53} encodes for a protein with a molecular weight 53 kilo Daltons (hence the name). It is believed that the protein produced by this gene helps DNA repair and suppresses cancer development. Certain damages that occur in DNA may lead to unlimited replication and uncontrolled multiplication of cells.

In such a situation, the protein encoded by p^{53} gene binds to DNA and blocks replication. Further, it facilitates the faulty DNA to get repaired. The result is that the cancerous cells are not allowed to establish and multiply. Thus, p^{53} is a cancer-suppressor gene and acts as a guardian of cellular DNA.

Any mutation in the gene p^{53} is likely to alter its tumor suppressor function that lead to cancer development. And in fact, the altered forms of p^{53} recovered from the various tumor cells (breast, bone, brain, colon, bladder, skin, lung) confirm the protective function of p^{53} gene against cancers.

It is believed that the environmental factors may cause mutations in p^{53} gene which may ultimately lead to cancer. Some of the mutations of p^{53} gene may be inherited, which probably explains the occurrence of certain cancers in some families.

Genes of breast cancer:

Two genes, namely BRCAI and BRCAII, implicated in certain hereditary forms of breast cancer in women, have been identified. It is estimated that about 80% of inherited breast cancers are due to mutations in either one of these two genes — BRCAI or BRCAII. In addition, there is a high risk for ovarian cancer due to mutations in BRCAI.

It is suggested that the normal genes BRCAI and BRCAII encode proteins (with 1863 and 3418 amino acids respectively) that function in a manner comparable to gene p^{53} protein (as described above). As such, BRCAI and BRCAII are DNA- repair and tumor-suppressor genes. Some researchers believe that these two proteins act as gene regulators. Diagnostic tests for the analysis of the genes BRCAI and BRCAII were developed. Unfortunately, their utility is very limited, since there could be hundreds of variations in the base sequence of these genes.

Genes of colon cancer:
The occurrence of colon cancer appears to be genetically linked since it runs in some families. Some researchers have identified a gene linked with hereditary non-polyposis colon cancer or HNPCC (sometimes called Lynch syndrome). This gene encoded a protein that acts as a guardian and brings about DNA repair whenever there is a damage to it.However, as and when there is a mutation to this protective gene, an altered protein is produced which cannot undo the damage done to DNA. This leads to HNPCC. It is estimated that the occurrence of this altered gene is one in every 200 people in general population.

Microsatellite marker genes:
Microsatellites refer to the short repetitive sequences of DNA that can be employed as markers for the identification of certain genes. For colon cancer, microsatellite marker genes have been identified on chromosome 2 in humans. There is a lot of variability in the sequence of microsatellites.

Early detection of the risk for colon cancer by DNA analysis is a boon for the would be victims of this disease. The suspected individuals can be periodically monitored for the signs and treated appropriately. Unlike many other cancers, the chances of cure for colon cancer are reasonably good.

Gene of retinoblastoma:
Retinoblastoma is a rare cancer of the eye. If detected early, it can be cured by radiation therapy and laser surgery or else the eyeball has to be removed. Scientists have identified a missing or a defective (mutated) gene on chromosome number 13, being responsible for retinoblastoma. The normal gene when present on chromosome 13 is anticancer and does not allow retinoblastoma to develop.

Diabetes:
Diabetes mellitus is a clinical condition characterized by increased blood glucose level (hyperglycemia) due to insufficient or inefficient (incompetent) insulin. In other words, individuals with diabetes cannot utilize glucose properly in their body.

A rare form of type II diabetes (i.e., non-insulin dependent diabetes mellitus, NIDDM) is maturity onset diabetes of the young (MODY). MODY, occurring in adolescents and teenagers is found to have a genetic basis. A gene, synthesizing the enzyme glucokinase, located on chromosome 7, is found to be defective in MODY patients. Glucokinase is a key enzyme in glucose metabolism. Besides its involvement in the metabolism, glucokinase in the pancreatic cells serves as a detector for glucose concentration in the blood. This detection stimulates β-cells of the pancreas to secrete insulin. A gene modification that results in a defective or an altered glucokinase hampers pancreatic insulin secretion. Later work has shown that glucokinase gene is defective in the common form of type II diabetes.

DNA probes for type II diabetes:
The glucokinase genes from normal and type II diabetes patients were cloned and scanned with DNA probes. It was found that a single base mutation of the gene led to a defective glucokinase production that is largely responsible for MODY, and also a majority of individuals with type II diabetes. Later, some workers reported a possibility of at least a dozen mutations in glucokinase gene for type II diabetes.

Genes responsible for type I diabetes:
Type I diabetes or insulin-dependent diabetes mellitus (IDDM) mainly occurs in childhood, particularly between 12-15 years of age. IDDM is characterized by almost total deficiency of insulin. Researchers have identified at least 18 different chromosome regions linked with type I diabetes. These DNA sequences are located on chromosomes 6, 11 and 18.

Obesity:
Obesity is an abnormal increase in the body weight due to fat deposition. Men and women are considered obese if their weight due to fat, respectively exceeds more than 20% and 25% of the body weight. Obesity increases the risk of high blood pressure, diabetes, atherosclerosis and other life-threatening conditions.

Although many believed that obesity could be genetically inherited, the molecular basis was not known for long. It was in 1994, a group of workers identified a mutated gene that caused obesity in mice. Later, a similar gene was found in humans also.

The gene designated ob (for obese) is located on chromosome 6 in mouse. The DNA of ob gene contains 650 kb and encodes a protein with 167 amino acids in adipose tissue. This protein is responsible to keep the weight of the animals under control.

The genetically obese mice have mutated ob gene and therefore the weight-control protein is not produced. It is believed that this protein functions like a hormone, acts on the hypothalamus, and controls the site of hunger and energy metabolism (these two factors are intimately linked with obesity).

With the discovery of ob gene, the treatment for inherited obesity may soon become a reality. In fact, one multinational biotechnology company has started producing ob protein that can be used for weight reduction in experimental mice. Besides the ob gene, a few other genes (fat gene, tub gene) that might be associated with obesity have also been discovered.

DNA Analysis for Other Human Diseases:
There is a continuous search for the identification of more and more genes that are responsible for human diseases. Such an approach will ultimately help in the specific diagnosis of these diseases before their actual occurrence. In addition to human diseases described above, some more are given below.

Deafness:
The deafness, inherited in some families, has genetic basis. A team of workers have identified a gene on chromosome 5, encoding a protein that facilitates the assembly of actin (protein) molecules in the cochlea of inner ear. The association of actin is very essential for the detection of sound waves by the ear.A mutation of the gene on chromosome 5 results in a defective protein synthesis and non-assembly of actin molecules which cause deafness. Some other genes, besides the one described here, have also been found to be associated with deafness.

Glaucoma:
Glaucoma is a disease of the eye that may often lead to blindness. It occurs as a result of damage to the optic nerve due to pressure that builds up in the eye. A gene responsible for the hereditary glaucoma in teenagers has been detected on chromosome 1. Another group of researchers have found a gene on chromosome 3 which is linked with the adult-onset glaucoma.

Baldness:
There is an inherited form of baldness, called alopecia universals. This is found to be associated with a gene located on chromosome 12.

Parkinson's disease:

Parkinson's disease is a common disorder in many elderly people, with about 1% of the population above 60 years being affected. It is characterized by muscular rigidity, tremors, expressionless face, lethargy, involuntary movements etc. In the victims of Parkinson's disease, there is degeneration of brain cells, besides a low concentration of dopamine (a neurotransmitter).

Researchers have identified that a gene-encoded protein namely α-synuclein plays a significant role in the development of Parkinson's disease. An altered form of α-synuclein (due to a mutation in the gene) accumulates in the brain as Lewy bodies. This is responsible for nerve cells degeneration and their death in the Parkinson's disease.

Hemochromatosis:

Hemochromatosis is an iron-overload disease in which iron is directly deposited in the tissues (liver, spleen, heart, pancreas and skin). An abnormal gene on chromosome 6 is linked with hemo-chromatosis. The amino acid tyrosine, in the normal protein encoded by this gene is replaced by cysteine. This abnormal protein is responsible for excessive iron absorption from the intestine which accumulates in the various tissues leading to their damage and malfunction.

Menke's disease:

Menke's disease, a copper deficiency disorder, is characterized by decreased copper in plasma, depigmentation of hair, degeneration of nerve cells and mental retardation. A gene located on X-chromosome, encoding a transport protein, is linked with Menke's disease. A defect in the gene, consequently in the protein, impairs copper absorption from the intestine.

Gene Banks—A Novel Concept:

As the search continues by scientists for the identification of more and more genes responsible for various diseases, the enlightened public (particularly in the developed countries), is very keen to enjoy the fruits of this research outcome. As of now, DNA probes are available for the detection a limited number of diseases. Researchers continue to develop DNA probes for a large number of genetically predisposed disorders.

Gene banks are the centres for the storage of individual's DMAs for future use to diagnose diseases. For this purpose, the DNA isolated from a person's cells (usually white blood cells) is stored. As and when a DNA probe for the detection of a specific disease is available, the stored DNA can be used for the diagnosis or risk assessment of the said genetic disease.

In fact, some institutions have established gene banks. They store the DNA samples of the interested customers at a fee (one firm was charging $ 200) for a specified period (say around 20-25 years). For the risk assessment of any disease, it is advisable to have the DNAs from close relatives of at least 2-3 generations.

Gene Therapy:

Gene therapy is a novel treatment method which utilizes genes or short oligonucleotide sequences as therapeutic molecules, instead of conventional drug compounds. This technique is widely used to treat those defective genes which contribute to disease development. Gene therapy involves the introduction of one or more foreign genes into an organism to treat hereditary or acquired genetic defects. In gene therapy, DNA encoding a therapeutic protein is packaged within a "vector", which transports the DNA inside cells within the body. The disease is treated with minimal toxicity, by the expression of the inserted DNA by the cell machinery. In 1990 FDA for the first time approved a gene therapy experiment on ADA-SCID in the United States after the treatment of Ashanti DeSilva. After that, approximately 1700 clinical trials on patients have been performed with various techniques and genes for numerous diseases.

Gene therapy is the process of inserting genes into cells to treat diseases. The newly introduced genes will encode proteins and correct the deficiencies that occur in genetic diseases. Thus, gene therapy primarily involves genetic manipulations in animals or humans to correct a disease, and keep the organism in good health. The initial experiments on gene therapy are carried out in animals, and then in humans. Obviously, the goal of the researchers is to benefit the mankind and improve their health.

An overview of gene therapy strategies is depicted in Fig. 13.1. In gene augmentation therapy, a DNA is inserted into the genome to replace the missing gene product. In case of gene inhibition therapy, the antisense gene inhibits the expression of the dominant gene.

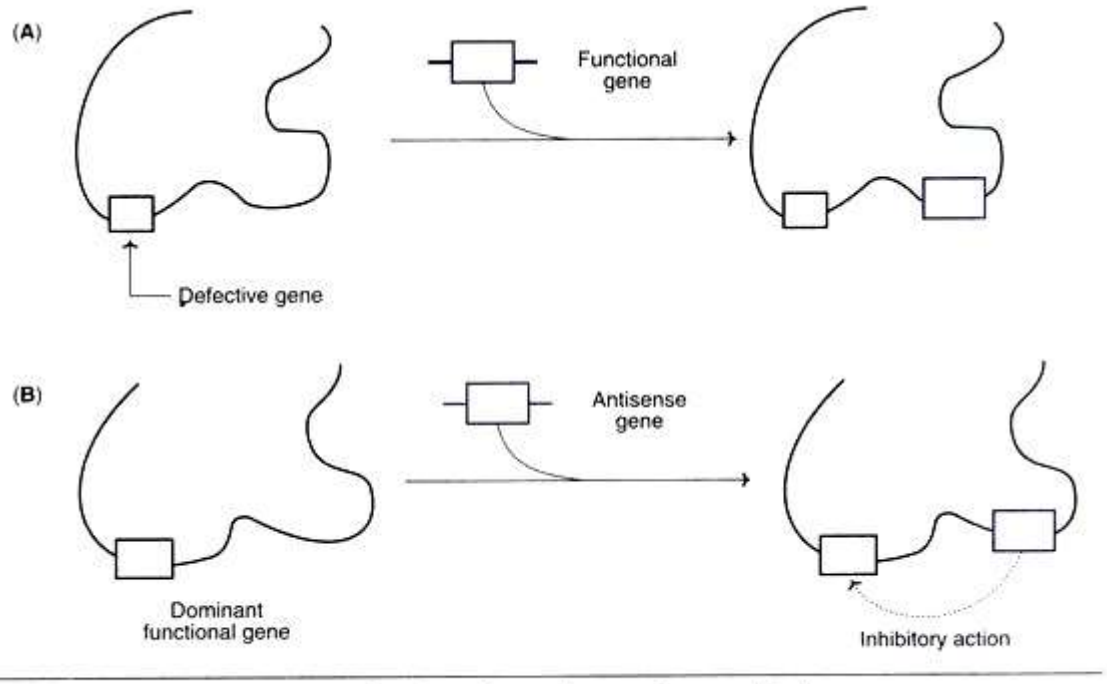


***Fig. 13.1** : Overview of two major gene therapy strategies (A) Gene augmentation therapy (B) Gene inhibition therapy.*

I. General gene therapy strategies

a. Gene Augmentation Therapy (GAT):

For diseases caused by loss of function of a gene, introducing extra copies of the normal gene may increase the amount of normal gene product to a level where the normal phenotype is restored (see Fig. 23.1). As a result GAT is targeted at clinical disorders where the pathogenesis is reversible.

It also helps to have no precise requirement for expression levels of the introduced gene and a clinical response at low expression levels. GAT has been particularly applied to autosomal recessive disorders where even modest expression levels of an introduced gene may make a substantial difference.

Dominantly inherited disorders are much less amendable to treatment; gain-of-function mutations are not treatable by this approach and, even if there is a loss-of- function mutation, high expression efficiency of the introduced gene is required: individuals with 50% of normal gene product are normally affected, and so the challenge is to increase the amount of gene product towards normal levels.

b. Targeted Killing of Specific Cells:
This general approach is popular in cancer gene therapies. Genes are directed to the target cells and then expressed so as to cause cell killing. Direct cell killing is possible if the inserted genes are expressed to produce a lethal toxin (suicide genes), or a gene encoding a pro drug is inserted, conferring susceptibility to killing by a subsequently administered drug. Indirect cell killing uses immunostimulatory genes to provoke or enhance an immune response against the target cell.

c. Targeted Mutation Correction:
If an inherited mutation produces a dominant-negative effect, gene augmentation is unlikely to help. Instead, the resident mutation must be corrected. Because of practical difficulties, this approach has yet to be applied but, in principle, it can be done at different levels: at the gene level (e.g. by gene targeting methods based on homologous recombination); or at the RNA transcript level (e.g. by using particular types of therapeutic ribozymes — or therapeutic RNA editing).

d. Targeted Inhibition of Gene Expression:
If disease cells display a novel gene product or inappropriate expression of a gene (as in the case of many cancers, infectious diseases, etc.), a variety of different systems can be used specifically to block the expression of a single gene at the DNA, RNA or protein levels. Allele-specific inhibition of expression may be possible in some cases, permitting therapies for some disorders resulting from dominant negative effects.

II. Approaches for Gene Therapy:
There are two approaches to achieve gene therapy.

1. Somatic Cell Gene Therapy:
The non- reproductive (non-sex) cells of an organism are referred to as somatic cells. These are the cells of an organism other than sperm or eggs cells, e.g., bone marrow cells, blood cells, skin cells, intestinal cells. At present, all the research on gene therapy is directed to correct the genetic defects in somatic cells. In essence, somatic cell gene therapy involves the insertion of a fully functional and expressible gene into a target somatic cell to correct a genetic disease permanently.

2. Germ Cell Gene Therapy:
The reproductive (sex) cells of an organism constitute germ cell line. Gene therapy involving the introduction of DNA into germ cells is passed on to the successive generations. For safety, ethical and technical reasons, germ cell gene therapy is not being attempted at present.

The genetic alterations in somatic cells are not carried to the next generations. Therefore, somatic cell gene therapy is preferred and extensively studied with an ultimate objective of correcting human diseases. Development of gene therapy in humans for any specific disease involves the following steps. In fact, this is a general format for introducing any therapeutic agent for human use.

a. In vitro experiments and research on laboratory animals (pre-clinical trials).

b. Phase I trials with a small number (5-10) of human subjects to test safety of the product.

c. Phase II trials with more human subjects to assess whether the product is helpful.

d. Phase III trials in large human samples for a final and comprehensive analysis of the safety and efficacy of the product.

As such, gene therapy involves a great risk. There are several regulatory agencies whose permission must be sought before undertaking any work related to gene therapy. Recombinant DNA Advisory Committee (RAC) is the supervisory body of the National Institute of Health, U.S.A., that clears

proposals on experiments involving gene therapy. A large number of genetic disorders and other diseases are currently at various stages of gene therapy trials. A selected list of some important ones is given in Table 13.1.

TABLE 13.1 Human gene therapy trials

Disease	*Gene therapy*
Severe combined immunodeficiency (SCID)	Adenosine deaminase (ADA).
Cystic fibrosis	Cystic fibrosis transmembrane regulator (CFTR).
Familial hypercholesterolemia	Low density lipoprotein (LDL) receptor.
Emphysema	α_1-Antitrypsin
Hemophilia B	Factor IX
Thalassemia	α- or β-Globin
Sickle-cell anemia	β-Globin
Lesch-Nyhan syndrome	Hypoxanthine-guanine phosphoribosyltransferase (HGPRT).
Gaucher's disease	Glucocerebrosidase
Peripheral artery disease	Vascular endothelial growth factor (VEGF)
Fanconi anemia	Fanconi anemia C
Melanoma	Tumor necrosis factor (TNF)
Melanoma, renal cancer	Interleukin-2 (IL-2)
Glioblastoma (brain tumor), AIDS, ovarian cancer	Thymidine kinase (herpes simplex virus)
Head and neck cancer	p^{53}
Breast cancer	Multidrug resistance I
AIDS	*rev* and *env*
Colorectal cancer, melanoma, renal cancer	Histocompatability locus antigen-B_7 (HLA-B_7)
Duchenne muscular dystrophy	Dystrophin
Short stature*	Growth hormone
Diabetes*	Glucose transporter-2, (GLUT-2), glucokinase
Phenylketonuria*	Phenylalanine hydroxylase
Citrullinemia*	Arginosuccinate synthetase

III. Methods of gene therapy:

There are mainly two approaches for the transfer of genes in gene therapy:

1. Transfer of genes into patient cells outside the body (ex vivo gene therapy)

2. Transfer of genes directly to cells inside the body (in vivo).

1. Ex vivo gene therapy:

The ex vivo gene therapy can be applied to only selected tissues (e.g., bone marrow) whose cells can be cultured in the laboratory. The technique of ex vivo gene therapy involves the following steps (Fig. 13.2).

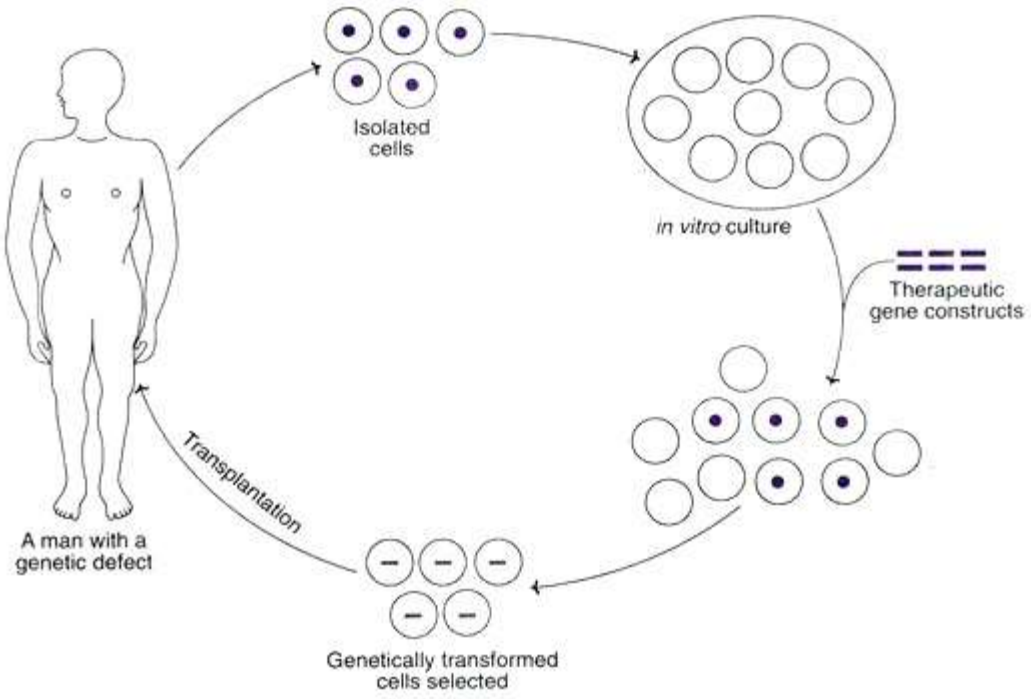


***Fig. 13.2** : The procedure for ex vivo gene therapy.*

1. Isolate cells with genetic defect from a patient.

2. Grow the cells in culture.

3. Introduce the therapeutic gene to correct gene defect.

4. Select the genetically corrected cells (stable trans-formants) and grow.

5. Transplant the modified cells to the patient.

The procedure basically involves the use of the patient's own cells for culture and genetic correction, and then their return back to the patient. This technique is therefore, not associated with adverse immunological responses after transplanting the cells. Ex vivo gene therapy is efficient only, if the therapeutic gene (remedial gene) is stably incorporated and continuously expressed. This can be achieved by use of vectors.

Vectors in Gene Therapy:

The carrier particles or molecules used to deliver genes to somatic cells are referred to as vectors. The important vectors employed in ex vivo gene therapy are listed below and briefly described next.

i. Viruses

ii. Human artificial chromosome

iii. Bone marrow cells.

i. Viruses:

The vectors frequently used in gene therapy are viruses, particularly retroviruses. RNA is the genetic material in retroviruses. As the retrovirus enters the host cell, it synthesizes DNA from RNA (by

reverse transcription). The so formed viral DNA (referred to as provirus) gets incorporated into the DNA of the host cell.

The proviruses are normally harmless. However, there is a tremendous risk, since some of the retroviruses can convert normal cells into cancerous ones. Therefore, it is absolutely essential to ensure that such a thing does not happen.

Making retroviruses harmless:

Researchers employ certain biochemical methods to convert harmful retroviruses to harmless ones, before using them as vectors. For instance, by artificially removing a gene that encodes for the viral envelope, the retrovirus can be crippled and made harmless. This is because, without the envelope, retrovirus cannot enter the host cell. The production of a large number (billions) of viral particles can be achieved, starting from a single envelope defective retrovirus (Fig. 13.3).

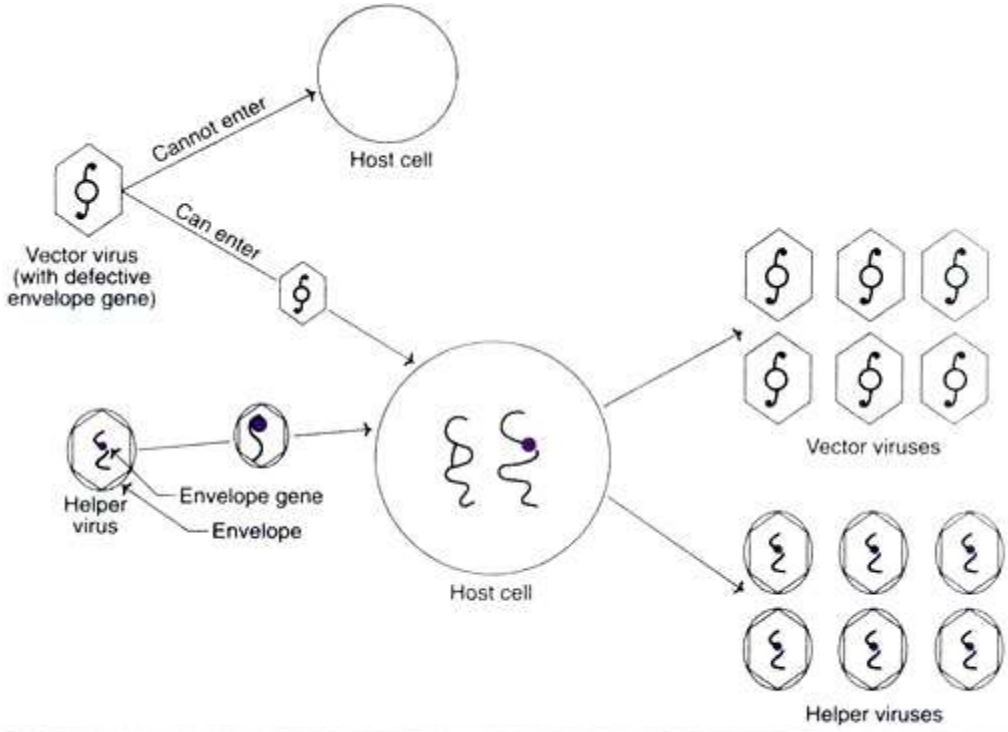


Fig. 13.3 : Large scale production of vector viruses by using helper viruses.

This is made possible by using helper viruses which contain normal gene for envelope formation. Along with the helper virus, the vector (with defective envelope gene) can enter the host cell and both of them multiply. By repeated multiplication in host cells, billions of vector and helper viruses are produced.

The vector viruses can be separated from the helper viruses and purified. Isolation of vector viruses, totally free from helper viruses, is absolutely essential. Contamination of helper viruses is a big threat to the health of the patients undergoing gene therapy.

Retroviruses in gene therapy:

The genetic map of a typical retrovirus is depicted in Fig. 13.4A. In general, the retrovirus particle has RNA as a genome organized into six regions. It has a 5′-long terminal repeat (5′-LTR), a non-coding sequence required for packaging RNA designated as psi (Ψ), a gene gag coding for structural protein, a gene pol that codes for reverse transcriptase, a gene env coding for envelope protein and a 3-LTR sequence.

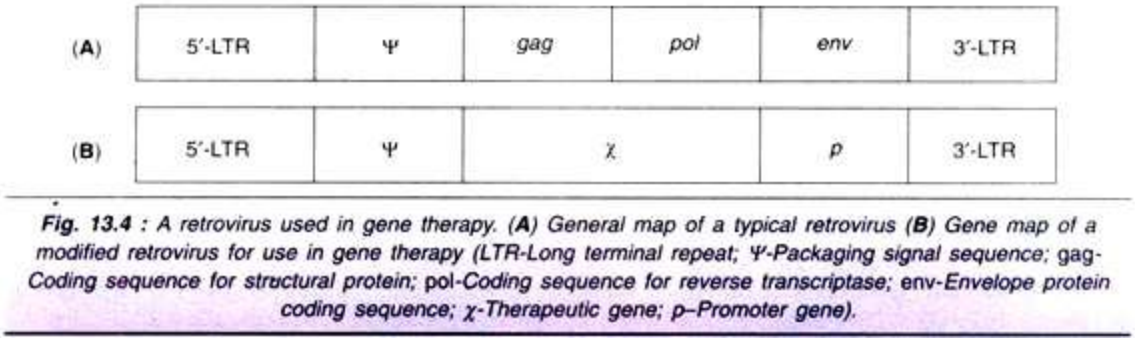


Fig. 13.4 : A retrovirus used in gene therapy. (A) General map of a typical retrovirus (B) Gene map of a modified retrovirus for use in gene therapy (LTR-Long terminal repeat; Ψ-Packaging signal sequence; gag-Coding sequence for structural protein; pol-Coding sequence for reverse transcriptase; env-Envelope protein coding sequence; χ-Therapeutic gene; p–Promoter gene).

For use of a retrovirus as a vector, the structural genes gag and pol are deleted. These genes are actually adjacent to Ψ region. In addition, a promoter gene is also included (Fig. 13.4B). This vector design allows the synthesis of cloned genes. A retroviral vector can carry a therapeutic DNA of maximum size of 8 kb.

A retroviral vector DNA can be used to transform the cells. However, the efficiency of delivery and integration of therapeutic DNA are very low. In recent years, techniques have been developed to deliver the vector RNA to host cells at a high frequency. For this purposes, packaged retroviral RNA particles are used. This technique allows a high efficiency of integration of pharmaceutical DNA into host genome.

Several modified viral vectors have been developed in recent years for gene therapy. These include oncoretrovirus, adenovirus, adenoassociated virus, herpes virus and a number of hybrid vectors combining the good characters of the parental vectors.

Murine leukaemia viruses in gene therapy:

This is a retrovirus that causes a type of leukaemia in mice. It can react with human cells as well as the mouse cells, due to a similarity in the surface receptor protein. Murine leukaemia virus (MLV) is frequently used in gene transfer.

AIDS virus in gene therapy?

It is suggested that the human immunodeficiency virus (HIV) can be used as a vector in gene transfer. But this is bound to create public uproar. Some workers have been successful in creating a harmless HIV (crippled HIV) by removing all the genes related to reproduction. At the same time, the essential genes required for gene transfer are retained. There is a distinct advantage with HIV when compared with MLV. MLV is capable of bringing out gene transfer only in dividing cells. HIV can infect even non-dividing cells (e.g., brain cells) and do the job of gene transfer effectively. However, it is doubtful whether HIV can ever be used as a vector.

ii. Human Artificial Chromosome:

The details of human artificial chromosome (HAC) are described elsewhere .HAC is a synthetic chromosome that can replicate with other chromosomes, besides encoding a human protein. As already discussed above, use of retroviruses as vectors in gene therapy is associated with a heavy risk. This problem can be overcome if HAC is used. Some success has been achieved in this direction.

iii. Bone Marrow Cells:

Bone marrow contains totipotent embryonic stem (ES) cells. These cells are capable of dividing and differentiating into various cell types (e.g., red blood cells, platelets, macrophages, osteoclasts, B- and T-lymphocytes). For this reason, bone marrow transplantation is the most widely used technique for several genetic diseases.

And there is every reason to believe that the genetic disorders that respond to bone marrow transplantation are likely to respond to ex vivo gene therapy also (Table 13.2). For instance, if there is

a gene mutation that interferes with the function of erythrocytes (e.g., sickle-cell anaemia), bone marrow transplantation is done. Bone marrow cells are the potential candidates for gene therapy of sickle-cell anaemia. However, this is not as simple as theoretically stated.

TABLE 13.2 Selected list of genetic diseases that are likely to be cured by using bone marrow cells (potential candidates for gene therapy)

Severe combined immunodeficiency (SCID)
Sickle-cell anemia
Fanconi anemia
Thalassemia
Gaucher's disease
Hunter disease
Hurler syndrome
Chronic granulomatous disease
Infantile agranulocytosis
Osteoporosis
X-linked agammaglobulinemia

Selected Examples of Ex Vivo Gene Therapy:

a. Therapy for Adenosine Deaminase Deficiency:

The first and the most publicized human gene therapy was carried out to correct the deficiency of the enzyme adenosine deaminase (ADA). This was done on September 14, 1990 by a team of workers led by Blaese and Anderson at the National Institute of Health, USA (The girl's name is Ashanti, 4 years old then).

b. Severe combined immunodeficiency (SCID):

This is rare inherited immune disorder associated with T-lymphocytes, and (to a lesser extent) B-lymphocytes dysfunction. About 50% of SCID patients have a defect in the gene (located on chromosome 20, and has 32,000 base pairs and 12 exons) that encodes for adenosine deaminase. In the deficiency of ADA, deoxyadenosine and its metabolites (primarily deoxyadenosine 5′-triphosphate) accumulate and destroy T-lymphocytes.

T-Lymphocytes are essential for body's immunity. Besides participating directly in body's defense, they promote the function of B-lymphocytes to produce antibodies. Thus, the patients of SCID (lacking ADA) suffer from infectious diseases and die at an young age. Previously, the children suffering from SCID were treated with conjugated bovine ADA, or by bone marrow transplantation.

c. Technique of therapy for ADA deficiency:

The general scheme of gene therapy adopted for introducing a defective gene in the patient has been depicted in Fig 13.2. The same procedure with suitable modifications can also be applied for other gene therapies.

A plasmid vector bearing a pro-viral DNA is selected. A part of the pro-viral DNA is replaced by the ADA gene and a gene (G 418) coding for antibiotic resistance, and then cloned. The antibiotic resistance gene will help to select the desired clones with ADA gene.A diagrammatic representation of the treatment of ADP deficient patient is depicted in Fig. 13.5.

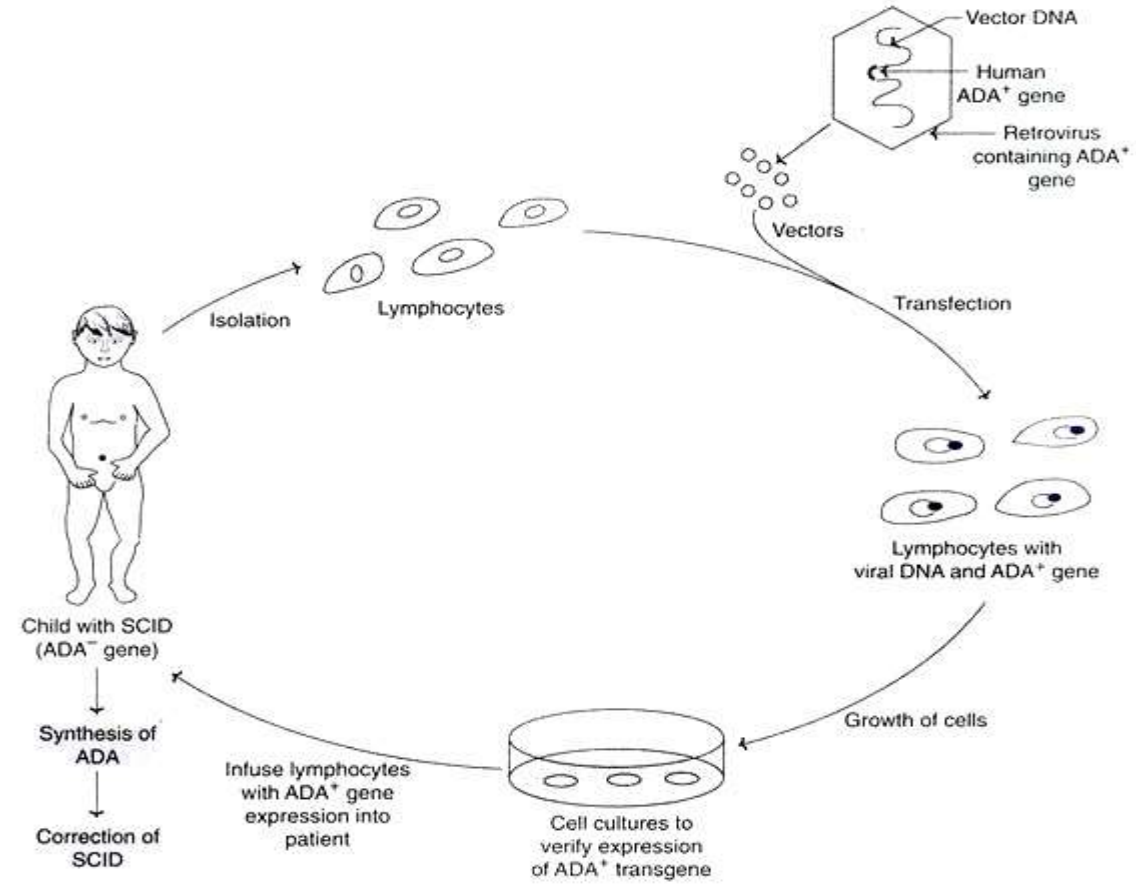


Fig. 13.5 : Treatment of adenosine deaminase (ADA) deficient patient by somatic ex vivo gene therapy (SCID-Severe combined immunodeficiency disease).

Circulating lymphocytes are removed from a patient suffering from ADA deficiency. These cells are transfected with ADA gene by exposing to billions of retroviruses carrying the said gene. The genetically-modified lymphocytes are grown in cultures to confirm the expression of ADA gene and returned to the patient. These lymphocytes persist in the circulation and synthesize ADA.

Consequently, the ability of the patient to produce antibodies is increased. However, there is a limitation. The lymphocytes have a short life span (just live for a few months), hence the transfusions have to be carried out frequently.

Transfer of ADA gene into stem cells:

In 1995, ADA gene was transferred into the stem cells, obtained from the umbilical cord blood, at the time of baby's delivery. Four days after birth, the infant received the modified cells back. By this way, a permanent population of ADA gene producing cells was established.

d. Therapy for Familial Hypercholesterolemia:

The patients of familial hypercholesterolemia lack the low density lipoprotein (LDL) receptors on their liver cells. As a result, LDL cholesterol is not metabolised in liver. The accumulated LDL-cholesterol builds up in the circulation, leading to arterial blockage and heart diseases.

Attempts are being made by gene therapists to help the victims of familial hypercholesterolemia. In fact, there is some success also. In a woman, 15% of the liver was removed. The hepatocytes were transduced with retroviruses carrying genes for LDL receptors. These genetically modified hepatocytes were infused into the patient's liver.

The hepatocytes established themselves in the liver and produced functional LDL-receptors. A significant improvement in the patient's condition, as assessed by estimating the lipid parameters in blood, was observed. Further, there were no antibodies produced against the LDL-receptor molecules, clearly showing that the genetically modified liver cells were accepted.

e. Therapy for Lesch-Nyhan Syndrome:

Lesch-Nyhan syndrome is an inborn error in purine metabolism due to a defect in a gene that encodes for the enzyme hypoxanthine-guanine phosphoribosyl transferase (HCPRT). In the absence of HGPRT, purine metabolism is disturbed and uric acid level builds up, resulting in severe gout and kidney damage. The victims of Lesch- Nyhan syndrome exhibit symptoms of mental retardation, besides an urge to bite lips and fingers, causing self-mutilation.

By using retroviral vector system, HGPRT producing genes were successfully inserted into cultured human bone marrow cells. The major problem in humans is the involvement of brain. Experiments conducted in animals are encouraging. However, it is doubtful whether good success can be achieved by gene therapy for Lesch-Nyhan syndrome in humans, in the near future.

f. Therapy for Haemophilia:

Haemophilia is a genetic disease due lack of a gene that encodes for clotting factor IX. It is characterized by excessive bleeding. By using a retroviral vector system, genes for the synthesis of factor IX were inserted into the liver cells of dogs. These dogs no longer displayed the symptoms of haemophilia.

g. Ex Vivo Gene Therapy with Non-Autologous Cells:

The ex vivo gene therapies described above are based on the transplantation of genetically modified cells for the production of desired proteins. However, there are several limitations in using the patient's own cells (autologous cells) for gene therapy. These include lack of enough cells from target tissues, defective uptake of genes and their inadequate expression. To overcome these problems, attempts are on to develop methods to use non-autologous cells (i.e., cells from other individuals or animals). The outline of the procedure is briefly described below.

Tissue-specific cells capable of growing in culture are selected. These include fibroblasts from skin, hepatocytes from liver, and myoblasts from muscle and astrocytes from brain. These cells are cultured and genetically modified with the therapeutic gene. They are then encapsulated in artificial membrane composed of a synthetic polymer (e.g., polyether sultone, alginase-poly L-lysine-alginate).The polymeric membranes are non-immunogenic, therefore the patient can accept non-autologous encapsulated cells. Further, being semipermeable in nature, these membranes allow the nutrients to enter in, and the encoded protein (by the therapeutic gene) to pass out.

Experiments conducted in animals have shown some encouraging results for using non-autologous cells in gene therapy. The encapsulated cells were found to proliferate and produce the required protein. However, the success has been very limited in human trials.

2. In Vivo Gene Therapy:

The direct delivery of the therapeutic gene (DNA) into the target cells of a particular tissue of a patient constitutes in vivo gene therapy (Fig. 13.6). Many tissues are the potential candidates for this approach. These include liver, muscle, skin, spleen, lung, brain and blood cells. Gene delivery can be carried out by viral or non- viral vector systems. The success of in vivo gene therapy mostly depends on the following parameters

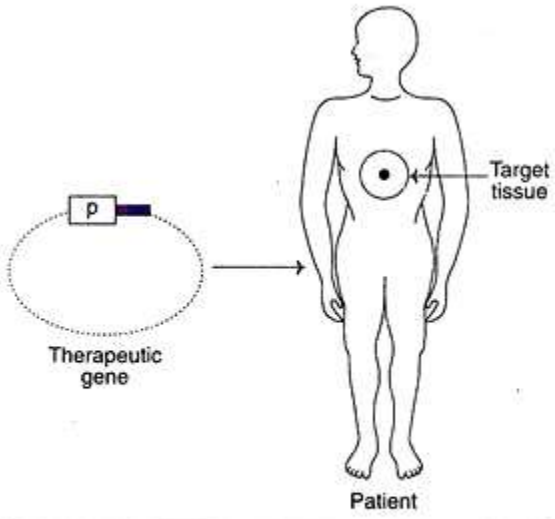


Fig. 13.6 : Diagrammatic representation of in vivo gene therapy. (p-Promoter gene specific for therapeutic gene)

i. The efficiency of the uptake of the remedial (therapeutic) gene by the target cells.

ii. Intracellular degradation of the gene and its uptake by nucleus.

iii. The expression capability of the gene.

In vivo gene therapy with special reference to gene delivery systems (viral, non-viral) with suitable examples is described.

Gene Delivery by Viruses:

Many viral vector systems have been developed for gene delivery. These include retroviruses, adenoviruses, adenoassociated viruses and herpes simplex virus.

Retrovirus vector system:

Replication defective retrovirus vectors that are harmless are being used. A plasmid in association with a retrovirus, a therapeutic gene and a promoter is referred to as plasmovirus. The plasmovirus is capable of carrying a DNA (therapeutic gene) of size less than 3.4 kb. Replication defective virus particles can be produced from the plasmovirus.

As such, for the delivery of genes by retroviral vectors, the target cells must be in a dividing stage. But majority of the body cells are quiescent. In recent years, viral vectors have been engineered to infect non-dividing cells. Further, attempts are on to include a DNA in the retroviral vectors (by engineering env gene) that encodes for cell receptor protein. If this is successfully achieved, the retroviral vector will specifically infect the target tissues.

Adenoviral vector system:

Adenoviruses (with a DNA genome) are considered to be good vectors for gene delivery because they can infect most of the non-dividing human cells. A common cold adenovirus is a frequently used vector. As the target cells are infected with a recombinant adenovirus, the therapeutic gene (DNA) enters the nucleus and expresses itself.

However, this DNA does not integrate into the host genome. Consequently, adenoviral based gene therapy required periodic administration of recombinant viruses. The efficiency of gene delivery by

adenoviruses can be enhanced by developing a virus that can specifically infect target cells. This is possible by incorporating a DNA encoding a cell receptor protein.

Adeno-associated virus vector system:

Adeno-associated virus is a human virus that can integrate into chromosome 19. It is a single-stranded, non-pathogenic small DNA virus (4.7 kb). As the adeno-associated virus enters the host cell, the DNA becomes double- stranded, gets integrated into chromosome and expresses.

Adeno-associated viruses can serve as good vectors for the delivery of therapeutic genes. Recombinant viruses are created by using two plasmids and an adenovirus (i.e., helper virus) by a special technique. Some attempts were made to use therapeutic genes for the treatment of the human diseases-haemophilia (for production of blood clotting factor IX) and cystic fibrosis (for synthesis of cystic fibrosis trans membrane regulator protein) by employing adeno-associated viruses.

Therapy for cystic fibrosis:

Cystic fibrosis (CF) is one of the most common (frequency 1: 2,500) and fatal genetic diseases. It is characterized by the accumulation of sticky, dehydrated mucus in the respiratory tract and lungs. Patients of CF are highly susceptible to bacterial infections in their lungs and most of them die before reaching the age of thirty. Cystic fibrosis can be traced in European folklore, the following statement used to be said **"Woe to that child which when kissed on the forehead tastes salty. He is be witched and soon must die".**

Biochemical basis:

In the normal persons the chloride ions of the cells are pushed out through the participation of a protein called cystic fibrosis trans membrane regulator (CFTR). In the patients of cystic fibrosis, the CFTR protein is not produced due to a gene defect. Consequently, the chloride ions concentrate within the cells which draw water from the surroundings. As a result, the respiratory tract and the lungs become dehydrated with sicky mucus, an ideal environment for bacterial infections.

Gene therapy for Cystic Fibrosis:

As the defective gene for cystic fibrosis was identified in 1989, researchers immediately started working on gene therapy for this disease. Adenoviral vector systems have been used, although the success has been limited. The major drawback is that the benefits are short-lived, since the adenoviruses do not integrate themselves into host cells. Multiple administration of recombinant adenovirus caused immunological responses that destroyed the cells.

By using adeno-associated virus vector system, some encouraging results were reported in the gene therapy of CF. In the phase I clinical trials with CF patients, the vector persisted for about 70 days and some improvement was observed in the patients. Some researchers are trying to insert CF gene into the developing foetal cells (in experimental animals such as mice) to produce CFTR protein. But a major breakthrough is yet to come.

Herpes simplex virus vector system:

The retroviruses and adenoviruses employed in in vivo gene therapy are engineered to infect specific target cells. There are some viruses which have a natural tendency to infect a particular type of cells. The best example is herpes simplex virus (HSV) type I, which infects and persists in non-dividing nerve cells. HSV is a human pathogen that causes (though rarely) cold sores and encephalitis.

These are a large number of diseases (metabolic, neurodegenerative, immunological, tumours) associated with nervous system. HSV is considered as an ideal vector for in vivo gene therapy of many nervous disorders. The HSV has a double-stranded DNA of about 152 kb length as its genome. About 30 kb of HSV genome can be replaced by a cloned DNA without loss of its basic

characteristics (replication, infection, packaging etc.). But there are some technical difficulties in dealing with large-sized DNAs in genetic engineering experiments. Some modified HSV vectors with reduced genomic sizes have been developed. Most of the work on the gene therapy, related to the use of HSV as a vector, is being conducted in experimental animals. And the results are quite encouraging. HSV vectors could deliver therapeutic genes to the brain and other parts of nervous system. These genes are well expressed and maintained for long periods. More research, however, is needed before going for human trials. If successful, HSV may help to treat many neurodegenerative syndromes such as Parkinson's disease and Alzheimer's disease by gene therapy.

Gene Delivery by Non-Viral Systems:

There are certain limitations in using viral vectors in gene therapy. In addition to the prohibitive cost of maintaining the viruses, the viral proteins often induce inflammatory responses in the host. Therefore, there is a continuous search by researchers to find alternatives to viral vector systems.

a. Pure DNA constructs:

The direct introduction of pure DNA constructs into the target tissue is quite simple. However, the efficiency of DNA uptake by the cells and its expression are rather low. Consequently, large quantities of DNA have to be injected periodically. The therapeutic genes produce the proteins in the target cells which enter the circulation and often get degraded.

b. Lipoplexes:

The lipid-DNA complexes are referred to as lipoplexes or more commonly liposomes. They have a DNA construct surrounded by artificial lipid layers. A large number of lipoplexes have been prepared and used. They are non-toxic and non-immunogenic.

The major limitation with the use of lipoplexes is that as the DNA is taken up by the cells, most of it gets degraded by the lysosomes. Thus, the efficiency of gene delivery by lipoplex is very low. Some clinical trials using liposome-CFTR gene complex showed that the gene expression was very short-lived.

c. DNA-molecular conjugates:

The use of DNA-molecular conjugates avoids the lysosomal breakdown of DNA. Another advantage of using conjugates is that large-sized therapeutic DNAs (> 10 kb) can be delivered to the target tissues. The most commonly used synthetic conjugate is poly-L-lysine, bound to a specific target cell receptor. The therapeutic DNA is then made to combine with the conjugate to form a complex (Fig. 13.7).

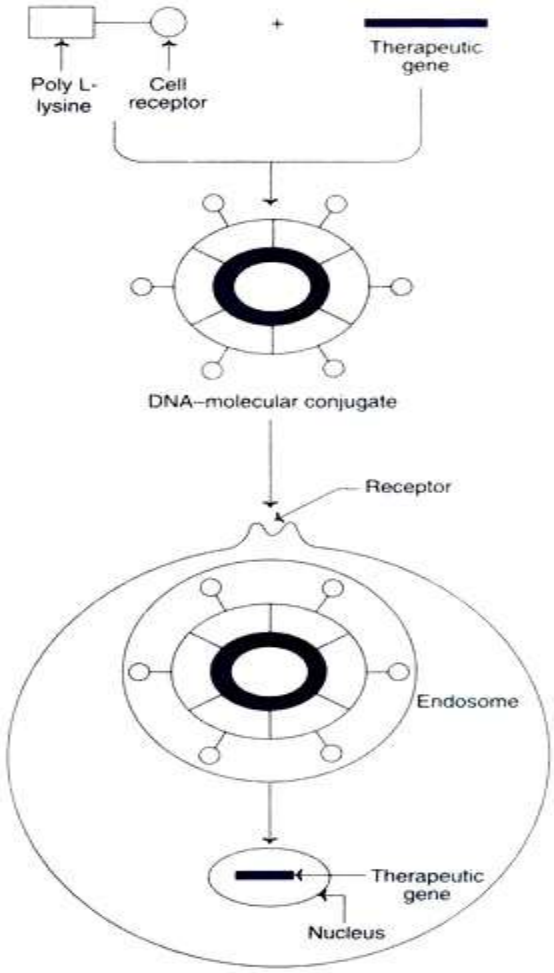


Fig. 13.7 : DNA-molecular conjugate in the delivery of therapeutic gene.

This DNA molecular conjugate binds to specific cell receptor on the target cells. It is engulfed by the cell membrane to form an endosome which protects the DNA from being degraded. The DNA released from the endosome enters the nucleus where the therapeutic gene is expressed.

d. Human artificial chromosome:

Human artificial chromosome (HAC) which can carry a large DNA one or more therapeutic genes with regulatory elements is a good and ideal vector. Studies conducted in cell cultures using HAC are encouraging. But the major problem is the delivery of the large-sized chromosome into the target cells. Researchers are working to produce cells containing genetically engineered HAC. There exists a possibility of encapsulating and implanting these cells in the target tissue. But a long way to go!

Efficiency of gene delivery by non-viral vectors:

Although the efforts are continuously on to find suitable non-viral vectors for gene delivery, the success has been very limited. This is mainly due to the following two reasons.

1. The efficiency of transfection is very low.

2. The expression of the therapeutic gene is for a very short period, consequently there is no effective treatment of the disease.

Gene Therapy Strategies for Cancer:

Cancer is the leading cause of death throughout the world, despite the intensive treatment strategies (surgery, chemotherapy, radiation therapy). Gene therapy is the latest and a new approach for cancer treatment. Some of the developments are briefly described hereunder.

Tumour necrosis factor gene therapy:

Tumour necrosis factor (TNF) is a protein produced by human macrophages. TNF provides defence against cancer cells. This is brought out by enhancing the cancer-fighting ability of tumour-infiltrating lymphocytes (TILs), a special type of immune cells.

The tumour-infiltrating lymphocytes were transformed with a TNF gene (along with a neomycin resistant gene) and used for the treatment of malignant melanoma (a cancer of melanin producing cells usually occurs in skin). TNF as such is highly toxic, and fortunately no toxic side effects were detected in the melanoma patients injected with genetically altered TILs with TNF gene. Some improvement in the cancer patients was observed.

Suicide gene therapy:

The gene encoding the enzyme thymidine kinase is often referred to as suicide gene, and is used for the treatment of certain cancers. Thymidine kinase (TK) phosphorylates nucleosides to form nucleotides which are used for the synthesis of DNA during cell division. The drug ganciclovir (GCV) bears a close structural resemblance to certain nucleosides (thymidine). By mistake, TK phosphorylates ganciclovir to form triphosphate-GCV, a false and unsuitable nucleotide for DNA synthesis. Triphosphate-GCV inhibits DMA polymerase (Fig. 13.8).

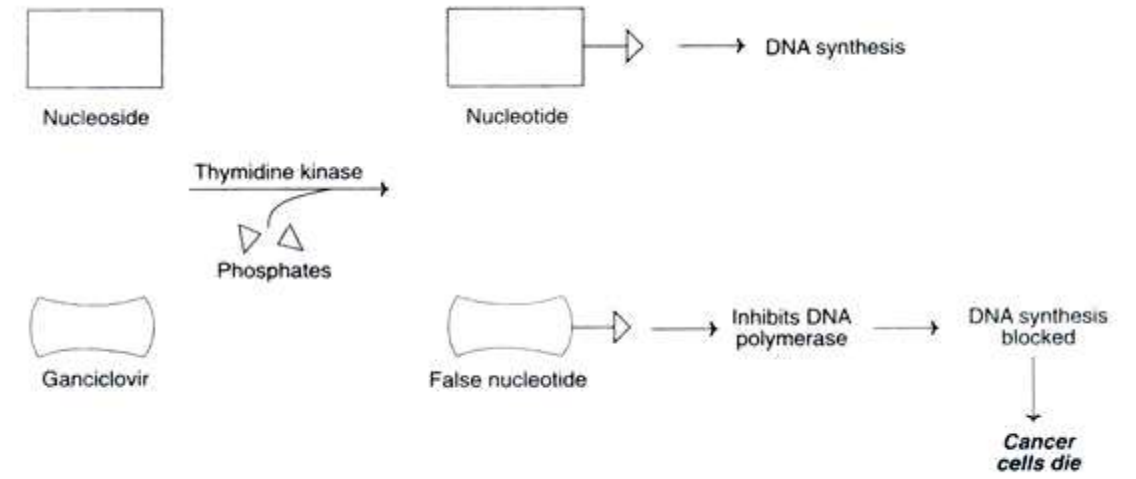


Fig. 13.8 : The action of ganciclovir mediated by thymidine kinase to inhibit the growth of cancer cells.

The result is that the elongation of the DNA molecule abruptly stops at a point containing the false nucleotide (of ganciclovir). Further, the triphosphate-GCV can enter and kill the neighbouring cancer cells, a phenomenon referred to as bystander effect. The ultimate result is that the cancer cells cannot multiply, and therefore die. Thus, the drug ganciclovir can be used to kill the cancer cells.

Ganciclovir is frequently referred to as a pro-drug and this type of approach is called pro-drug activation gene therapy. Ganciclovir has been used for treatment of brain tumours (e.g., glioblastoma, a cancer of glial cells in brain), although with a limited success.

In the suicide gene therapy, the vector used is herpes simplex virus (HSV) with a gene for thymidine kinase (TK) inserted in its genome. Normal brain cells do not divide while the brain tumour cells go

on dividing unchecked. Thus, there is a continuous DNA replication in tumour cells. By using GCV-HSVTK suicide gene therapy, some reduction in proliferating tumour cells was reported. Several new strategies are being developed to increase the delivery of HSVTK gene to all the cells throughout a tumour.

Two-gene cancer therapy:

For treatment of certain cancers, two gene systems are put together and used. For instance, TK suicide gene (i.e., GCV-HSVTK) is clubbed with interleukin-2 gene (i.e. a gene promoting immunotherapy). Interleukin-2 produced mobilizes immune response. It is believed that certain proteins are released from the tumour cells on their death.

These proteins, in association with immune cells, reach the tumour and initiate immunological reactions directed against the cancer cells. Two-gene therapies have been carried out in experimental animals with colon cancer and liver cancer, and the results are encouraging.

Gene replacement therapy:

A gene named p^{53} codes for a protein with a molecular weight of 53 kilo Daltons (hence p^{53}). p^{53} is considered to be a tumour-suppressor gene, since the protein it encodes binds with DNA and inhibits replication. The tumour cells of several tissues (breast, brain, lung, skin, bladder, colon, bone) were found to have altered genes of p^{53}(mutated p^{53}), synthesizing different proteins from the original.
These altered proteins cannot inhibit DNA replication. It is believed that the damaged p^{53} gene may be a causative factor in tumour development. Some workers have tried to replace the damaged p^{53}gene by a normal gene by employing adenovirus vector systems .There are some encouraging results in the patients with liver cancer.

Gene Therapy for AIDS:

AIDS is a global disease with an alarming increase in the incidence every year. It is invariably fatal, since there is no cure. Attempts are being made to relieve the effects of AIDS by gene therapy. Some of the approaches are discussed hereunder.

a. rev and env genes:

A mutant strain of human immunodeficiency virus (HIV), lacking rev and env genes has been developed. The regulatory and envelope proteins of HIV are respectively produced by rev and env genes. Due to lack of these genes, the virus cannot replicate.

Researchers have used HIV lacking rev and env genes for therapeutic purposes. T-Lymphocytes from HIV-infected patients are removed, and mutant viruses are inserted into them. The modified T-lymphocytes are cultivated and injected into the patients. Due to lack of essential genes, the viruses (HIV) cannot multiply, but they can stimulate the production of CD_8 (cluster determinant antigen 8) cells of T-lymphocytes. CD_8 cells are the killer lymphocytes. It is proved in the laboratory studies that these lymphocytes destroy the HIV-infected cells.

b. Genes of HIV proteins:

Some genes synthesizing HIV proteins are attached to DNA of mouse viruses. These genetically-modified viruses are injected to AIDS patients with clinical manifestations of the disease. It is believed that the HIV genes stimulate normal body cells to produce HIV proteins. The latter in turn stimulate the production of anti-HIV antibodies which prevent the HIV replication in AIDS patients.

c. Gene to inactivate gp120:

gp120 is a glycoprotein (molecular weight 120 kilo Daltons) present in the envelope of HIV. It is absolutely essential for binding of virus to the host cell and to bring replication. Researchers have synthesized a gene (called F105) to produce an antibody that can inactivate gp120.

In the anti- AIDS therapy, HIV-infected cells are engineered to produce anti-HIV antibodies when injected into the organism. Studies conducted in experimental animals showed a drastic reduction in the synthesis of gp120 due to anti-AIDS therapy. The production of F1IV particles was also very reduced. There are some attempts to prevent AIDS by antisense therapy.

Advantages of Gene Therapy

Gene therapy can cure genetic diseases by addition of gene or by removal of gene or by replacing a mutated gene with corrected gene.

Gene therapy can be used for cancer treatment to kill the cancerous cells.

Gene expression can be controlled.

Therapeutic protein is continuously produced inside the body which also reduces the cost of treatment in long term.

The Future of Gene Therapy:

Theoretically, gene therapy is the permanent solution for genetic diseases. But it is not as simple as it appears since gene therapy has several inbuilt complexities. Gene therapy broadly involves isolation of a specific gene, making its copies, inserting them into target tissue cells to make the desired protein. The story does not end here.

It is absolutely essential to ensure that the gene is harmless to the patient and it is appropriately expressed (too much or too little will be no good). Another concern in gene therapy is the body's immune system which reacts to the foreign proteins produced by the new genes.The public, in general, have exaggerated expectations on gene therapy. The researchers, at least for the present, are unable to satisfy them. As per the records, by 1999 about 1000 Americans had undergone clinical trials involving various gene therapies.

Unfortunately, the gene therapists are unable to categorically claim that gene therapy has permanently cured any one of these patients. Some people in the media (leading newspapers and magazines) have openly questioned whether it is worth to continue research on gene therapy. It may be true that as of now, gene therapy due to several limitations, has not progressed the way it should, despite intensive research. But a breakthrough may come anytime, and of course, this is only possible with persistent research. And a day may come (it might take some years) when almost every disease will have a gene therapy, as one of the treatment modalities. And gene therapy will revolutionize the practice of medicine.

Probable Questions:

1. Describe different steps of gene cloning with suitable diagram.
2. What is the difference between adaptor and linker?
3. What is homopolymer tailing?
4. How recombinant cells are detected?
5. What are the characteristics of a ideal vector?
6. Describe the role of plasmid as vector.

7. What is the difference between cloning vector and expression vector?

8. What is shuttle vector?

9. Describe the role of cosmids as vector.

10. Describe the role of bacteriophage as vector.

11. Define Gene therapy.

12. Describe different strategies of gene therapy ?

13. Define somatic cell gene therapy and germ cell gene therapy?

14. What is ex vivo gene therapy and in vivo gene therapy?

15. How retroviruses are used in gene therapy?

16. How gene therapy is used in treatment of Cystic fibrosis ?

17. What is suicide gene therapy?

18. Describe gene therapy treatments for AIDS?

19. What is are the advantages of gene therapy?

20. Write about the future of gene therapy?

21. Describe the procedure of cloning in details with suitable diagram.

22. How DNA markers are used in disease diagnosis? Give examples.

Suggested Readings:

1. Biotechnology by P.K. Gupta

2. Gene Cloning by T. Brown.

3. Biotechnology by N. Kumarsen.

4. Biotechnology by B.D. Singh

Unit-IV

Transgenic Technology in Animals

Objective: In this unit you will learn about TransgenicTechnology used in animal science.

Features of Transgenic Technology:

Important points related to gene technology are briefly presented as follows:

i. Direct Gene Transfer:
Gene technology permits direct gene transfer into the recipient parent bypassing sexual process. In other words, there is no need of union of male and female gametes in gene technology. The gene of interest can be directly inserted into the cell of recipient parent.

ii. Single Gene Transfer:
Gene technology permits transfer of one or two genes from donor species or organism to the recipient organisms. It hybridization method hundreds of genes are transferred to the recipient parent which are eliminated by repeated back, crossing from the recipient or recurrent parent.

iii. Rapid and Accurate Technique:
Gene technology is a rapid method of crop improvement. It takes 4-5 years for release of new cultivar against 10-12 years by conventional breeding method. Moreover, it is highly accurate and reliable technique.

iv. Free Gene Transfer:
Gene technology permits gene transfer between two totally unrelated organisms i.e. from bacteria to higher plant cell and even from animals to plants. Thus gene technology has overcome the natural barriers of gene transfer.

Steps in Transgenic Technology:

Development of transgenic (genetically engineered) plant is a lengthy process which consists of following important steps:

i. Identification of useful Genes:
The desirable genes may be located in wild species, unrelated plant species, unrelated organisms i.e., micro-organisms (bacteria, viruses or fungi) and animals. This work is carried out in the research laboratory.

ii. Designing Gene for Insertion:
The gene of interest is isolated from the donor source and cloned in the laboratory. The cloning is done generally using plasmids.

Introduction to Transgenesis:

Conventional animal husbandry involves the proper feeding, caring, management and breeding methods for the increased production of their yield which may be in the form of meat, milk, eggs, etc. In animal husbandry, selective breeding aims to increase the frequency of desired genes and the desired phenotype. For many farm animals, the conventional breeding has already achieved high

yielding animals but by this procedure, it seems that the productivity would soon be approaching a plateau.To sustain an ever increasing world population, new methods must be developed to meet this increasing demand for animal products. Secondly, selective breeding is a painfully slow process and, especially with larger animals with long gestation period, can take many years to establish desired phenotypic changes.

The advent of technology of transgenesis also called transfection and its application to animal breeding programmes may greatly increase the speed and range of selective breeding. The transgenesis involves the transfer of desired isolated gene or gene fragments or individual chromosome or chromosomal fragments, or isolated nuclei from one organism to another organism.The first recorded examples of the transfer of a foreign gene into an animal by recombinant DNA technology was the insertion and expression of a rat gene for growth hormone (rGH) into the mouse metallothionein (mMT) gene in 1882. The subsequent progeny were all much larger than, the parents and the transgenic mouse was called **"super mouse"** (Fig. 12.11). Since then, many transgenic animals including cattie, goats, pigs, rabbits, chickens and fish have been produced (Table 12.2).

Table 12.2. Examples of transgenic animals.

Transgenic animals	Genes transferred
1. Mouse	mMT/rGH; mMT/bGH (b=bovine) ; mMT/oGH (o= ovine); mMT/hGH(h=human); mMT/hGRF; mMT/hf IX(factor IX).
2. Chickens	ALV (Avian Leukosis Virus); REV (Raticulo Endotheliosis Virus)
3. Cow	BPV (Bovine Papilloma Virus);Lactoferin.
4. Fish	hGH; Cd-crystallin(c= chicken); AFP(Anti-Freeze Protein).
5. Pig	hMT/pGH (p= porcine); MLV(Moloneymurine Leukemia Virus)/ rGH; bPRL (prolactin)/bGH.
6. Rabbit	hMT/hGH; rbEu (Immunoglobulin heavy chain)/rb.
7. Sheep	mMT/TK (Thymidine kinase); oMT/ oGH; oBLG (β-Lactoglobulin)/hf IX.
8. Goat	A varient of tPA gene (human tissue-type plasminogen activator).

About 95 % of the existing transgenic animals are mice.

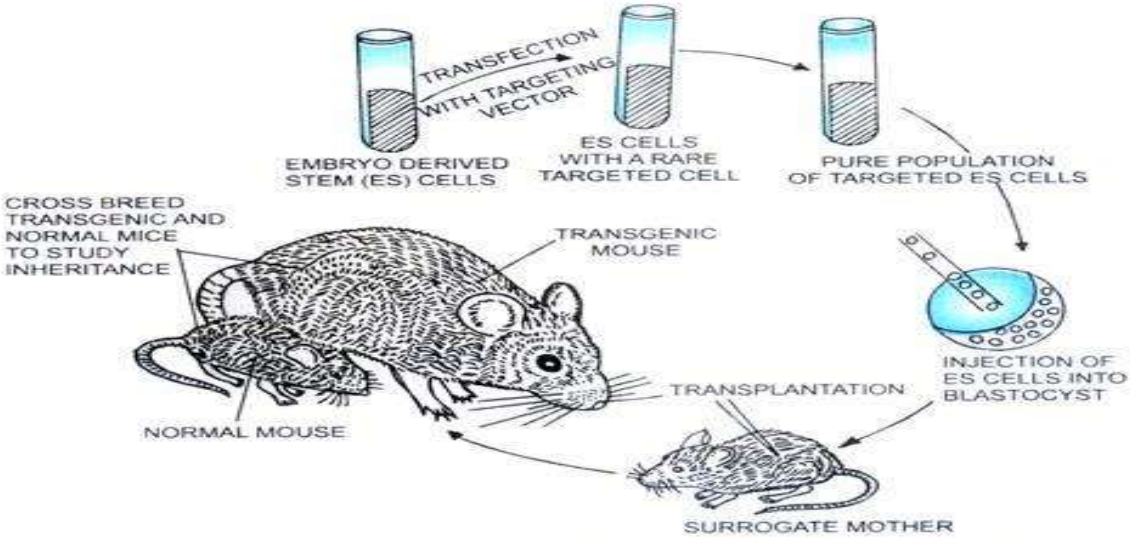


Fig. 12.11. Gene targeting using embryonic stem cells.

Mechanism of Transgenesis in Animals:

Transgenesis involved a number of methods like:

1. Transfer of whole nucleus from a somatic cell of a superior donor into the enucleated egg of recipient animal.

2. Transfer of a part of dissected embryo into the enucleated unfertilized egg.

3. Transfer of a chromosome or chromosomal fragments.

4. DNA microinjection technique.

5. Gene targeting using embryonic stem cells.

Frequency of Success of Transgenesis:

Now the transgenic pigs, sheep and cattle have been produced, although the frequency of success is only about 1% compared to 2-5% in mice while successful fish transgenics can be as high as 70 %.

Significance of Transgenesis:

1. Role of Transgenesis in Molecular Farming:

Molecular farming involves the extraction of useful proteins and drugs from the milk, food and urine of hansgenic animals which may be used as bioreactors e.g:

(i) Transgenic goats with LAtPA protein were produced by microinjecting murine whey acid promotor (WAP) carrying a c-DNA with LAtPA protein coding gene. The LAtPA protein dissolves the blood clot and is useful for treating coronary thrombosis.

(ii) (ii) Transgenic sheep with human antihaemophillic factor IX gene was produced with the help of BLG-gene (β-Lactoglobulin gene), which acts as vector gene Transgenic ewes secrete human factor IX in their milk.

(iii) Transgenic sheep having human α_1 antitrypsin gene (hα_1 AT) was produced with the help of ovine β- lactoglobulin gene promoter. Transgenic ewes produce h α_1 AT protein in their milk and can be used against the emphysema.

Thus by transgenesis the animals are genetically modified in such a way that they start acting bioreactorsproducing useful products in abundance and continuously.

Study of Diseases and Gene Therapy :

Many transgenic animals help us to understand the following facts:

(i) How do genes contribute to the development of disease?

(ii) These act as models for human diseases like cancer, cystic fibrosis, rheumatoid arthritis, Alzheimer's disease, etc., and their possible new methods of their treatment.

(iii) Transfections of cultured mammalian cells have been used extensively for detecting the cancer genes (oncogenes) and their gene therapy. In these retroviruses, adeno- associated virus (AAVs) and naked DNA have been used as vectors and gene therapy.

(iv) In 1991, the transgenic cow with a bovine alpha- Si casein promotor driving a c-DNA was produced. This foreign gene encodes for the lactoferrin (a iron- binding protein) which has the antibacterial properties.

Increased Production of Biological Products:

(i) Transgenic sheep with genes like cysE and cysM (coding for two enzymes—serine acetyl transferase and o-acetyl serine sulphhydrylase) have been produced. In the transgenic sheep, wool production was found to much more than the non-transgenic sheep because these two enzymes are essential for the biosynthesis of the amino acids involved in the formation of wool.

(ii) In 1985, the transgenic fishes of many species like common carp, catfish, goldfish, salmon, Tilapia, rainbow trout and zebra fish have been produced by microinjection of genes coding for rat or human growth hormone (rGH or hGH). It was found that transgenic fish with hGH gene was found to be twice in size than the non- transgenic fish.

(iii) In 1997, first transgenic cow, named Rosie, with human alpha-lactalbumin gene was produced. The milk of transgenic cow contained about 2.4 grams of human protein per litre of milk and was found to be more nutritionally balanced product for human babies than that of natural cow milk. Such human milk proteins can be extracted and used pharmaceutically.

For Study of Normal Physiology and Development:

Transgenic animals have been successfully utilized to understand:
(i) Mechanism of regulation of genes.

(ii) Mode of effects of genes on the normal functions of the body and its development e.g. study of biological role of insulin-like growth factor in regulating the body's growth.

Vaccine Safety Testing:
Transgenic mice are first to be used as laboratory animals to test the efficacy of a newly discovered vaccine before it is used on human beings e.g. polio vaccine. If such vaccines are found satisfactory and reliable on mice, then these are tested on the monkeys much closely related to man.

Chemical Safety Testing:
For this transgenic animals with foreign genes are produced so that the transgenic animals become more sensitive to the toxic chemicals of them, the non-transgenic animals. Then these animals are exposed to toxic chemicals and their effects are observed. The time required to obtain the results is less.

So contrary to some popular view points, transgenic animal studies are not about producing animal monsters but rather introducing specific and economically significant traits into livestock that will have benefits to mankind. These encouraging results have made transfection and production of transgenic animals a fascinating thrust of research.

Importance of Transgenic Animals-General:

Trans-genesis has now become a powerful tool for studying the gene expression and developmental processes in higher organisms, besides the improvement in their genetic characteristics. Transgenic animals serve as good models for understanding the human diseases.

Further, several proteins produced by transgenic animals are important for medical and pharmaceutical applications. Thus, the transgenic farm animals are a part of the lucrative world-wide biotechnology industry, with great benefits to mankind. Trans-genesis is important for improving the quality and quantity of milk, meat, eggs and wool production, besides creating drug resistant animals.

Milk as the Medium of Protein Production:

Milk is the secretion of mammary glands that can be collected frequently without causing any harm to the animal. Thus, milk from the transgenic animals can serve as a good and authenticated source of human proteins for a wide range of applications. Another advantage with milk is that it contains only a few proteins (casein, lactalbumin, immunoglobulin etc.) in the native state, therefore isolation and purification of a new protein from milk is easy.

Commonly used Animals for Trans-genesis:

The first animals used for trans-genesis was a mouse. The 'Super Mouse', was created by inserting a rat gene for growth hormone into the mouse genome. The offspring was much larger than the parents. Super Mouse attracted a lot of public attention, since it was a product of genetic manipulation rather than the normal route of sexual reproduction. Mouse continues to be an animal of choice for most transgenic experiments. The other animals used for trans-genesis include rat, rabbit, pig, cow, goat, sheep and fish.

Position Effects:

Position effect is the phenomenon of different levels of gene expression that is observed after insertion of a new gene at different position in the eukaryotic genome. This is commonly observed in transgenic animals as well as plants. These transgenic organisms show variable levels and patterns of transgene expression. In a majority of cases, position effects are dependent on the site of transgene integration. In general, the defective expression is due to the insertion of transgene into a region of highly packed chromatin. The transgene will be more active if inserted into an area of open chromatin.

The positional effects are overcome by a group of DNA sequences called insulators. The sequences referred to as specialized chromatin structure (SCS) are known to perform the functions of insulators. It has been demonstrated that the expression of the gene is appropriate if the transgene is flanked by insulators.

Animal Bioreactors:

Trans-genesis is wonderfully utilized for production proteins of pharmaceutical and medical use. In fact, any protein synthesized in the human body can be made in the transgenic animals, provided that the genes are correctly programmed. The advantage with transgenic animals is to produce scarce human proteins in huge quantities. Thus, the animals serving as factories for production of biologically important products are referred to as animal bioreactors or sometimes pharm animals. Frankly speaking, transgenic animals as bioreactors can be commercially exploited for the benefit of mankind.Once developed, animal bioreactors are cost- effective for the production of large quantities of human proteins. Routine breeding and healthful living conditions are enough to maintain transgenic animals.

Transgenic Animals in Xenotransplantation:

Organ transplantation (kidney, liver, heart etc.) in humans has now become one of the advanced surgical practices to replace the defective, nonfunctional or severally damaged organs. The major limitation of transplantation is the shortage of organ donors. This often results in long waiting times and many unnecessary deaths of organ failure patients.

Xenotransplantation refers to the replacement of failed human organs by the functional animal organs. The major limitation of xenotransplantation is the phenomenon of hyper acute organ rejection due to host immune system.

The organ rejections is mainly due to the following two causes:
i. The antibodies raised against the foreign organ.

ii. Activation of host's complement system.

Pigs in Xenotransplantation?
Some workers are actively conducting research to utilize organs of pigs in xenotransplantation. It is now identified that the major reason for rejection of pig organs by primates is due to the presence of a special group of disaccharides (Gal-α 1, 3-Gal) in pigs, and not in primates.

The enzyme responsible for the synthesis of specific disaccharides in pigs has been identified. It is α 1, 3-galactosyltransferase, present in pigs and not in primates. Scientists are optimistic that knockout pigs lacking the gene encoding the enzyme α 1, 3-galactosyltransferase can be developed in the next few years. Another approach is to introduce genes in primates that can degrade or modify Gal-α 1, 3-Gal disaccharide groups (of pigs). This will reduce immunogenicity.Besides the above, there are other strategies to avoid hyperactive organ rejection by the hosts in xenotransplantation.

i. Expression of antibodies against the pig disaccharides.

ii. Expression of complement— inactivating protein on the cell surfaces.

By the above approaches, it may be possible to overcome immediate hyperactive rejection of organs. The next problem is the delayed rejection which involves the macrophages and natural killer cells of the host.

Another concern of xenotransplantation is that the endogenous pig retroviruses could get activated after organ transplantation. This may lead to new genetic changes with unknown consequences.The use of transgenic animals in xenotransplantation is only at the laboratory experimental stages, involving animals. It is doubtful whether this will become a reality in the near future.There is a vigorous debate concerning the ethics of xenotransplantation and the majority of general public are against it.

Transgenesis in large animals:

In general, trans-genesis in large animals is more difficult than with mice. There are several factors for the lower efficiency of trans-genesis in large animals.These include less number of eggs they produce and technical difficulties in handling, besides long gestational periods to get the offspring (It takes about 2 years to produce a calf from a fertilized egg).

Some of the early experiments to produce transgenic large animals were far from satisfactory. For instance, transgenic sheep overproducing growth hormone grow leaner with increased feed efficiency. But they are more susceptible to infection, become infertile and tend to die at young age. All this might be due to ineffective control of gene regulation. Several improvements have been made to produce transgenic animals with desirable characters.Biotechnologists are particularly interested to improve the quality of animals, with improved resistance to diseases, besides enhancing their ability produce foods. 'Building a better animal', being the motto. Further, production of commercial and pharmaceutical compounds by transgenic animals is also gaining importance in recent years. The protocol adopted for producing other transgenic animals is comparable with that already described for transgenic mice, with certain modifications.

Transgenic Cattle:

The mammary gland of the dairy cattle is an ideal bioreactor for producing several new proteins (of pharmaceutical importance), besides improving the quality and quantity of the existing ones. For instance, a transgenic cow, with an over-expressed casein transgene, can give milk with higher content of casein.

If lactase transgene is introduced and expressed in the mammary gland, milk free from lactose will be secreted. Such a milk will be a boon for lactose intolerant people who experience indigestion and other complications, after consuming normal milk and milk products.Some success has been achieved in creating transgenic cattle with improved resistance to viral, bacterial and parasitic diseases. However, this is not an easy job due to the complexity of genetic control to combat the disease-producing organisms.

Attempts have been made in recent years to produce cattle with inherited immunological protection by trans-genesis. Introduction of genes that code for heavy and light chains of monoclonal antibodies has met with some success in this direction.In vivo immunization of an animal although not yet fully successful, is ideal for disease protection. In vivo immunization primarily involves the insertion of a transgene for an antibody that specifically binds to an antigen.

Transgenic Sheep and Goats:

Trans-genesis experiments in sheep and goats mostly involve the development of mammary glands as bioreactors for the production of proteins for pharmaceutical use. This is possible despite the fact that quantity of milk produced by sheep and goats is less than that of dairy cattle (cow, buffalo). Some proteins produced by sheep and goats have good pharmaceutical use (Table 41.2).

TABLE 41.2 Transgenic animals as bioreactors for the production of therapeutically important proteins

Transgenic animal	*Protein product*	*Biological importance*
Cow	Lactoferrin	Promotes intestinal iron absorption and hence can be used to overcome iron-deficiency anemias. Possesses antibacterial activity
Cow	Interferon	Provides resistance against viral infections
Sheep	α_1-Antitrypsin	Used in the treatment of emphysema (promotes the exchange of gases in lungs)
Goat	Cystic fibrosis transmembrane regulator (CFTR)	For the treatment of patients suffering from cystic fibrosis (promotes transport of ions)
Goat	Tissue plasminogen activator (tPA)	Used in treating the patients of myocardial infarction (dissolves blood clots)
Goat	Antithrombin III	Regulates blood clotting
Rabbits	α-Glucosidase	Treatment of Pompe's disease (a genetic disorder characterized by block in glycogen degradation)
Mouse	Urokinase	For dissolving blood clots
Mouse	Immunoglobulins (antibodies)	Administration enhances immunity
Pig	Hemoglobin	Blood transfusion
Goat and other animals	Vaccines (?)	To immunize against various diseases

Transgenic sheep with increased wool production:

Keratin is the wool protein with highly cross- linked disulfide bridges. For good production of quality wool, the amino acid cysteine (or its precursor methionine) is required in large quantities. However, cysteine supply to sheep is always inadequate, since the microbes harboring the rumen utilize it and release in the form of sulfide. This problem can be overcome by producing transgenic sheep

containing bacterial genes for the synthesis of cysteine. The two enzymes, synthesized by the transgenes, are capable of trapping the hydrogen sulfide liberated in the intestine to produce cysteine. Thus, good supply of cysteine to the sheep improves the quality and quantity of wool.

Transgenic Pigs:

Transgenic pigs that can produce human haemoglobin have been successfully developed. This human haemoglobin can be separated from pig haemoglobin by simple analytical techniques. Hemoglobin, the oxygen carrying protein of RBC, can be used as a substitute in blood transfusion experiments.

In fact, haemoglobin can be stored for longer period (a few months) than whole blood (weeks only). Further, there is no problem of contamination (like HIV) as is the case with whole blood. However, the free haemoglobin (naked haemoglobin) cannot transport oxygen as effectively as the haemoglobin of RBC.In addition, naked haemoglobin is easily degraded and the breakdown products cause damage to kidney. There also exists a risk of contamination by pig viruses and other compounds to cause allergic reactions. With these limitations, the initial enthusiasm for substituting blood transfusion with free haemoglobin has remained short-lived. It is now advised not to use naked haemoglobin for transfusion, when there is a heavy blood loss. However, it can be used during major surgeries for supplementing the whole blood transfusion.

Pig in organ farms:

The human organs such as heart, liver, pancreas, kidney and lungs are in great demand for transplantation surgery. The shortage of these transplantable organs can be overcome by developing them in animals. Pig is a favourite animal for harvesting human organs. This is because the physiology of pigs is close to that of humans.

Further, pigs do not carry any major infectious diseases transmissible to humans. The use of pigs in organ farming is still at the experimental stages. In the preliminary experiments, organ transplantation from transgenic pigs into primates showed some promising results. The day may not be very far for utilizing transgenic pigs as donors of human organs.

Transgenic Chickens:

The production of transgenic chickens (or other birds) is rather complicated. This is mainly because during fertilization in chickens, several sperms enter the ovum instead of one. This is in contrast to mammals where usually only one sperm enters the egg. The identification of male pronuclei that will fuse with female pronuclei is quite difficult. Further, embryonic stem (ES) cells have not been identified in chicken. Despite all these limitations, transgenic chickens have been developed.

The blastoderm cells (from an egg) can be removed from a donor chicken. They are transfected with transgenes (usually by lipofection with liposomes). The so modified blastoderm cells are reintroduced into the sub-germinal space of irradiated blastoderm of freshly laid eggs. Some of the resulting chickens may carry the transgene. Transgenic lines of chickens can be established.Trans-genesis in chicken can be used to develop low fat and cholesterol, and high protein containing eggs. Transgenic chickens that are resistant to viral and bacterial diseases have also been developed. Some attempts have also been made to develop pharmaceutical proteins in the eggs of transgenic chickens.

Transgenic Fish:

Several transgenic fish (catfish, salmon, trout etc.) have been developed with increase in their growth and size. This was carried out by introducing growth hormone transgene (by microinjection or electroporation). The fertilized eggs with inserted transgene are incubated in temperature-regulated holding tanks. (Note: The fish egg development is external in contrast to the mammalian embryogenesis). The efficiency of fish trans-genesis is as high as 70%. It was found that the transgenic salmon fish (with growth hormone transgene) were 10 times heavier than the normal ones, at the end of one year.

Aquatic animals are being engineered to increase aquaculture production, for medical and industrial research, and for ornamental reasons (Fig. 18.7). Genes inserted to promote disease resistance may allow transgenic fish to absorb higher levels of toxic substances, including heavy metals. In turn, consumers of these fish may be ingesting higher amounts of substances such as mercury and selenium.

Transgenic fish that have genes from species such as peanuts or shellfish that are common causes of allergic reactions in humans may prompt allergic reactions in an unsuspecting consumer. Transgenic species may behave much like invasive species when interacting with the natural environment.They may compete with native species for resources and pose a threat to the genetic diversity of native populations, especially when genetic modifications such as a rapid growth rate offer advantages over slower-developing native species. Despite industry assurances that transgenic fish would be unable to naturally reproduce or significantly threaten the environment, some scientists are far more doubtful.

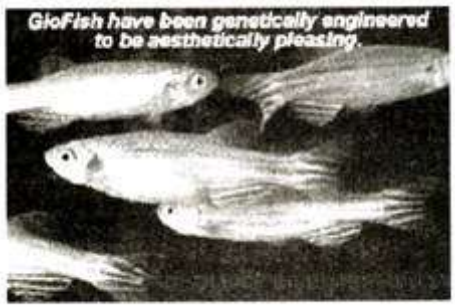


Fig. 18.7. Transgenic fish.

The sample bill included in this package addresses these concerns by banning the importation, transportation, possession, spawning, incubation, cultivation, or release of aquatic transgenic animals except under a permit.

Fluorescent Cat:

Recently South Korean scientist produced transgenic white Turkish angora cats to glow red under ultraviolet light these cats contain a fluorescent gene for flu protein and expressed under skin. Subsequently they produced a number of cloned cats from the skin cells of transformed mother cat. They proposed that such cat could be beneficial in diagnosis of genetic diseases and also showed a way to produce endangered animal by cloning.

Probable Questions:

1. What are the main features of transgenic technology.
2. Write down the steps of transgenic technology.
3. What are the significance of transgenesis.
4. What are the importance of transgenic animals?
5. What are the implication of transgenic cattle?
6. What are the implication of transgenic pig?
7. What are the implication of transgenic chicken?
8. What are the implication of transgenic fish?

9. What are the implication of transgenic sheep?

10. what is fluorescent cat?

11. What is animal bioreactor?

12. define xenotransplantation.

Suggested readings:

1. Biotechnology by P.K. Gupta

2. Gene Cloning by T. Brown.

3. Biotechnology by N. Kumarsen.

4. Biotechnology by B.D. Singh

Disclaimer:

The study materials of this book have been collected from various books, e-books, journals and other e sources.