B. Sc III YEAR VI SEMESTER

TOOLS AND TECHNIQUES IN BIOLOGY

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PREFACE

ABOUT THE SUBJECT

The test book Tools & Techniques in Biology caters to undergraduate students of Biological Sciences In this book I have tried to include topics that ranges from basic histology to the latest techniques of biological sciences like recombinant DNA technology, PCR, ELISA and RIA. The basic concepts of Biostatistics are also explained in the book in an easier way.

This book is intended to give the fundamental ideas on tools and techniques which are commonly used in Biology. It is written in a simple and lucid style. At the same time all the relevant ideas with up to date knowledge are incorporated in each and every chapter.

ABOUT THE BOOK

The book entitled "**TOOLS AND TECHNIQUES IN BIOLOGY**" is designed for **B.Sc III-Year, VI-Semester** students. The contents provided in the book are strictly as per the syllabus prescribed by different Universities of Telangana state.

The information given in the book has been explained in a simple and lucid manner, along with diagrams for the convenience of the students.

A brief introduction has been provided prior to every unit, to help students get basic idea about the forthcoming topics.

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- **Unit** I explains about Microscopy, Histopathology and Centrifugation techniques.
- **Unit II** explains about Spectrophotometry, Chromatography and Electrophoresis.
- **Unit III** explains about Immunoassay principles. PCR and RIA.
- **Unit IV** explains about Various statistical tools used in Biology research

Important questions of all the units have been provided at the end of the book, to help students prepare for exams.

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TOOLS AND TECHNIQUES IN BIOLOGY SYLLABUS

UNIT-I

1. MICROSCOPY CENTRIFUGATION

    1.1. Microscopy

    1.2. Histopathological techniques

    1.3. Centrifugation

UNIT-II

2. SEPERATION TECHNIQUES

    2.1. Colorimetry and spectrophotometry

    2.2. Chromatography

    2.3. Electrophoresis

UNIT-III

3. ADVANCED TECHNIQUES

    3.1. Immonoassay

    3.2. PCR Techniques

    3.3. RIA

UNIT - IV

4. STATISTICAL TOOLS

    4.1. Data

    4.2. Representing data

    4.3. Non parametric tests

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UNIT I: MICROSCOPY CENTRIFUGATION

1.1 MICROSCOPY

- Microscope is the most important instrument used in microbiological laboratory to observe microorganisms.
- The main function of a microscope is magnification of the object.
- Depending on the principle on which the magnification is based, microscopes can be classified into two types.
    1. Light microscope
    2. Electron microscope

Light or optical microscopes

- In light microscopes, the magnification is obtained by a system of optical lenses using light waves.
- Light microscopes are classified into following types.

A. Bright field microscopes
B. Dark field microscopes
C. Fluorescent microscopes
D. Phase contrast microscopes

A. Bright field microscopes

- It is generally referred as student's compound microscope.
- These are commonly used to observe microorganisms in all Microbiology laboratories.
- In bright field microscopy, the area observed is brightly lighted and the microorganisms appear dark because they absorb some light.
- Generally microorganisms do not absorb any light but staining them with a dye greatly increases their light absorbing ability, resulting in greater contrast and color differentiation.

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- These microscopes consist of a strong metal stand with broad base or foot.
- From the foot rises a short stout pillar supporting an upright curved arm.
- A strong light source either an adjustable built in electric lamp or a mirror is situated at the base.
- Attached to the pillar above a light source is a system of one or more horizontal iris or condenser diaphragm. This is used to regulate the passage of light and to eliminate undesirable peripheral rays from the light source.
- Above the condenser, there is a horizontal stage which is square or circular plate of 3-4 inches size with a central opening, the window to admit light from the condenser.
- Objects to be examined are centered on the stage over the condenser.
- Attached to the curved upright arm of the microscope is the body tube or barrel. This may be single or double (binocular) and contains a system of prisms and reflectors that permit tilting of the barrel for easy viewing.
- The body tube is mounted on a rack and pinion mechanism for vertical course and fine adjustment for focusing.
- At the lower end of the body tube is the objective lens system fitted onto a revolving nose piece turret.
- The objective lens may be of 10X, 45X or 100X for oil immersion. The 10X is commonly used without oil immersion.
- The objective lens produces a real image within the instrument.
- At the top of the barrel is the ocular lens system or eye piece. These may be 5X, 10X or 15X and magnify the real image to produce a greatly enlarged virtual image.
- The efficiency of light microscope doesn't depend on the magnification power but depends on the resolving power.

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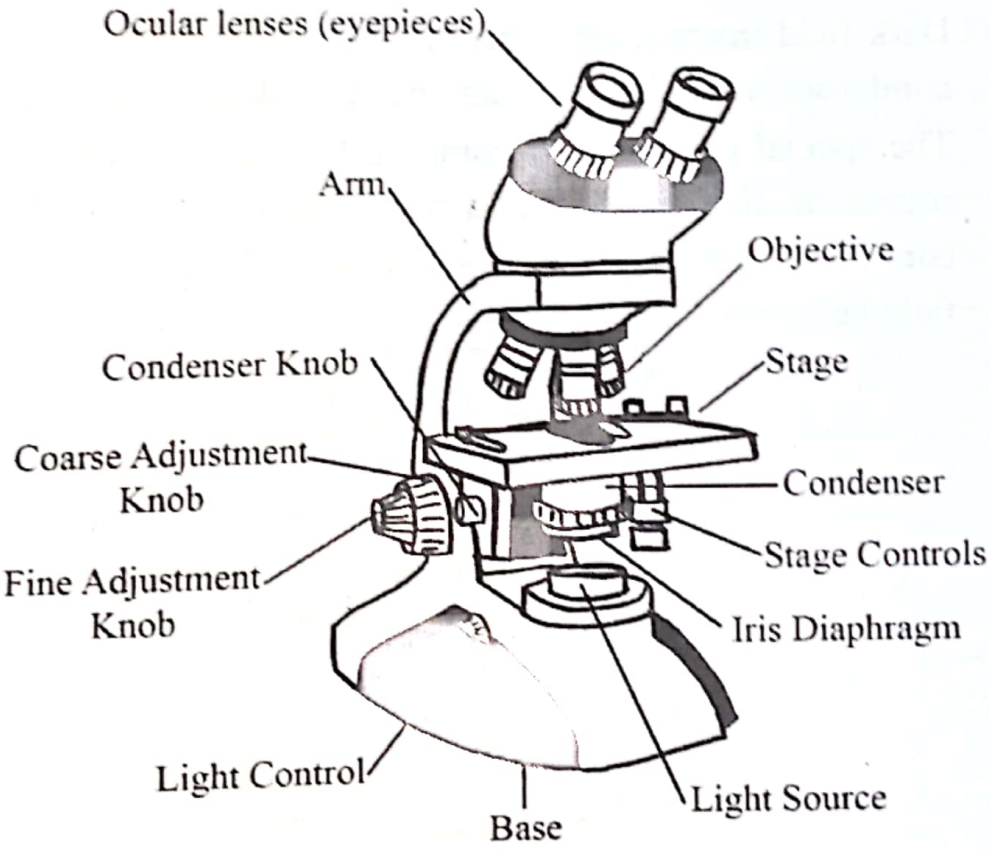
- The resolving power of the microscope can be defined as the ability to distinguish two adjacent points as distinct and separate points.
- The resolving power of a microscope is a function of a wavelength of light used and the numerical aperture of the lens system.
- The numerical aperture is always engraved on the objective lens itself.
- The greatest resolution in light microscope is obtained with the short wavelength of visible light and an objective with the maximum numerical aperture.
- The total magnification of the system is determined by multiplying the magnifying power of the objective by that of the eye piece.



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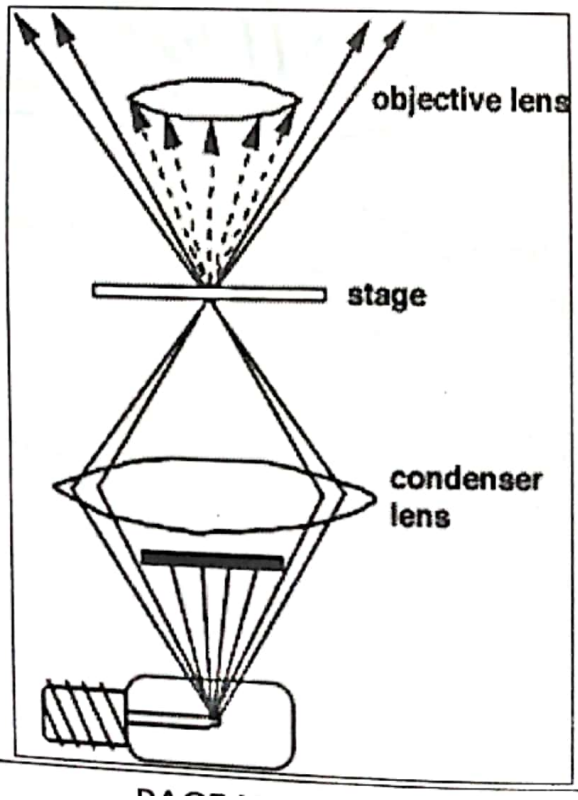
B. Dark field microscope

- This microscope is most useful for observing bacteria less than 0.2 micrometer in width or very thin microorganisms which are otherwise invisible in a bright field microscope and in living and stained preparations.
- In dark field microscope, organisms appear as bright spots against a dark background just like stars in a dark night.
- With dark field illumination, the observer sees only the light scattered by the specimen.
- This technique is ideal for living unstained microorganisms suspended in fluids such as wet mount and hanging drop preparations.
- Dark field microscope consists of a special condenser called abbe condenser which is having an opaque disk or dark field spot.
- The special condenser transmits a hollow cone of light from the source of illumination. As a result the light directed through the condenser does not enter the objective and thereby the microscopic field becomes dark.



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C. Fluorescence Microscope

- Certain substances absorb light of particular wavelength and energy, after sometime emits light of longer wavelength and lesser energy. Such substances are called fluorescent compounds and phenomenon is called fluorescence.
- Application of fluorescence is the basic principle in fluorescence microscope.
- In practice microorganisms are stained with fluorescent dyes and then illuminated with blue light or UV light.
- When exposed to UV light, organism stained with quinine sulphate fluoresces yellow. Sometimes microorganisms themselves may contain fluorescent compounds.
- Fluorescence microscope is commonly used for cytological investigations and in diagnostic microbiology for bacterial identification.
- Fluorescence microscope has special light sources that illuminate the specimen with a short wavelength light that will be absorbed by fluorescent compound. The specimen then emits fluorescence that forms the image by the observer.
- The fluorescence is seen against a dark background created by dark field illumination. Because UV light causes eye damage, the fluorescence microscope contains a barrier filter in its light path that absorbs the short wavelength excitation light.
- The Tuberculus bacilli are observed with fluorescence microscope.
- A high intensity mercury lamp is used as the light source and emits white light. The exciter filter transimits only blue light to the specimen and block out all other colors.
- The blue light is reflected downward to the specimen by a dichroic mirror.

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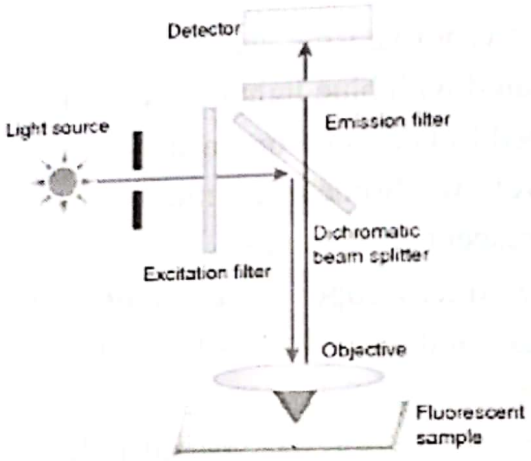
- The specimen stained with fluorescent stains absorbs blue light and emit green light which passes upwards, penetrates the dichroic mirror and reaches the barrier filter.
- The filter allows the green light to pass to eye, however it blocks residual blue light from the specimen. Thus the eye perceives the stained portions of the specimen glowing green against a black background.



D. PHASE CONTRAST MICROSCOPY

- 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.
- Phase-contrast microscopy is an optical microscopy technique that converts phase shifts in the light passing through a transparent specimen to brightness changes in the image. It was first described in 1934 by Dutch physicist Frits Zernike.
- 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.

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Working Procedure of Phase Contrast Microscopy

- Partially coherent illumination produced by the tungsten-halogen lamp is directed through a collector lens and focused on a specialized annulus (labeled condenser annulus) positioned in the sub stage condenser front focal plane.
- Wave fronts passing through the annulus illuminate the specimen and either passes through undeviated or are diffracted and retarded in phase by structures and phase gradients present in the specimen.
- Undeviated and diffracted light collected by the objective is segregated at the rear focal plane by a phase plate and focused at the intermediate image plane to form the final phase-contrast image observed in the eyepieces.

Instrumentation:

Phase-contrast microscopy is basically a specially designed light microscope with all the basic parts in addition to which an annular phase plate and annular diaphragm are fitted.

The annular diaphragm

- It is situated below the condenser.
- It is made up of a circular disc having a circular annular groove.
- The light rays are allowed to pass through the annular groove.
- Through the annular groove of the annular diaphragm, the light rays fall on the specimen or object to be studied.
- At the back focal plane of the objective develops an image.
- The annular phase plate is placed at this back focal plane.

The phase plate

- It is either a negative phase plate having a thick circular area or a positive phase plate having a thin circular groove.
- This thick or thin area in the phase plate is called the conjugate area.
- The phase plate is a transparent disc.
- With the help of the annular diaphragm and the phase plate, the phase contrast is obtained in this microscope.
- This is obtained by separating the direct rays from the diffracted rays.
- The direct light rays pass through the annular groove whereas the diffracted light rays pass through the region outside the groove.

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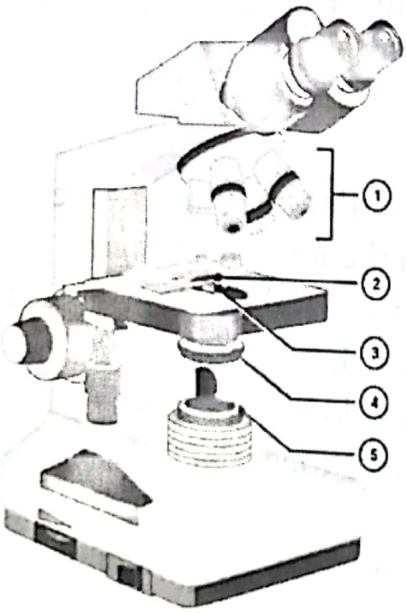
- Depending upon the different refractive indices of different cell components, the object to be studied shows a different degree of contrast in this microscope.

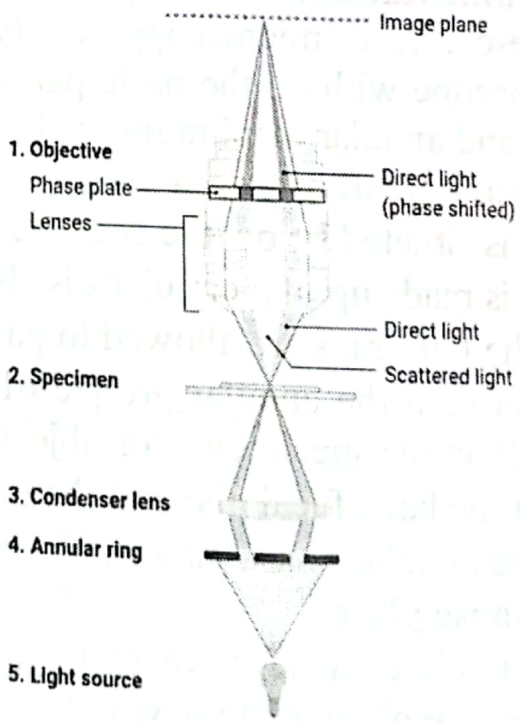
Applications:

To produce high-contrast images of transparent specimens, such as

1. living cells (usually in culture),
2. microorganisms,
3. thin tissue slices,
4. lithographic patterns,
5. fibers,
6. latex dispersions,
7. glass fragments, and
8. Sub cellular particles (including nuclei and other organelles).

Phase Contrast Microscopy





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ELECTRON MICROSCOPY

- Electron microscope is a type of microscope that provides an electronically magnified image of a specimen of detailed observations.
- Electron microscope uses a particle beam of electrons to illuminate the specimen and create a magnified image of it.
- Electron microscope has a greater resolving power than light microscopes because it uses electrons that have wavelength shorter than visible light.
- Electron microscope can magnify images up to 1,00,000X.
- Electron microscope uses electrostatic and electromagnetic lenses to control electron beam and focus it to form image.
- In 1931, German engineers Ernst Ruska and Max Knoll constructed the prototype of electron microscope.
- Electron microscope was constructed by Ernst Ruska in 1933.
- In 1933, Ernst Ruska constructed electron microscope that exceeded the resolution attainable with optical microscope.
- Two types of electron microscope are in use today.
    A. Transmission Electron Microscope
    B. Scanning Electron microscope

A. Transmission Electron Microscope

- It is the original form of electron microscope.
- It is modern and sophisticated microscope.
- It is very useful in observing the internal structure of prokaryotic cells.
- In this microscope, electrons work in place of light and magnetic fields in place of lenses.
- The whole system works in vacuum to prevent the collision of electrons with air particles.

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- The source of electrons in TEM is electron gun which is made up of tungsten filament.
- On heating the tungsten filament generates a beam of electrons which will be focused on the specimen by the magnetic fields.
- The specimen scatters the electrons passing through it. Then the beam is focused by objective magnet lens and projector magnet lens to form an enlarged visible image of the specimen on fluorescent screen.
- The magnified image can also be recorded on a photographic plate by a camera built in the instrument or by means of lens optical electron beam or by charge coupled device (CCD).
- The penetration power of electrons through solid matter is weak so the specimens are either thin films or thin sections. To increase the contrast of these thin preparations of the biological specimens, they are treated with special electron microscopic stains.
- The important stains are osmic acid, permanganate, uranium lanthanum and lead.
- Typically thin sections are stained for several minutes.

Freeze-fracture or freeze-etch:

- It is a preparation method which is particularly useful for examining lipid membranes and their incorporated proteins in face on view.
- The fresh tissue or cell suspension is frozen rapidly, and then fractured by simply breaking or by using microtome while maintained at liquid nitrogen temperature.
- The cold fractured surface is then shadowed with evaporated platinum or gold at an angle of 45 degrees in high vacuum conditions.

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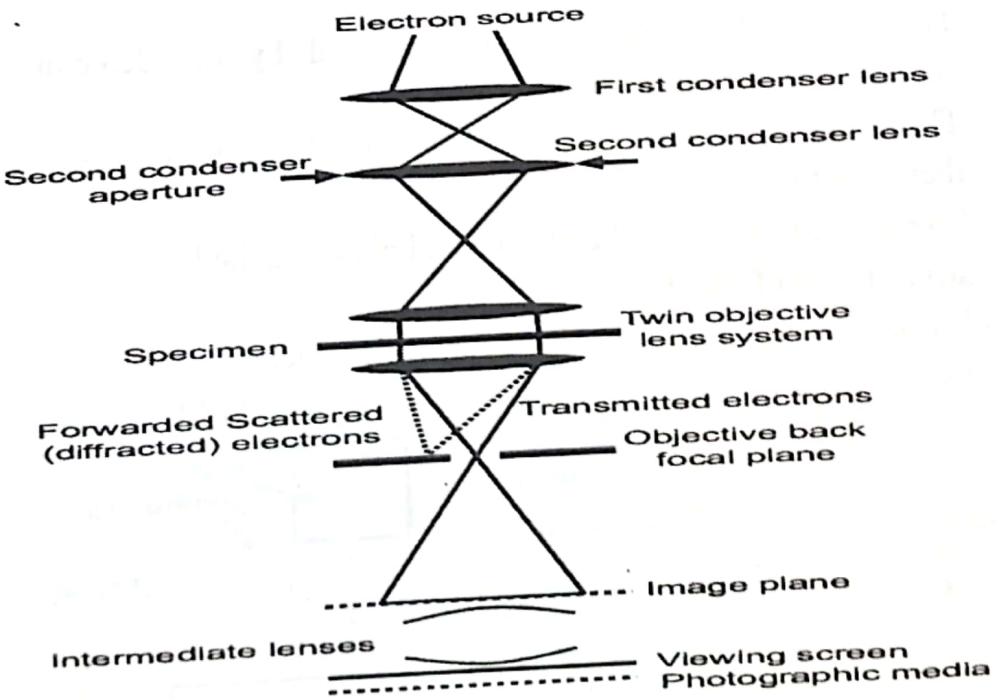
- A second coat of carbon is evaporated perpendicular to the surface plane is performed to improve stability of the replica coating.
- Now the specimen is brought to room temperature, the surface of the specimen is released from the biological material by careful chemical digestion with acids and viewed in the TEM.

Disadvantages of TEM:

- It is expensive to build and maintain.
- Specimens must be dehydrated before observing under TEM. This may bring changes in the structure of specimen.
- It needs very thin sections of specimens which are difficult to prepare.

›f Transmission Electron Microscopy



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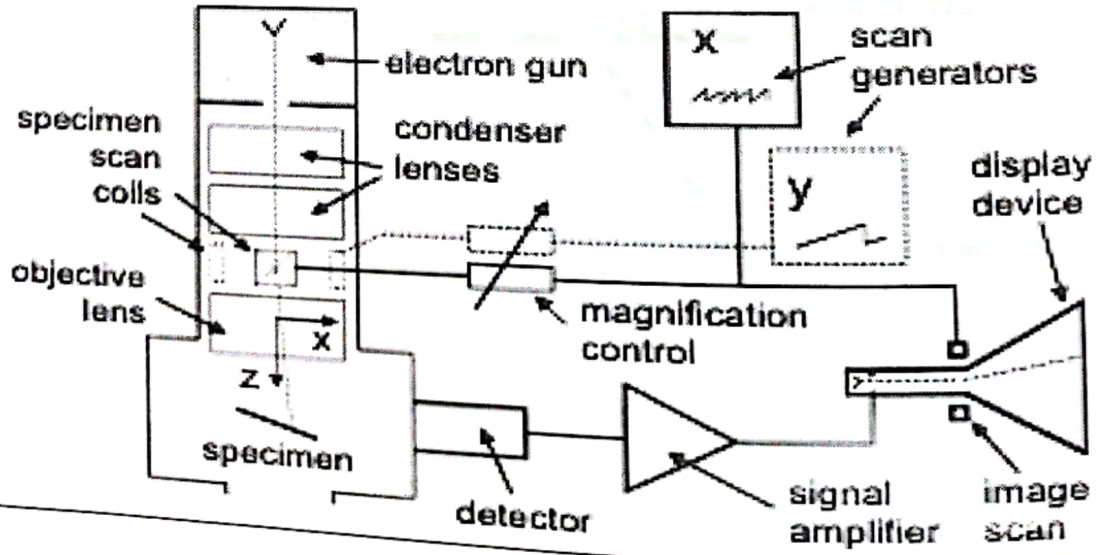
B. Scanning Electron Microscope (SEM)

- SEM is comparatively new type of electron microscope which is different from TEM in principle and applications.
- SEM gives 3D images of specimens.
- SEM is useful to study the surface structures of microoraganisms.
- SEM consists of a detector, photomultiplier, cathode ray tubes for viewing and photography as special components.
- In SEM, the sample preparation is comparatively simple and the material to be observed is coated with a thin layer of heavy metals such as gold.
- In SEM, the beam of electrons released from electric gun strike the specimen and scans the surface which releases the secondary electrons.
- These secondary electrons are collected by the detector and generates electronic signal.
- Thus generated electronic signals are amplified by multiplier and then sent to the system.
- The formed image can be viewed and photographed.

Disadvantages of SEM

- It is useful only for the study of surface characters.
- Its resolving power is comparatively lower than TEM.



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Applications of electron microscope

- Following are the important applications of electron microscopes

1. **In semiconductor and data storage**
   - Electron microscope is useful to edit the circuits, analysis and defects and failures in semiconductors.
2. **In research**
   - Electron microscope is useful for observing samples, nanoprototyping, nanomateriology, device testing and characterization.
3. **In biology and life sciences**
   - Biological applications of electron microscopy include
     a) Diagnosis
     b) Cryobiology
     c) Protein localization
     d) Electron tomography
     e) Cellular tomography
     f) Cryo electron microscopy
     g) Toxicology
     h) Biology production and viral load monitoring
     i) Particle analysis
     j) Pharmaceutical quality control
     k) In structural biology
     l) 3D tissue imaging
4. **In industry**
   - Industrial applications of electron microscopy include
     a) High resolution imaging
     b) 2D and 3D micro – characterization
     c) Particle detection and characterization

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d) Direct beam –writing fabrication
e) Mining
f) petrochemical

1.2 HISTOPATHOLOGICAL TECHNIQUES

HISTOPATHOLOGY

- Histopathology is the diagnosis and study of diseased tissue which involves examining tissues or cells under a microscope.
- The word Histopathology is a Greek term, histo means tissue, patho means disease, logy means study.
- The main purpose of histopathology is to differentiate normal tissue and abnormal tissue.
- Normal tissue cells have nucleus smaller in size than cytoplasm. But in abnormal tissue nucleus becomes larger in size and cytoplasm is shorter than nucleus

HISTOPATHOLOGICAL TECHNIQUES

Histopathological techniques include four steps.

1. Collection of tissues
2. Processing of tissue
3. Staining of processed tissue
4. Mounting

1. Collection of tissue

- Histopathological examination of tissue can be done by biopsy or autopsy.
- Biopsy: It is a procedure of removing a piece of tissue from a living being.
- Autopsy : It is a procedure of removing large tissue or organ from dead body

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2. Processing of tissue

The steps in processing of tissue include fixation, dehydration, clearing, Infiltrations, embedding, section cutting and deparaffinization.

a. Fixation

- Fixation is a process to prevent tissue from autolysis and putrefaction.
- Fixative is used to preserve tissue.
- Most common fixative used in lab is 10% formalin.
- Other fixatives are aldehydes, mercurials, alcohols, oxidizing agents, picrates.

b. Dehydration

- The water from the tissue must be removed by dehydration.
- This process is carried out by immersing specimen in a series of ethanol solutions of increasing concentrations.
- A typical dehydration sequence would be 30%, 50%, 70%, 90% and absolute ethanol.
- Other dehydrating agents are acetone, dioxane etc.

c. Clearing

- Clearing consists of removal of dehydrant from the tissue with the substance that will be miscible with embedding medium.
- The most commonly used clearing agent is xylene.
- The other clearing agents are toluene, chloroform, methyl salicylate.

d. Infilteration

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- In this the tissue is kept in a jar of molten paraffin wax.
- Entering the wax inside the tissue provides internal strength to the tissue.
- The melting point of paraffin wax is 56 degree C.

e. Embedding

- It is a process in which tissue is surrounded by paraffin wax.
- This paraffin wax provide external support to the tissue.
- Embedding provide hardness to the tissue for proper section cutting.
- Other embedding medium are carbowax, paraplast, and celloidin.

f. Section cutting

- This involves cutting of tissue into very thin slices. This can be done by section cutting machine called microtome.
- The thickness of the section cut should be 3-5 micrometer.
- Then the section cut is kept in warm water bath.
- This is done to remove fold in section.
- Then section cut is put on microscopic slide coated with Mayer's egg albumin, which help to adhere section cut to slide.

g. Deparaffinization

- The section of tissue having paraffin wax around is slightly warmed so that the paraffin gets melted. This is called deparaffinization.
- The slide containing tissue cut is dipped in xylene jar to remove paraffin wax completely.

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- Then hydration of the slide should be done from higher to lower concentration by using ethanol and then with distilled water.

3. Staining of processed tissue

- In this step, stain the section cut slide with hematoxin and eosin staining.
- Some other special stains such as PAS (Peroidic Acid Schiff) stain, orcein stain or reticulin stain can be used.
- After staining dehydration of the tissue slide is done for long term storage of tissue slide.
- Now tissue slide is again kept in xylene jar.

4. Mounting

- Mount the tissue slide with Dpx i.e ., Distyrene plasticizer xylene.
- Mounting will help the tissue to preserve long storage without any harm to the tissue.
- Finally observe the slide under microscope.

ADVANTAGES

- This is a least expensive method.
- It is the best technique for examining the internal structure of various cells and tissues.
- This technique can preserve the tissue for atleast one year.
- It is used for diagnosis by examining a small piece of tissue from various organs.

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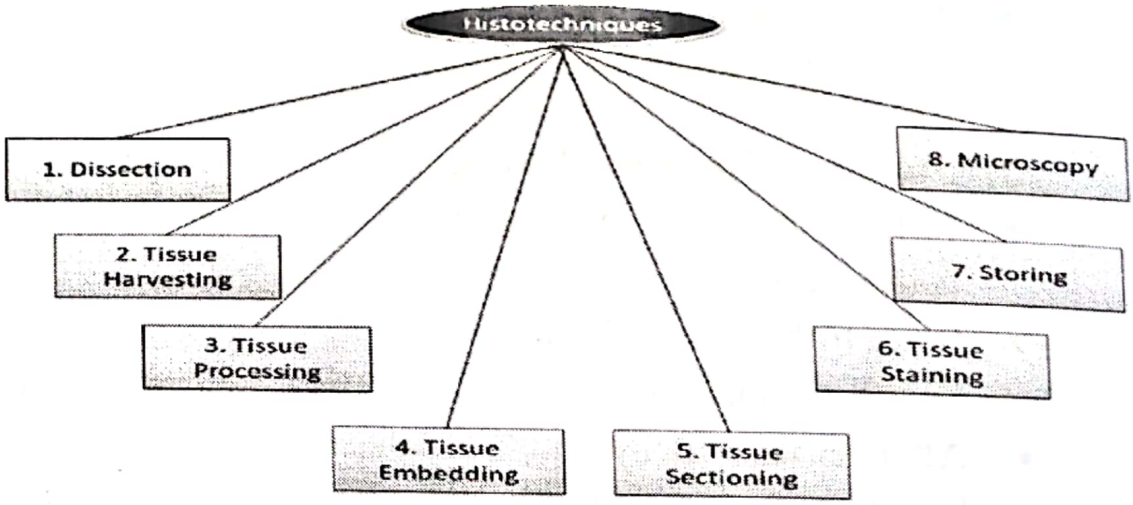

1.3 CENTRIFUGATION

- Centrifugation is a technique used for the separation of constituents or particles present in the solution with the help of centrifugal force.
- In this technique, particles separate from a solution according to their size, shape, density, viscosity of the medium and rotor speed.
- Centrifugation technique is carried out in centrifuge.
- The centrifuge is commonly used in laboratories for the separation of biological molecules from a crude extract.
- In a centrifuge, the sample is kept in a rotor that is rotated about a fixed point (axis), resulting in strong force perpendicular to the axis.
- There are different types of centrifuge used for the separation of different molecules.
- There are four major types of centrifuges. They are small bench centrifuges, large capacity refrigerated centrifuges, high speed refrigerated centrifuges and Ultra Centrifuges.

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Small bench centrifuge

- Small bench centrifuges are used to separate a small amount of material that sediments quickly.
- They are used to collect small amount of material that rapidly sediment like yeast cells, erythrocytes etc.
- They have maximum relative centrifugal field of 3000-7000 g.

Large capacity refrigerated centrifuge

- Large capacity refrigerated centrifuges have capacity to change rotor chambers with varying sizes.
- These types of centrifuges can go to a maximum of up to 65,000g.
- They are typically used for collecting substances that sediment rapidly such as yeast cells, chloroplast and erythrocytes.

High speed refrigerated centrifuge

- They can generate speed of about 60000g and are used to collect micro-organism, cellular debris, larger cellular organelles and proteins precipitated by ammonium sulphate.

Ultra Centrifuges

- The ultracentrifuge is a centrifuge optimized for spinning a rotor at very high speeds, capable of generating acceleration as high as 10,00000 g .
- There are two kinds of ultracentrifuges, the preparative and the analytical ultracentrifuge.

PRINCIPLE:

- A centrifuge works by using the principle of sedimentation. Under the influence of gravitational force, substances separate according to their density.
- Microcentrifugation, high-velocity centrifugation, and ultracentrifugation are the three types of centrifugation based on the volume and speed used in the process.

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- Among other centrifugation methods, ultracentrifugation uses the maximum angular velocity.
- The rotating speed of ultracentrifugation is as high as 1,000 000 g. Thus, ultracentrifugation is used to isolate small particles such as ribosomes, proteins, and viruses.
- Preparative and analytical centrifugation is the two types of ultracentrifugation methods.

PREPARATIVE CENTRIFUGATION

- Preparative centrifugation refers to a high-velocity centrifuge used in the separation of small submicroscopic particles.
- It separates small particles such as viruses, viral particles, protein complexes, proteins, lipoproteins, RNA, and plasmid DNA.
- Beckman-Coulter preparative centrifuge is used for preparative centrifugation.
- The preparative centrifugation can also be used as a density gradient centrifugation method.

Density gradient centrifugation method

- In density gradient centrifugation also called isopycnic centrifugation in which particles are separated only on the basis of their density.

- Isopycnic centrifugation is also termed the equilibrium centrifugation as the separation of particles takes place particularly on the basis of their densities and not on their sizes.
- The density of the particle becomes equal to the density of the surrounding medium.
- The density in the gradient increases as we move down the tube towards the bottom. As a result, the particles with higher densities settle down at the bottom, followed by less dense particles that form bands above the denser particles.

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- It is considered as a true equilibrium as this depends directly on the buoyant densities and not the sizes of the particles.

- The sucrose gradient can be used to separate cellular organelles.
- Caesium gradient can be used to separate nucleic acids.
- Preparative ultracentrifuges can be equipped with a wide range of different types of rotors, which can spin samples at different angles, and at different speeds.

ANALYTICAL CENTRIFUGATION

- Analytical centrifugation is an analytical technique which combines an ultracentrifuge with optical monitoring systems.
- Analytical centrifugation is versatile and is used for the quantitative analysis of macromolecules in solution.
- Analytical ultracentrifuges include a scanning visible ultraviolet light-based optical detection system for real-time monitoring of the sample's progress during a spin.

STEPS INVOVLED IN ANALYTICAL CENTRIFUGATION

- Sample taken in analytical cells is to be placed inside the ultracentrifuge.

- The ultracentrifuge is then operated so that the centrifugal force causes a migration of the randomly distributed biomolecules through the solvent radially outwards from the center of rotation.
- The distance of the molecules from the center is determined through the Schlieren optical system.
- A graph is drawn from the solute concentration versus the squared radial distance from the center of rotation, based on which the molecular mass is determined.

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APPLICATIONS OF CENTRIFUGATION

- The centrifugation method has a wide variety of industrial and laboratorial applications.
- Centrifugation method is used to analyze the hydrodynamic properties of macromolecules.
- Mammalian cells can be purified with the help of a special type of centrifuge.
- Centrifugation is known to have a vital role in the fractionation of many subcellular organelles, fractionation of membrane fractions and membranes.
- Centrifugation plays an important role in the pharmaceutical industry in the production of bulk drugs, biological products, and determination of molecular weight of colloids and in the evaluation of suspensions and emulsions.
- Skimmed milk is a form of milk that has a lower amount of dissolved fats. Skimmed milk can be obtained from regular milk with the help of the process of centrifugation.
- Centrifugation in the food industry include: milk separation, cheese production, pulp control in juices, edible oil production, essential oil recovery, and production of starch and yeast.
- It is also used in the clarification and stabilization of wine.
- Centrifugation is widely used in the In forensic and research laboratories. It can be used in the separation of blood components from blood samples of urine and blood components.

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SYNOPSIS UNIT - I

1.1 MICROSCOPY: Microscope is the most important instrument used in microbiological laboratory to observe microorganisms.

- Two types of microscopes
  Light microscope
  Electron microscope

LIGHT MICROSCOPE

E. Bright field microscopes

- student's compound microscope.
- Area observed is brightly lighted and the microorganisms appear dark.
- The objective lens may be of 10X, 45X or 100X for oil immersion.

F. Dark field microscopes

- observing bacteria less than 0.2 micrometer in width
- organisms appear as bright spots against a dark background

G. Fluorescent microscopes

- uses fluorescence to generate an image
- examine material that fluoresces under ultraviolet light.

ELECTRON MICROSCOPE

- provides an electronically magnified image of a specimen
- uses a particle beam of electrons to illuminate the specimen
- can magnify images upto 1,00,000X.
- uses electrostatic and electromagnetic lenses to control electron beam and focus it to form image.
- Two types 1. TEM
  2. SEM

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1. **TEM**

- *Observing the internal structure of prokaryotic cells.*
- *Electrons work in place of light and magnetic fields in place of lenses.*

2. ***SEM***

- *Gives 3D images of specimens.*
- *used to study the surface structures of microorganisms*

1.2 HISTOPATHOLOGICAL TECHNIQUES

- *diagnosis and study of diseased tissue*
- *Differentiate normal tissue and abnormal tissue.*
- *These techniques include four steps.*

1. *Collection of tissues*
2. *Processing of tissue*
3. *Staining of processed tissue*
4. *Mounting*

- *Collection of tissues can be done by biopsy or autopsy.*
- *Processing of tissue include fixation, dehydration, clearing, Infilteration, embedding ,section cutting and deparaffinization*
- *Stain the slide with hematoxin and eosin staining.*
- *Mount the tissue slide with Dpx i.e ., Distyrene plasticizer xylene.*
- *Mounting helps tissue to preserve long storage without any harm to the tissue.*

1.3 CENTRIFUGATION

- *Separation of constituents or particles present in the solution.*

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- *Particles separate from a solution according to their size, shape, density, viscosity of the medium and rotor speed.*
- *Carried out in centrifuge.*
- *Used in laboratories for the separation of biological molecules.*
- *Four major types of centrifuges.*
    1. *small bench centrifuges*
    2. *large capacity refrigerated centrifuges*
    3. *high speed refrigerated centrifuges*
    4. *Ultra Centrifuges.*
- *Works by using the principle of sedimentation.*
- *Two types ultracentrifugation methods.*
    1. *Preparative*
    2. *analytical*

1. *Preparative centrifugation*

- *Separates small particles such as viruses, viral particles, protein complexes, proteins, lipoproteins, RNA, and plasmid DNA.*
- *Used as a density gradient centrifugation method.*

2. *Analytical centrifugation*

- *Combines an ultracentrifuge with optical monitoring systems.*
- *Quantitative analysis of macromolecules in solution.*

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UNIT II: SEPERATION TECHNIQUES

2.1 SPECTROPHOTOMETRY

- The instrument used to study the absorption or emission of electromagnetic radiation as function of wavelength is called **spectrophotometer** or **spectrometer**.
- (Colorimeter – if the instrument applies wavelength only in visible range).

PRINCIPLE OF SPECTROPHOTOMETER:

- Spectrophotometer works on the principle of laws of absorption which include
  1. Beer's law
  2. Lambert's law
- **Lambert's law** states "the amount of light absorbed is proportional to the thickness of the absorbing material".

$$\log \frac{I_0}{I} \alpha\, b$$

Where I_0 = initial intensity of light

I = intensity of transmitted light

b = thickness of absorbing material

- **Beer's law** states that "the amount of light absorbed by the material is proportional to concentration".

$$\log \frac{I_0}{I} \alpha\ c$$

- If we combine the above two equation we get the following

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$$\log \frac{I_0}{I} \alpha\, b\, c$$

$$\log \frac{I_0}{I} = a\, b\, c$$

$$absorbance = abc$$

- This equation is called Beer – Lambert law. This combined law states that the amount of light absorbed is proportional to the concentration of absorbing substance and to the thickness of the absorbing material.
- With the help of the above equation we can find out the unknown concentration of substance by using the following formula.

INSTRUMENTATION OF SPECTROPHOTOMETER

- The essential components of spectrophotometer include
  1. Stable and cheap radiant energy source
  2. Monochromator
  3. Sample holder
  4. Detector
  5. Amplifier and Recorder

1. Radiant energy sources:

- Materials which can be excited to high energy states by a high voltage electric discharge or by electrical heating serve as excellent radiant sources.
- Different sources are available for different regions of wavelengths.
  - Sources of ultraviolet radiation
  - Sources of visible radiation

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- Sources of infrared radiation

Sources of ultraviolet radiation: Most commonly used sources of ultraviolet radiation are hydrogen lamp, deuterium lamp and Xenon lamp may also be used.

Sources of visible radiation: Tungsten lamp is most commonly used source of visible radiation. Carbon arc which provides more intense visible radiation is used in small number of commercially available instruments.

Sources of infrared radiation: Nernst Glower and Globar are most satisfactory sources of infrared radiation. Nernst glower consists of hollow rod of zirconium and yetrium. The Globar consists of silicon carbide rod which when heated emits radiation in the 1 – 40 micron region. Globar is more stable than Nernst glower.

2. Monochromator:

- As the name suggests, a monochromator converts a polychromatic radiation into its individual wavelengths and isolates these wavelengths into very narrow bands.
- The components of monochromator are
  - An entrance slit which admits polychromatic light from the source.
  - A collimating lens which collimates the polychromatic radiation on to the dispersion device.
  - A prism or a grating which breaks the radiation into component wavelength.
  - A focusing lens or a mirror which focus the light on to the sample holder.
  - An exit slit which allows the monochromatic beam to escape.
- The entire assembly is mounted in a light tight box.

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- Gratings are often used in place of monochromator of spectrophotometers operating in ultraviolet, visible and infrared regions.
- The grating consists of highly aluminized surface etched with a large number of parallel grooves which are equally spaced.

3. Sample holder:

- Sample to be studied in ultraviolet or visible region are usually gases or solutions and are put in cells known as cuvettes.
- Cuvettes meant for the visible region are made of either glass or sometimes quartz. Since glass absorbs in ultraviolet region, quartz or silica cells are used in this region.
- Standard width of cuvettes is usually 1 cm.

4. Detector device:

- Most of the detectors depend on the photoelectric effect where incident light liberates electrons from a metal or other material surface. Some sort of external circuits collects these electrons and measures their number as current.
- The current is then proportional to the light intensity and therefore measure of it.
- Following are the important requirements for a detector include
  - High sensitivity to the detection of low levels of radiant energy.
  - Short response time
  - Long term stability
  - Electronic signal which is easily amplified for typical readout apparatus.
- Most commoly used detectors are
  - Photovoltaic or barrier layer cell

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- Phototubes
- Photomultipliers
- Photo diodes

6. Amplification and readout device :

- Radiation detectors generate electronic signals which are proportional to the transmitted light.
- These signals need to be translated into a form that is easy to interpret.
- This is accomplished by using amplifiers, ammeters, potentiometers and potentiometric recorders.

APPLICATIONS OF SPECTROPHOTOMETRY:

- Spectrophotometry is a versatile technique, has diverse applications.
- Following are the important applications of spectrophotometers
    1) In qualitative analysis
    2) In quantitative analysis
    3) In enzyme assay
    4) In molecular weight determination
    5) In study of cis trans isomerism
    6) Other physicochemical studies
    7) In control of purification

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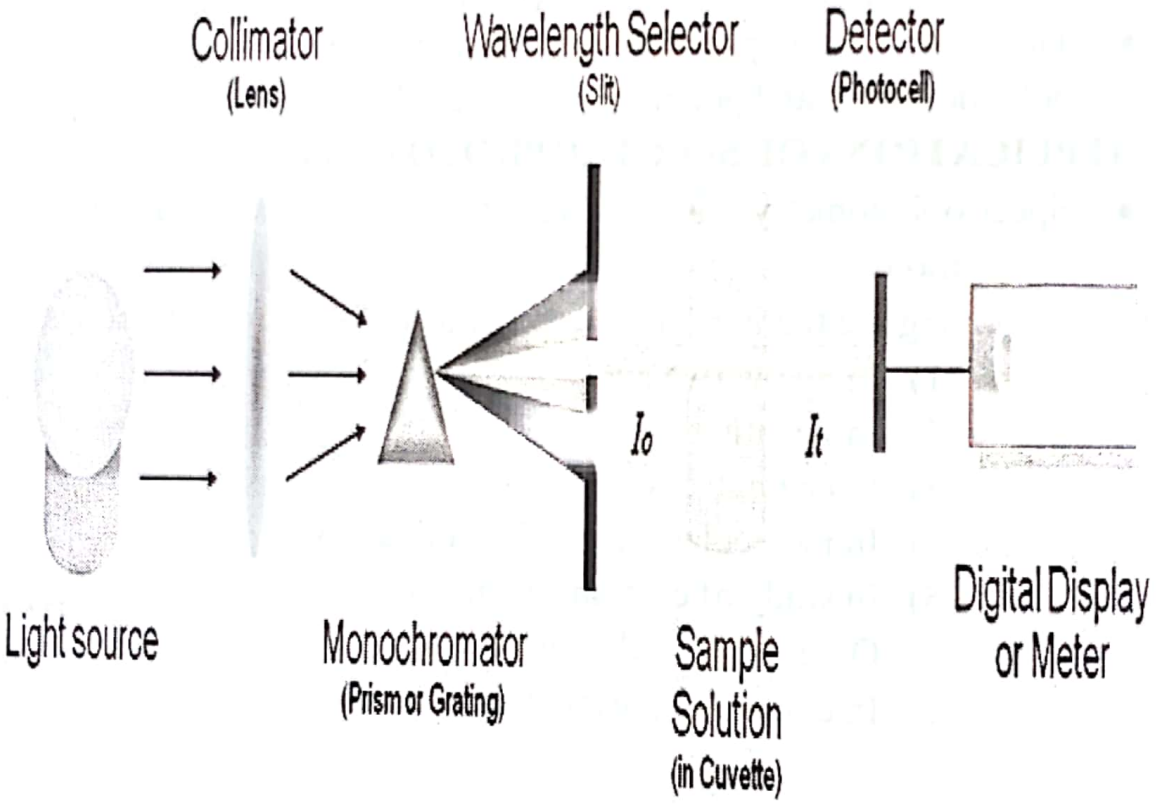
Spectrophotometer

Principle, Instrumentation, Applications



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2.2 CHROMATOGRAPHY

- Chromatography is a biochemical technique introduced by Michael Tswett in 1906.
- Originally this technique is used to separate the different colored pigments of a mixture. (chroma = color, graphy = to write)
- This technique has undergone various modifications and now this technique can be used to separate all compounds whether colored or colorless.
- Chromatography is defined as the technique of separation of substances according to their solubilities in two immiscible phases. This procedure of separation is called chromatography and the preparation is called chromatogram.
- Chromatography is a separation technique used in chemical and biology.
- This technique is used for the separation and identification of mixtures of chemical compounds and biological molecules.

Components of chromatography include the following

- Sample to be separated
- Stationary phase
- Moving phase

In chromatography separation of molecules is based on the following principle.

- Separation of differential solubilities of samples in stationary phase and in moving phase.
- All the chromatography techniques fall into two main categories based on the nature of stationary phase.
- They are 1. Adsorption chromatography
  2. Partition chromatography

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1. **Adsorption chromatography**

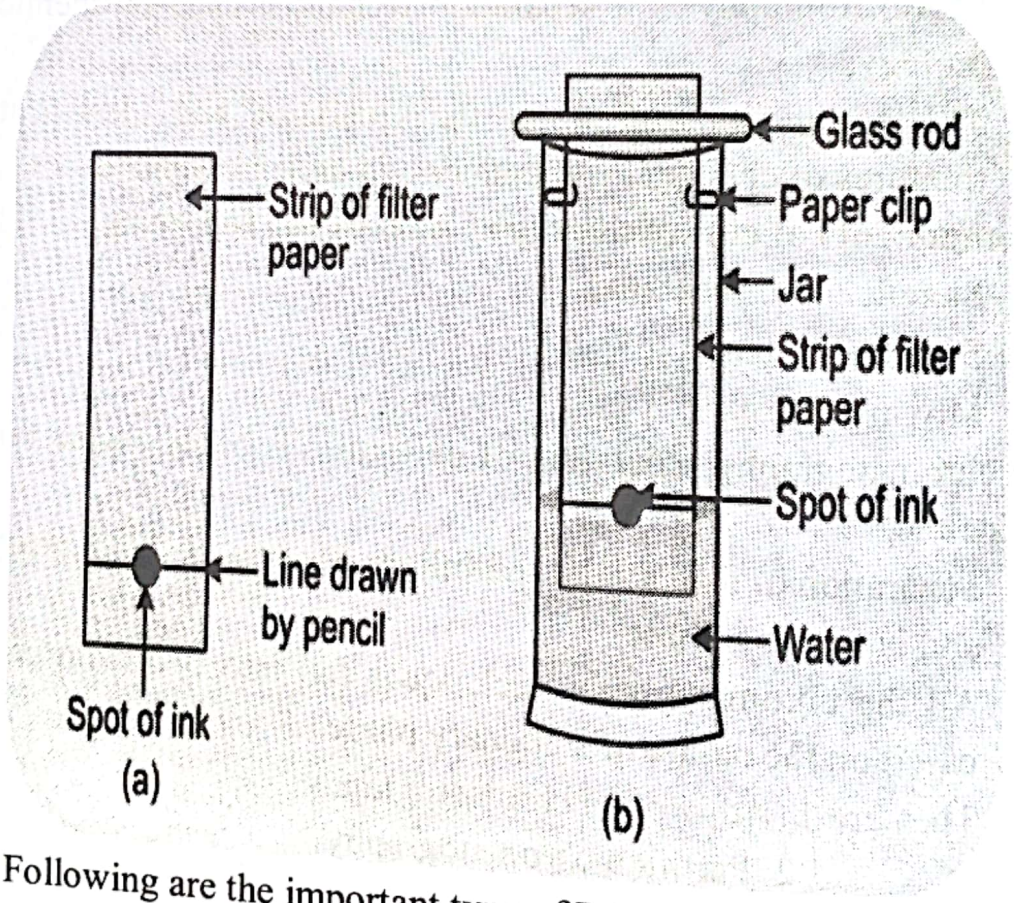
- In this type of chromatography the stationary phase is a solid such as alumina, silica gel and the mobile phase may be liquid or gas.

2. **Partition chromatography**

- In this chromatography the stationary phase is a liquid and mobile phase may be liquid or gas.

1. Paper chromatography (PC)

- This technique was introduced by Schreiber and Imzilov in 1938.
- It is a type of partition chromatography.
- In this technique a strip of paper is used to support the stationary water phase hence the name PC.
- The mobile phase is also a liquid.
- Separation is based on liquid-liquid partition of the compounds.



- Following are the important type of PC.

1. Ascending PC

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2. Descending PC
3. Radial PC

Ascending paper chromatography

- In this technique following steps are involved.
- Take whattman no. 1 filter paper of the size 2X10 cm and draw a line 3 cm from one end.
- Place a very small spot of mixture to be separated at the centre of the line.
- Solvent system (moving phase) is prepared and taken in the chromatography chamber.
- Suspend the paper strip in a trough in such a way that the paper edge is just below the solvent level and the pencil line should be above the solvent.
- The paper should not touch the sides and it should not be slanted.
- Keep this setup undisturbed for sometime till the solvent rises and reaches the top.
- Use the formula and calculate the Rf.

$$R_f = \frac{\text{Distance travelled by the sample}}{\text{Distance travelled by the solvent}}$$

- Each spot can be identified by comparing the Rf value with the standard Rf value.
- Most of the compounds are colorless and a location reagent is applied by spraying on the paper to detect the spots.

2. Thin layer chromatography

- This is a recent modification of column and paper chromatography.
- In this technique a thin uniform film of adsorbent (silica or alumina) containing a binder ($CaSO_4$) is spread onto a plate.
- The thin layer is allowed to dry at room temperature and is then activated by heating in an oven between 100-250 temperatures.

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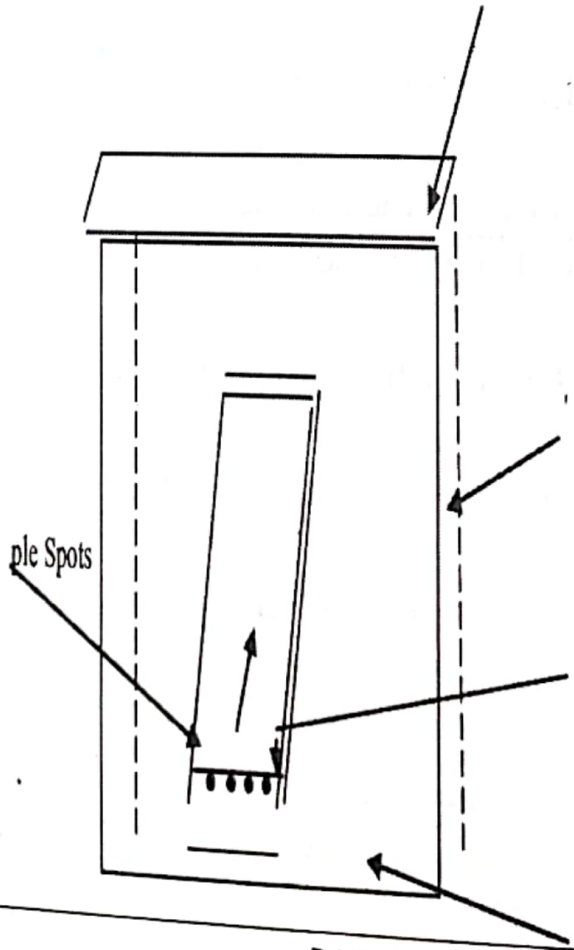
- The activated plate is then placed flat and samples are spotted with the micropipette carefully on the surface of thin layer.
- After the sample placement, the plate is placed vertically in glass tank containing a suitable solvent.
- With in the short time various components get separated by the solvent rising through the thin layer.
- The glass plate is then taken out from the tank allowed to dry and the spots are detected by spraying the plate with a variety of reagents.

The superiority of TLC over PC lies in the following facts.

- PC require long duration for separation where as TLC requires short duration for separation.
- TLC is extremely sensitive which require only small amounts of samples.
- TLC efficiency is more than PC.



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3. Column chromatography

- All the major types of chromatography are routinely carried out using the columns.
- Long columns (20X1 cm) give good resolution of components.
- Wider columns are preferred for large quantities of samples.
- An insoluble material is packed on the column and the molecules to be separated are added to the top of the column.
- The migration of the molecules is started by adding a solvent.
- The separation depends on the choice of the solvent and carrier material.
- This technique is important for the separation of enzymes such as cytochromes.

4. Ion exchange chromatography

- Ion exchange is a process in which ions of insoluble substances exchange ions in a surrounding solution.
- The insoluble substances are called as ion exchangers.
- Proteins, resins, cellulose, bone, living cell and silicate minerals are good examples for ion exchangers.
- Ion exchange acts as either cationic or anionic exchangers.
- The biological molecules exist in electrically charged forms under different conditions and these are made use of in their separation by ion exchange chromatography.
- Aminoacids may have negative or positive charge and can be separated by using ion exchange resins in ion exchange chromatography.

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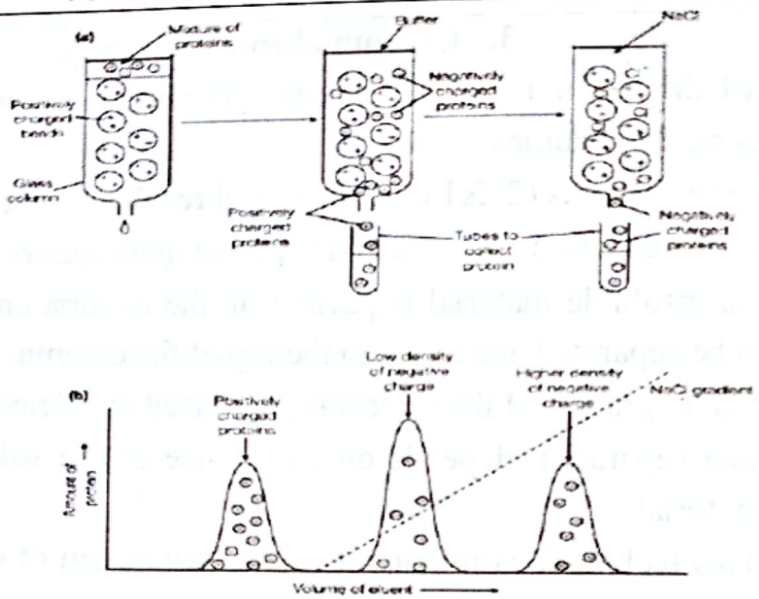

5. Affinity chromatography

- This is a type of adsorption chromatography.
- There is a high degree of specificity in the interaction between the adsorbent and the compound to be separated.
- In this technique for separation of antibodies we have to use antigens and vice versa.
- For the separation of enzymes we have to use substrates and vice versa.
- This technique is highly specific and rapid separation is achieved.

6. Gel permeation chromatography

- This technique is also called as gel filtration chromatography or molecular sieving.
- Molecules are also separated on the basis of differences in their size and weight by passing them down into a column containing swollen particles of gel.
- Small molecules can enter the gel but larger molecules are excluded from the cross linked network.
- The smaller molecules diffuse into the gel and follow a longer path than the larger molecules and leave the column first.

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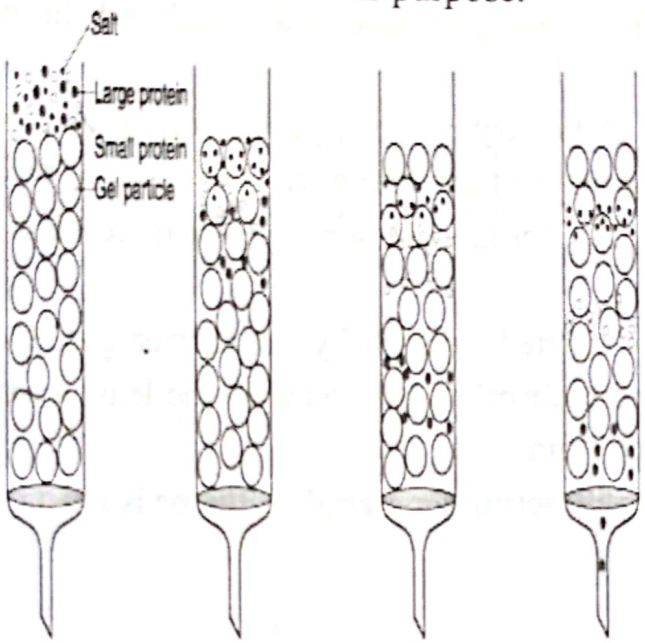
- Various gels are available for this purpose.



7. Gas chromatography

- Gas chromatography is a separation technique involving passage of gas through a column containing fixed phase.
- Gas chromatography is of two types.

1. Gas solid chromatography
2. Gas liquid chromatography

Gas solid chromatography (GSC)

- In GSC the stationary phase is a solid and the mobile phase is a gas.
- The solid stationary phase is packed in a column and an inert gas like nitrogen is passed through as carrier.
- The mixture of gases to be separated is injected into the one end of the column.
- As they are carried through by the carrier gas, they get separated, released and reabsorbed the gas with the least absorptivity emerges out of the column.
- To detect the emerging material, detector is used.

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Gas liquid chromatography (GLC)

- In GLC the stationary phase is a liquid and the mobile phase is a gas.
- The liquid stationary phase is packed on an inert solid support in a column and an inert gas like nitrogen is passed through as carrier.
- The mixture of gases to be separated is injected into the one end of the column.
- As they are carried through by the carrier gas, they get separated; released and reabsorbed the gas with the least adsorptivity emerges out of the column.
- To detect the emerging material, detector is used.

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ELECTROPHORESIS

- Electrophoresis is the migration of charged particles or molecules in a medium under the influence of an applied electric field.
- Electrophoresis is based upon the size, shape and molecular weight of the molecules. Most of the biological molecules like carbohydrates, proteins, nucleic acids etc. are separated by this technique.
- Consider a molecule is placed in an electric field. The force acting upon the molecules (F) is given as

$$F = \frac{\Delta E}{d} q$$

Where ,

$\frac{\Delta E}{d}$ = Field strength applied

q = charge of the molecule.

- Since the particle is accelerating in the solution, a force called frictional force (f) acts upon the molecule in opposite direction. It is given by stokes equation.

$$F = 6\pi rv$$

Where,

y= viscosity

r = radius of the molecule

v = velocity of the molecule

- Equating the accelerating force with the equation we get, i.e., F = f

$$\frac{\Delta E}{d} q = 6\pi yrv$$

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- Thus it can be said that the velocity of the molecule is directly proportional to the field strength, charge inversely proportional to the viscosity and radius

Factors affecting electrophoretic mobility

- Factors affecting electrophoretic mobility are
- Sample
- Electric field
- Buffer
- Medium

1. **Sample:** The charge, size and shape of the molecule of the sample dictates the electrophoretic mobility.
   a. Charge: The higher is the charge the greater is the electrophoretic mobility and vice versa. The charge depends upon the pH of the medium.
   b. Size: The bigger molecules have greater frictional forces and electrostatic forces acting upon them by the medium of suspension thus they have smaller electrophoretic mobility when compared to smaller particles.
   c. Shape: Less frictional and electrostatic forces act on spherical molecules when compared to other molecules. Thus globular proteins move faster than fibrous proteins.
2. **Electric field:** Increased potential gradient increases the rate of migration of ions.
   For example, when 100 mv of electric field is applied, the ions migrate faster than at 50 mv.
3. **Buffer:** Buffer helps in maintaining constant pH of the supporting medium. The commonly used buffers are EDTA, formate, citrate etc. Generally 0.05 to 0.1 m ionic strength of buffer is used.
4. **Medium:** The supporting medium can exert adsorption and molecular effects on the particle thus affecting the migration of ions.

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a. **Adsorption:** Adsorption means retention of a component on the surface of supporting medium. Thus the component does not form a sharp band but it contains a tail. The rate of migration is reduced.

b. **Molecular sieving:** Supporting media such as polyacrylamide, agar, sephadex etc. have cross linked structure which is used for the separation of the molecules so that large size molecules cannot enter the pores thus they migrate fast. But in case of polyacrylamide and agar , larger molecules are made squeeze through the process. The smaller molecules migrate faster than the larger through re process. Thus molecules affect the rate of migration.

Types of electrophoresis

- Electrophoresis is of two types.

1. Free electrophoresis: It is a type of electrophoresis without stabilizing media.
2. Zone electrophoresis: It is a type of electrophoresis with stabilizing media.

1. Free electrophoresis: Free electrophoresis has two main techniques.
   a. Micro electrophoresis
   b. Moving boundary electrophoresis

a. Micro electrophoresis

- Micro electrophoresis technique involves the observation of motion of small particles in an electric field with a microscope.
- Any microscope with a graduated fine foreseeing adjustment and an ocular micrometer may be used in conjunction with fast electrophoretic cells.

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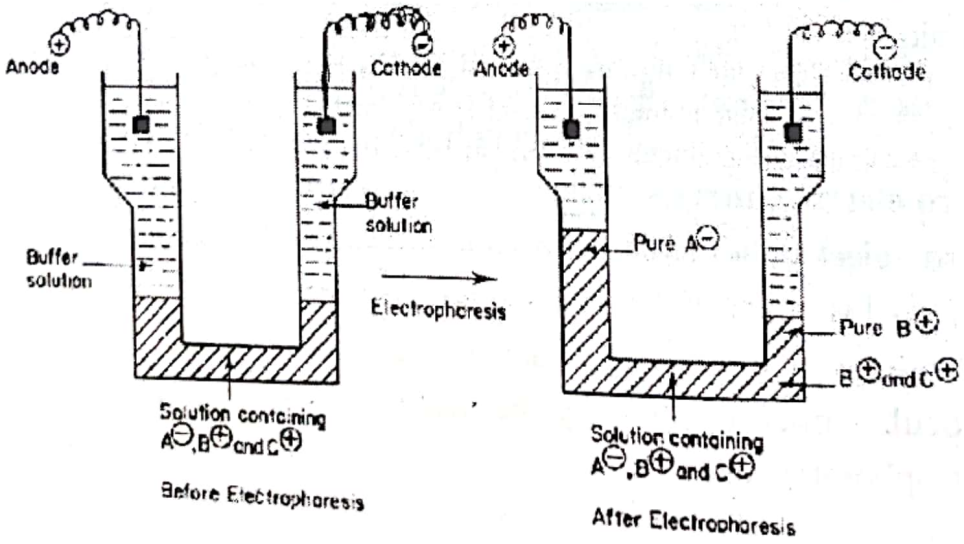
- The ocular mobilizing server for the measurement of the electrophoresis is mobilizing the microscopic particles in conjunction with a stop watch.
- This technique is applied only for measuring the zeta potential of cells, such as RBC's, neutrophils, bacteria etc.

b. Moving boundary electrophoresis

- This is the prototype of all modern methods of electrophoresis and was first developed by Tiseluis.
- It consists of a U shaped tube filled with buffer solution in which the sample is mixed.
- The ends of the tube consists of two electrodes, when electric field is applied the negative charged ions or anions will move.

Types of electrophoresis- Moving boundary Electrophoresis



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2. Zone electrophoresis

- Zone electrophoresis is of following types.
  1. Paper electrophoresis
  2. Gel electrophoresis
- It is classified based on the type of stabilizing media.

Apparatus: The equipment required for zone electrophoresis consists of basically two items.

a. Power pack
b. Electrophoretic cell

a. Power pack: It provides a stabilized direct current and controls both voltage and current output. Power packs of 0 – 500v and 0 – 150mA are available.

b. Electrophoretic cell: The electrophoretic cell generally contains electrodes and buffer reservoir. But in paper electrophoresis as the stabilizing media is filter paper, in addition to electrodes and buffer reservoirs, supports for paper, transparent insulating cover is present. The electrodes are made up of platinum.

In gel electrophoresis, the stabilizing media is gel. In order to support the gel two glass plates are present in addition to electrodes and buffer reservoirs in the electrophoresis cell.

1. Paper electrophoresis

- In this type paper is the stabilizing media.
- The paper is kept between the two buffer reservoirs.
- The sample application is important. It can be applied as spot or as a narrow uniform streak. When the sample is applied the current is switched on. The sample gets separated within two hours.
- In horizontal electrophoresis, the paper is kept horizontally between the buffers reservoirs.
- In vertical paper electrophoresis, the paper is placed vertically between the two buffers reservoirs. One buffer reservoir is at the top of the electrophoresis cell and other at the bottom of the cell.

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2. **Gel electrophoresis**

- In this type of electrophoresis, stabilizing media is gel.
- Based on the type of the gel used, it is of two types.

a. Agarose gel electrophoresis
b. SDS – PAGE (Sodium Dodecyl sulphates – Polyacrylamide gel electrophoresis.

APPLICATIONS

- Electrophoresis is used in analysis of PCR products, e.g. in molecular genetic diagnosis or genetic fingerprinting
- It is used in the separation of DNA fragments for DNA fingerprinting to investigate crime scenes. To analyze results of polymerase chain reaction.
- It is used to analyze genes associated with a particular illness.
- It is used in DNA profiling for taxonomy studies to distinguish different species.
- The application of electrophoresis in the food industry is to safeguard the quality of food products.

- It is used for separation of DNA fragments for extraction and purification.
- Electrophoresis is also incredibly useful in the determination of the strength of the antibiotic.
- Muscle protein (Myosin), egg protein (albumin), milk protein (casein), snake and insect venoms have been analysed using paper electrophoresis.

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SYNOPIS *UNIT - II*

2.1 SPECTROPHOTOMETRY

- *Absorption or emission of electromagnetic radiation as function of wavelength.*
- *works on the principle of laws of absorption which include*

3. *Beer's law*
4. *Lambert's law*

1. ***Lambert's law*** *states "the amount of light absorbed is proportional to the thickness of the absorbing material".*

$$I_o/I \propto b \quad — \quad \text{equation 1}$$

- ***Beer's law*** *states that "the amount of light absorbed by the material is proportional to concentration".*

$$I_o/I \propto c \quad — \quad \text{equation 2}$$

- *If we combine the above two equation we get the following*

$$I_o/I \propto bc$$

$$I_o/I = abc$$

This equation is called Beer – Lambert law.

- *Instrumentation* ***of spectrophotometer***
- *components of spectrophotometer include*

7. *Radiant energy source*

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8. *Monochromators*
9. *Sample holder*
10. *Detector*
11. *Amplifier and Recorder*

a. CHROMATOGRAPHY

- *Biochemical technique introduced by Michael Tswett in 1906.*
- *Technique of separation of substances according to their solubilities in two immiscible phases.*
- *Components of chromatography include the following*
    1. *Sample to be separated*
    2. *Stationary phase*
    3. *Moving phase*
- ***Stationary phase** remains stationary while the other phase moves.*
- ***Mobile phase** is the solvent that carries the mixture as it moves down the stationary phase.*
- *Two main categories based on the nature of stationary phase.*
- *They are 1. Adsorption chromatography*
  2. Partition chromatography

3. *Adsorption chromatography*

- *Stationary phase is a solid such as alumina, silica gel and the mobile phase may be liquid or gas.*

4. *Partition chromatography*

- *Stationary phase is a liquid and mobile phase may be liquid or gas.*

8. *Paper chromatography (PC)*

- *Type of partition chromatography.*
- *a strip of paper is used to support the stationary water phase*
- *The mobile phase is also a liquid.*

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- *Important type of PC.*
    4. *Ascending PC*
    5. *Descending PC*
    6. *Radial PC*
- ***Ascending paper chromatography***

$$Rf = \frac{\text{Distance travelled by the sample}}{\text{Distance travelled by the solvent}}$$

9. *Thin layer chromatography*
10. *Column chromatography*
11. *Ion exchange chromatography*
12. *Affinity chromatography*
13. *Gel permeation chromatography*
14. *Gas chromatography is of two types.*
    1. *Gas solid chromatography (GSC)*
    2. *Gas liquid chromatography (GLC)*

b. ELECTROPHORESIS

- *Migration of charged particles or molecules in a medium under the influence of an applied electric field.*
- *Is based upon the size, shape and molecular weight of the molecules.*
- ***WRITE EQUATION***
- *Factors affecting electrophoretic mobility are*

- *Sample*
- *Electric field*
- *Buffer*
- *Medium*

- ***Types of electrophoresis***

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Electrophoresis is of two types.

3. ***Free electrophoresis*** *is electrophoresis without stabilizing media.*
4. ***Zone electrophoresis*** *is electrophoresis with stabilizing media.*
3. *Free electrophoresis is of following types.*
    c. *Micro electrophoresis*
    d. *Moving boundary electrophoresis*
4. *Zone electrophoresis is of following types.*
    a. *Paper electrophoresis*
    b. *Gel electrophoresis*

- *The equipment required for zone electrophoresis consists of two items.*

c. *Power pack*
d. *Electrophoretic cell*

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UNIT III: ADVANCED TECNIQUES

3.1 IMMUNO ASSAY

- It is a biochemical test that measures the presence or concentration of a substance in a body fluid through the use antibody or immunoglobulin.

ELISA (ENZYME LINKED IMMUNOSORBENT ASSAY)

- It is an immunological assay.
- ELISA is used to detect antigens present in the patient's blood or serum.
- It was discovered by Engvall and Perlmann.

PRINCIPLE

- Enzyme linked antibody reacts with colorless substrate to produce colored product.
- Antigen + Antibody (enzyme linked) ⇨ Ag - Ab (enzyme linked)
- Ag – Ab (enzyme linked) +substrate ⇨ Ag –Ab (enzyme linked) substrate (coloured product)

PROCEDURE

- Take a polystyrene microtitre plate and add antigen coating sample. Antigen will be attached on the surface.
- Wash the microtitre plate with by using triphosphate buffer with PH 7.4 and detergent tween 20
- Add enzyme linked antibody to microtitre plate.
- Then wash the plate by using buffer solution.

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- Add the specified substrate which will react with enzyme linked antibody.
- Enzyme and substrate will react and forms a product which is blue in colour.
- Then sulphuric acid is added and the colour will be changed to yellow.
- This yellow colour substance is observed by spectrophotometry.

TYPES OF ELISA

- There are four types of ELISA.
  - Direct ELISA
  - Indirect ELISA
  - Sandwich ELISA
  - Competetive ELISA

Direct ELISA

- In direct ELISA, an antigen is directly placed on microtitre plate and enzyme linked antibody is added.
- Substrate is then added. This substrate changes color upon reaction with the enzyme.

Indirect ELISA

- Indirect ELISA detects the presence of an antibody in a sample.
- The antigen is attached to the wells of the microtitre plate.
- A sample containing the antibodies is added to the antigen-coated wells for binding with the antigen.
- The free primary antibodies are washed away and the antigen-antibody complex is detected by adding a secondary

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antibody conjugated with an enzyme that can bind with the primary antibody.

- All the free secondary antibodies are washed away. A specific substrate is added which gives a coloured product.
- The absorbance of the coloured product is measured by spectrophotometry.

Sandwich ELISA

- The sandwich ELISA uses two antibodies: a capture antibody and a detection antibody.
- It is called a sandwich because antigen is bound between antibodies.

Competitive ELISA

- Competitive ELISA helps to detect antigen concentration in a sample.
- The microtitre wells are coated with the antigen.
- Antibodies are incubated in a solution having the antigen.
- The solution of the antigen-antibody complex is added to the microtitre wells. The well is then washed to remove any unbound antibodies.
- More the concentration of antigen in the sample, lesser the free antibodies available to interact with the antigen, which is coated in the well.
- The enzyme-linked secondary antibody is added to detect the number of primary antibodies present in the well.
- The concentration is then determined by spectrophotometry.

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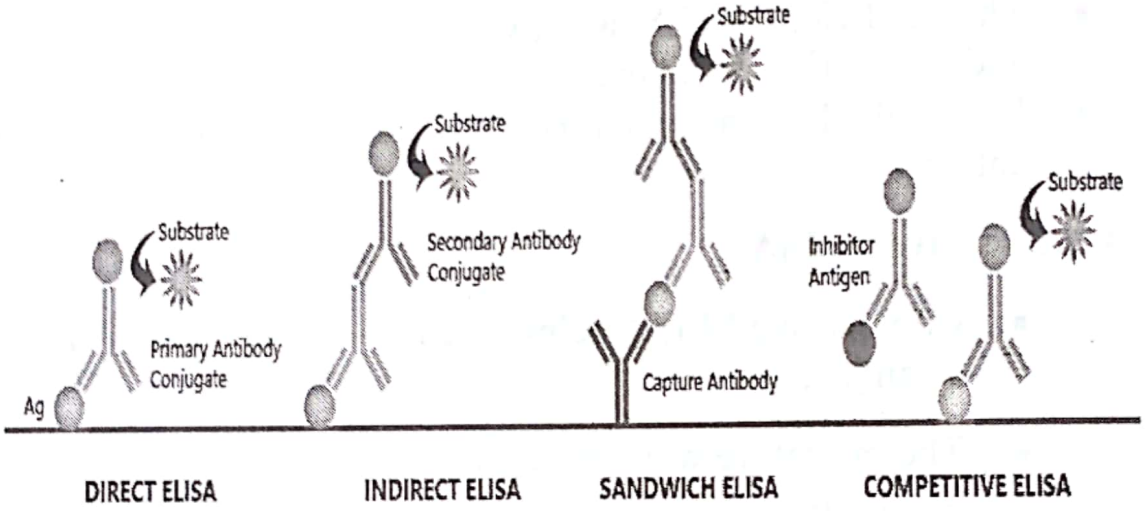
APPLICATIONS

- ELISA is used to determine serum antibody concentration.
- It is used to detect potential food allergins.
- It is used for tracking the spread of diseases such as HIV, Bird flu, common cold, cholera, STD etc.
- It is used in toxicology for screening of drugs.



3.2 POLYMERASE CHAIN REACTION

- Polymerase chain is the invitro amplification of DNA to produce multiple copies from the template DNA.
- In PCR, the reaction is repeatedly cycled through a series of temperature changes, which allow many copies of the target region to be produced.
- This technique was developed by Kary Mullis.

Requirements in polymerase chain reaction.

- Taq DNA polymerase is isolated from from the thermophilic bacterium, Thermus aquaticus (Taq).

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- DNA Primers: primers are short pieces of single-stranded DNA, usually around 20 nucleotides in length.
- template DNA and
- nucleotides

- The three steps to each amplification cycle include denaturation, annealing and extension.

Denaturation

- Firstly, the DNA is denatured by heating to 90-95 °C, which separtates double-stranded DNA (dsDNA) to single-stranded DNA.
- The temperature at which 50% of the dsDNA is denatured is known as the melting temperature (T_m).
- Each DNA strand act as templates for the production of new strands of DNA.

Annealing

- In this step, the sample is cooled to 40-60 °C, allowing the primers to attach to the target DNA.

Extension

- The temperature is then raised slightly to 70-75°C.
- During this stage, DNA polymerase extends the DNA from the primers, creating new dsDNA with one old strand and one new strand.
- This process of denaturation, annealing and extension is repeated 25-35 times to exponentially to replicate the target DNA of interest.
- This process takes 2 - 4 hours, depending on the length of the DNA region being copied.

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- This entire reaction used to be easily done in a programmed thermocycler.
- The results of a PCR reaction are usually visualized using gel electrophoresis.

Gel electrophoresis :

- It is a technique used to separate DNA fragments according to their size.
- DNA samples are loaded into wells at one end of a gel, and an electric current is applied to pull them through the gel.
- DNA fragments are negatively charged, so they move towards the positive electrode. Because all DNA fragments have the same amount of charge per mass, small fragments move through the gel faster than large ones.
- When a gel is stained with a DNA-binding dye, the DNA fragments can be seen as bands, each representing a group of same-sized DNA fragments.

APPLICATIONS OF PCR

- PCR has been used in the detection and quantification of anaerobic bacteria.
- PCR is used to quantify the HIV in blood.
- PCR is used to study the impact of antiviral drugs and also to screen donor blood samples for HIV.
- PCR is used to analyze sickle cell anemia.

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- The most widely used application of PCR in forensic labs is the amplification of short tandem repeat (STR) loci used in DNA typing.
- PCR can be used as a tool in genetic fingerprinting.
- PCR is now commonly used for a wide variety of applications including genotyping, cloning, mutation detection, sequencing, microarrays, forensics, and paternity testing.
- PCR is used for the sensitive detection of pathogenic microorganisms.
- It is used for analysis of DNA of arachaeological specimens.

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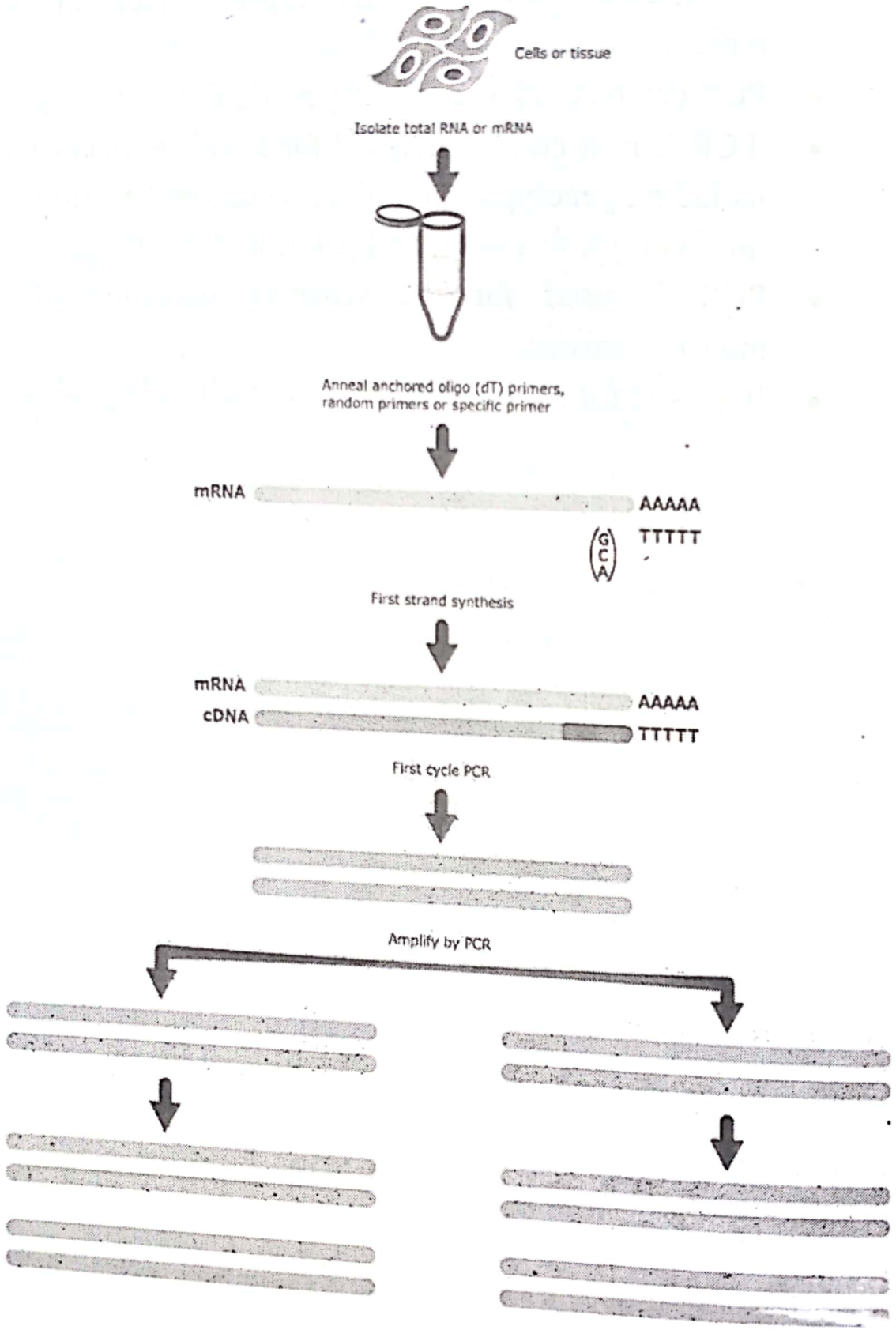
RT - PCR



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3.3 RADIOIMMUNO ASSAY (RIA)

- Radioimmuno assay (RIA) is used to determine the concentration of antigen present in blood or serum of the patient.
- **PRINCIPLE**
- It is based on competitive binding between radiolabelled antigen (hot antigen) and unlabelled antigen (cold antigen) with selected antibody and ultimately radioactivity is determined.
- Radioactive materials which are used for radiolabelling in radio immuno assay are iodine 125, tritium, carbon 14
- The two methods used in radioimmuno assay are
  1. Qualitative method and
  2. Quantitative method.

QUALITATIVE METHOD

- Take a micro titre plate with pre coated antibody.
- Then add radiolabelled antigen.(hot antigen)
- The hot antigen will covalently bind with precoated antibody.
- Remove the unbound antigen by washing with buffer solution.
- The buffer solutions used are buffer A i.e., trifluoraacetic acid and buffer B i.e., 60% acetonitrile , 1% TFA and 39% distilled water.
- Now add the patient sample which contain cold antigen.(unlabelled antigen from patient serum)
- This cold antigen will compete with radiolabelled antigen and replace them.
- This replacement of cold antigen releases hot antigen away from antibody.
- Then the microtitre plate is washed with buffer solution to remove unbound antigen.
- Then the washed solution is centrifuged and supernatant liquid will be analyzed for radioactivity in radioactive counters.

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- The radioactive counters used are
  1. Gamma counters.
  2. Scintillation counters.

QUANTITATIVE METHOD

- Take a microtitre plate containing antibody.
- Add standard cold antigen solution.
- Wash the microtitre plate with buffer solution.
- Then the washed solution is centrifuged and supernatant liquid will be analyzed for radioactivity.

ADVANTAGES

- Radioimmunoassay has become a major tool in clinical laboratory.
- It is used to assay
  1. Plasma levels of hormones
  2. Drugs like digitoxin
- It is used to assay infectious diseases like HBs Ag, HIV, TORCH.
- It is used for measuring toxins in contaminated food.
- Radioimmunoassay is widely used because of its great sensitivity.
- Using antibodies of high affinity, it is possible to detect a picograms of antigen in the tube.
- The greater the specificity of the antiserum, the greater the specificity of assay.

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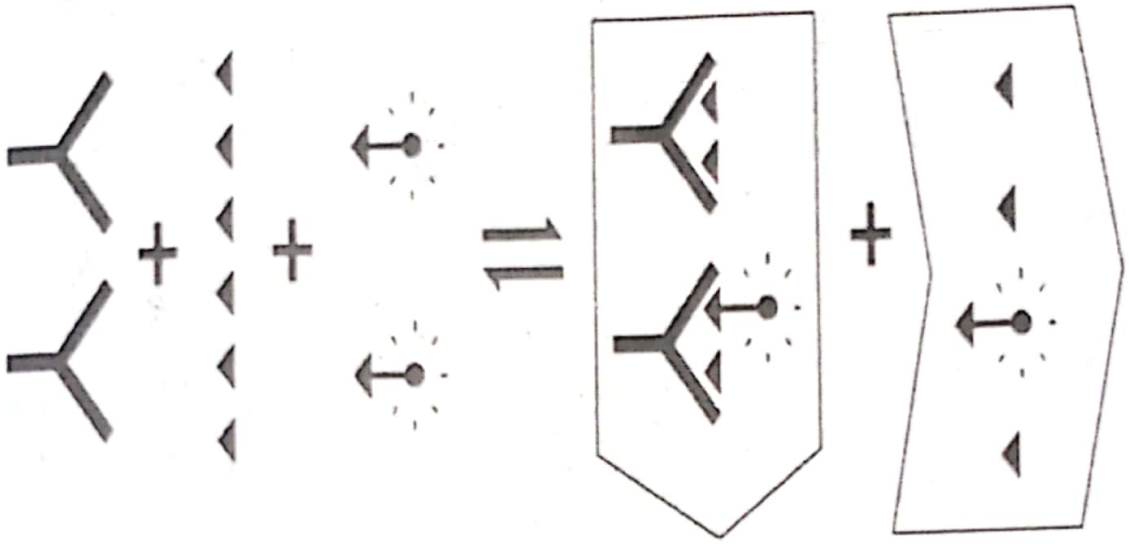

Antibody | Analyte in sample | Labeled analyte (tracer) | Separation of antibody-bound and free fractions e.g. by precipitation of antibody, centrifugation and decantation of free fraction

SYNOPSIS UNIT III

3.1 IMMUNOASSAY

- *Biochemical test that measures the concentration of a substance in a body fluid through the use antibody*

ELISA (ENZYME LINKED IMMUNOSORBENT ASSAY)

- *It is an immunological assay.*
- *Used to detect antigens present in the patient's blood or serum.*
- *Principle: Enzyme linked antibody reacts with colourless substrate to produce coloured product.*

TYPES OF ELISA

- *There are four types of ELISA.*
  1. *Direct ELISA*
  2. *Indirect ELISA*

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3. *Sandwich ELISA*
4. *Competetive ELISA*

3.2 PCR TECHNIQUES

- *Invitro amplification of DNA to produce multiple copies from the template DNA.*
- *The reaction is repeatedly cycled through a series of temperature changes, which allow many copies of the target region to be produced.*
- *This technique was developed by Kary Mullis.*
- ***Requirements in polymerase chain reaction.***

    1. *Taq DNA polymerase is isolated from from the thermophilic bacterium, Thermus aquaticus (Taq).*
    2. *DNA Primers : primers are short pieces of single-stranded DNA, usually around 20 nucleotides in length.*
    3. *template DNA and*
    4. *nucleotides*

- *Three steps to each amplification cycle include denaturation, annealing and extension.*
- *This process takes 2 - 4 hours, depending on the length of the DNA region being copied.*
- *This entire reaction used to be easily done in a programmed thermocycler.*
- *The results of a PCR reaction are usually visualized using gel electrophoresis.*

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3.3 RADIO IMMUNOASSAY

- *Used to determine the concentration of antigen present in blood or serum of the patient.*
- *It is based on competitive binding between radiolabelled antigen and unlabelled antigen with selected antibody and ultimately radioactivity is determined.*
- *Radio active materials used for radiolabelling are iodine 125, tritium, carbon 14*
- *Two methods used in radioimmuno assay*
  1. *Qualitative method*
  2. *Quantitative method.*

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UNIT IV: STATISTICAL TOOLS

Introduction to Biostatistics

- Biostatistics refers to the subject of scientific activity
- Modern statistical methods are being found increasingly useful in research in different fields Statistics refers to the theories and methods of **collection processing presentation analysis** and **interpretation of data.**
- The term Biostatistics is used when tools of statistics are applied to the data that is related to Biological Sciences
- Biostatistics can be defined as the Science dealing with the **C**ollection, **P**rocessing **P**resentation, **A**nalysis and **I**nterpretation of **Biological data** (**CPPAI** OF BIOLOGICAL DATA)
- Modern biological investigations how become quantitative in which biological observations of numerical facts called data.
- As the biological observations are measured OR counted it become clear that the object to methods are necessary to help Biologist in presenting and verifying the research data
- The statistical methods apply to biological problems is called as Biostatistics.
- It is also called as Biometry or Biological Measurement.
- Bio statistics is not only useful but necessary hence it can be rightly remarked **"Science without Statistics bear no fruits, Statistics without Science have no roots"**

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4.1 DATA

- The values recorded in an experiment or observations are called data.
- Data is raw, unorganized facts that need to be processed.
- When data is processed, organized, structured or presented in a given context so as to make it useful, it is called information.

TYPES OF DATA

- The data is of two types. 1.Primary data
  2. Secondary data

1. Primary data

- The data collected by an investigator is called primary data.
- It is the first hand information.
- The person collecting the data is called investigator.
- Primary data gives first hand information.
- It is original in nature.
- It is accurate and reliable.
- It consumes more money and time.
- The amount of oxygen estimated by a student by titration is a primary data.
- The rainfall recorded by PWD department is a primary data.

Merits of primary data

- It is first hand information.
- It is original.
- It is accurate and reliable.

Demerits of primary data

- It consumes more money.
- It requires more time.
- It requires more labour.

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- The investigator must be well trained.
- Personal bias may creep in.

2. Secondary data

- The data obtained from already collected data is called secondary data.
- Eg. Data collected from newspaper, journals.
- They are secondary in nature.
- They are economical.
- It is not reliable.
- The amount of oxygen estimated by a student by titration is a primary data.
- When this data is used by a teacher, it becomes secondary data.
- The rainfall data obtained from a PWD department is a secondary data.

Merits of secondary data

- They are obtained at minimum cost.
- They require less labour.
- They are quickly obtained.

Demerits of secondary data

- They are not reliable.
- Errors may be there.
- The method of collection is not known.
- They are secondary in character.

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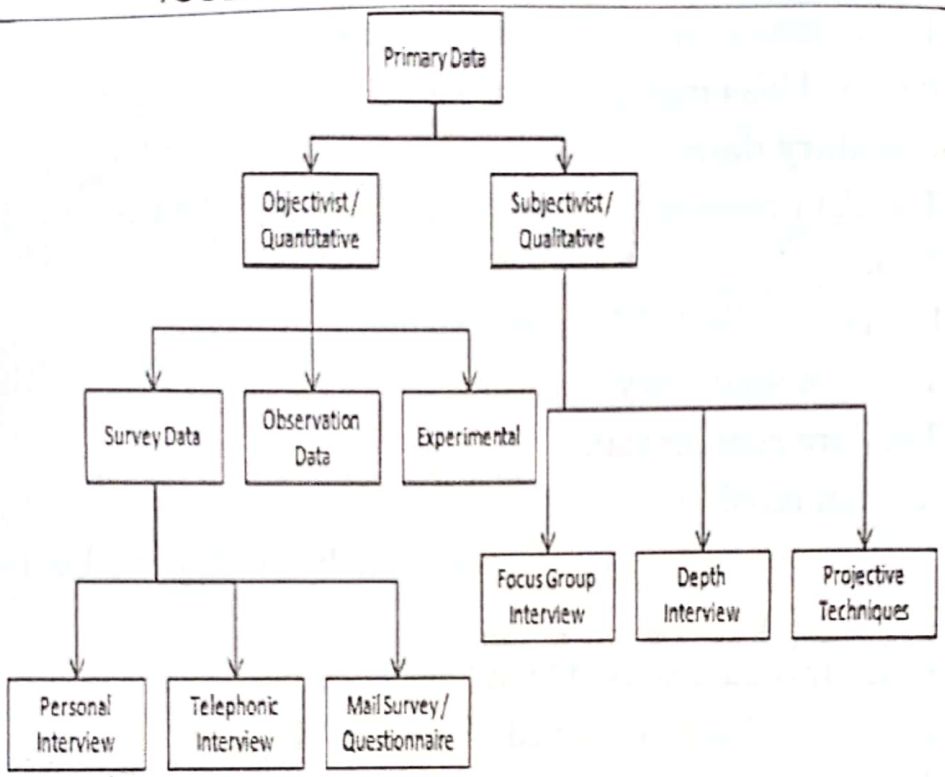

CONCEPTS OF VARIABLES

- Variables are things you measure, manipulate and control in statistics and research.
- A variable may also be called a data item.
- Researchers and statisticians use variables to describe and measure the items, places, people or ideas they are studying.
- Age, sex, business income and expenses, country of birth, capital expenditure, class grades, eye color are examples of variables.
- A strong understanding of variables can lead to more accurate statistical analyses and results.

TYPES OF VARIABLES

- There are different types of variables according to the ways they can be studied, measured, and presented.

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- They are numerical variables, categorical variables, independent variables and dependent variables.

1. Numerical variables

- Numerical variables have values that describe a measurable quantity as a number.
- Examples include height, distance or number of items. Therefore numeric al variables are quantitative variables.
- Numerical variables may be further categorized as continuous or discrete.
- **Continuous**: Continuous variable is a variable whose value is obtained by measuring.
- Examples are height of students in class, weight of students in class
- **Discrete**: Discrete variable is a variable whose value is obtained by counting.
- Examples are number of students present, students grades.

2. Categorical variables

- Categorical variables have values that describe a quality or characteristic of a data unit.
- In categorical, variables are non-numerical values.
- Categorical variables are qualitative variables.
- Categorical variables may be further categorized as ordinal or nominal
- **Ordinal** : Observations can take a value that can be logically ordered or ranked.
- Examples of ordinal categorical variables include academic grades , clothing size and attitudes).
- **Nominal variable** : Observations can take a value that is not able to be organized in a logical sequence. Examples of nominal

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categorical variables include sex, business type, eye color, religion and brand.

- The data collected for a categorical variable are qualitative data.

3. Independent variables

- Independent variables are the ones that you include in the model to explain or predict changes in the dependent variable.
- The name helps you understand their role in statistical analysis. These variables are independent.
- Independent indicates that they stand alone and other variables in the model do not influence them.
- The researchers are not seeking to understand what causes the independent variables to change.
- Example : Your test score affects how long you sleep.
- Independent variables are also known as predictors, factors, treatment variables, explanatory variables, input variables, x-variables, and right-hand variables.

4. Dependent variables

- The dependent variable is what you want to use the model to explain or predict. The values of this variable depend on other variables. It is the outcome that you're studying. It is also known as the response variable or outcome variable.
- A grade on an exam is an example of a dependent variable because it depends on factors such as how much sleep you got and how long you studied.
- Independent variables can influence dependent variables, but dependent variables cannot influence independent variables.

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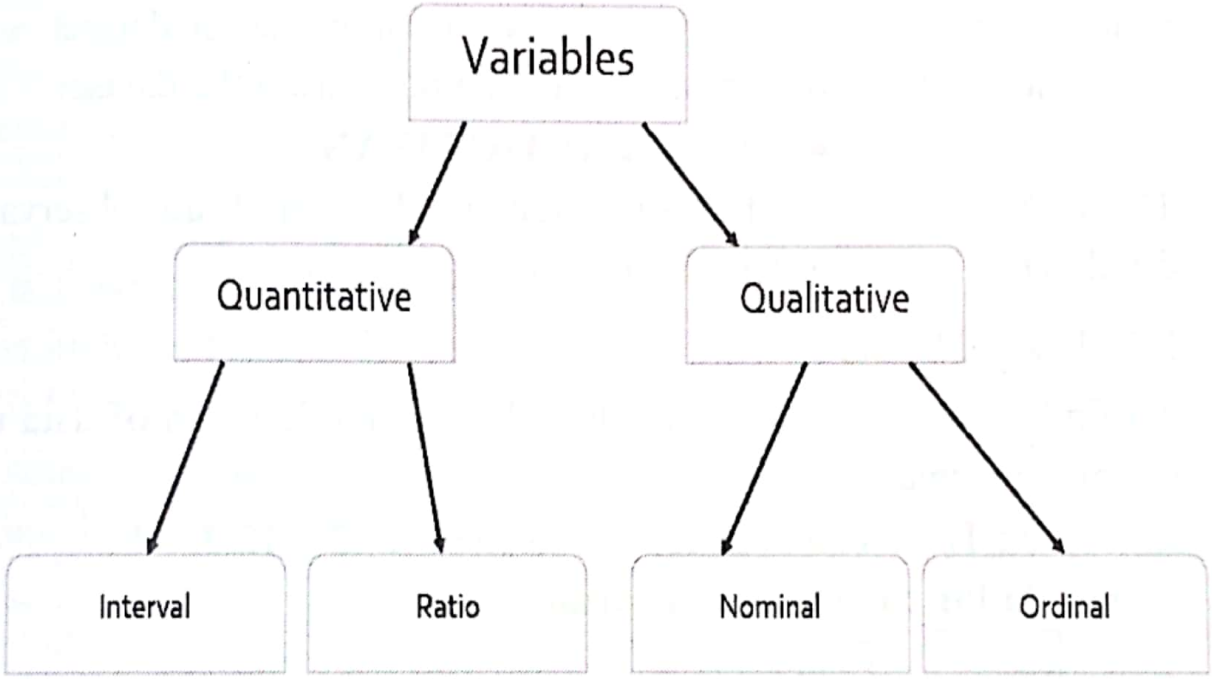
- For example, the time you spent studying (dependent) can affect the grade on your test (independent) but the grade on your test does not affect the time you spent studying.



SUMMARISING DATA: AVERAGES

- The term data summarization refers to presenting the summary of generated data in an easily comprehensible and informative manner.
- The word average denotes a representative of a whole set of observations.
- Average is a single figure which describes the entire series of observations with their varying sizes.
- Average is a central value of series.
- Average is the central part of the distribution and therefore they are also called the measure of central tendency.
- **Measures of central tendency** are grouped into two types. They are
  1. Mathematical averages
  2. Positional averages

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Mathematical averages include 1. Arithmetic Mean

Positional averages include 2. Median

3. Mode

- Mean, median, and mode are different measures of central tendency in a numerical data set. They each try to summarize a dataset with a single number to represent a typical data point from the dataset.

• ARITHMETIC MEAN

- The arithmetic mean of a given data is the sum of all observations divided by the number of observations.
- It is denoted by $\bar{X}$.
- To find his average , we calculate the arithmetic mean of data using the mean formula:
- Example : The mean of 5, 1, and 6 is (5+1+6)/3 = 12/3 = 4
- **Formula for calculation of Mean**
- **Mean** $\overline{X} = A \pm \frac{\sum fd}{N} xC$
- **Where** A= Assumed Mean
- f= frequency

  N- Sum of frequencies

  d=deviation

  C= height of class interval

Merits of Mean

- It is the one central value, most commonly used in biology
- It is easy to understand
- It is easy to calculate.
- It is easy for further calculations.
- It is not affected by sampling fluctuations.
- Mean value is dependent on every item in the series.
- It gives overall trend of the series. It describes the centre of a series.

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- It is a measure of central tendency or concentration of all other observations around the central value.
- It helps to find which group is better off by comparing the average of one group with that of the other
- Mean can be used further in the test of significance.

Demerits of Mean

- Mean is not a positional average like median and mode.
- It is upward biased as it yields more importance to bigger values and less important to smaller values.
- It is highly influenced by every small and every big numbers.
- It sometimes may give absurd results.
- Sometimes mean may give false conclusions.
- It sometimes gives ridiculous results.

Uses of Mean

- It is used in practical statistics.
- Estimates are always obtained by using mean Common people uses mean for calculating average marks obtained by students.
- It is commonly used in biology research

• MEDIAN

- The value of the middlemost observation, obtained after arranging the data in ascending or descending order, is called the median of the data.

- For example, consider the data: 4, 4, 6, 3, 2.

- Let's arrange this data in ascending order: 2, 3, 4, 4, 6.

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- There are 5 observations. Thus, median = middle value i.e. 4.

Ungrouped data

- Arrange the data in ascending or descending order.
- Let the total number of observations be n.
- To find the median, we need to consider if n is even or odd. If n is odd number, then use the formula:

Median = $(n + 1)/2^{nd}$ observation

- Example 1: Let's consider the data: 56, 67, 54, 34, 78, 43, 23.
- Arranging in ascending order, we get: 23, 34, 43, 54, 56, 67, 78. Here, n (number of observations) = 7

So, $(7 + 1)/2 = 4$

Therefore Median = 4^{th} observation

Median = 54

- If n is even number, then use the formula :

Median = [$(n/2)^{nd}$ observation+ $((n/2) + 1)^{nd}$ observation]/2

- Let's consider the data: 50, 67, 24, 34, 78, 43. What is the median?

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- Arranging in ascending order, we get 24, 34, 43, 50, 67, 78.
- Here, n (no.of observations) = 6

  6/2 = 3

  Using the median formula, Median = (3^{rd} obs. + 4^{th} obs.)/2= (43 + 50)/2 Median = 46.5

Formula for calculation of Median

$$Md = l + \frac{h}{d}(N/2 - C)$$

Where l = lower limit of median class h=height of class interval

f= frequency of median class

N= Sum of frequencies

C= cumulative frequency of class preceeding median class

Merits of median

- It is simple to calculate
- It is easy to understand.
- It is regarded as an inspectional value
- Its values are not affected by extreme values.

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- Value if median may be further used in statistical calculations
- Its value generally lies in the distribution.

Demerits of Median

- Median value is affected by sampling fluctuations.
- It is not a familiar average like mean.
- Since median is a positional average, its values are influenced by each and every observation.
- Calculations of median become tedious in the large ungrouped data.
- When all the observations of a variable are arranged in either ascending or descending order, the middle observation is known as Median.
- Actually median is better indicator of an average than me when one or more of the lowest or the highest observations are wide apart or not so evenly distributed.

MODE

- The mode is the value that appears most often in a set of data values.
- The observation with the highest frequency is called a mode of data.

Ungrouped Data

- For ungrouped data, we need to identify the observation which occurs maximum times.

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"Mode = Observation with maximum frequency"

- For example in the data : 6, 8, 9, 3, 4, 6, 7, 6, 3, the value 6 appears the most number of times.
- Thus, mode = 6.
- A data may have no mode, 1 mode, or more than 1 mode.
- Depending upon the number of modes the data has, it can be called unimodal, bimodal, trimodal or multimodal.

Grouped Data

- When the data is continuous, the mode can be found using the following steps:
- Find modal class i.e. the class with maximum frequency.
- Find mode using the following formula:
- $$\boxed{M_0 = l + \frac{h(f_1 - f_0)}{2f_1 - f_0 - f_2}}$$

Where,

- l = lower limit of modal class,
- f_1 = frequency of modal class,
- f_0 = frequency of class preceding modal class,
- f_2 = frequency of class succeeding modal class,
- h = height of class interval

Merits of Mode

- It can be obtained by inspection.
- It is not affected by extreme values. This average can be calculated from open end classes.
- It can be easily understood.
- It can be used to describe qualitative phenomenon.

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- Its value can also found graphically.

Demerits of Mode

- Mode has no significance unless a large number of observations are available.
- It cannot be treated algebraically.
- It is a peculiar measure of central tendency.
- For the calculation of mode the data must be arranged in the form of frequency distribution.
- It is not rigidly defined.

MEASURES OF DISPERSION OR MEASURES OF DEVIATION

- Measure of dispersion gives you an idea about the variation of the variable from a central value.
- It is useful for the determination of reliability of an average.
- It will be useful to compare two or more series with regard to the variability.
- Measure of dispersion of various observations can be studied by using

1. Range
2. Mean deviation
3. Quartile deviation
4. Standard deviation

RANGE

- Range is the difference between the lowest value and highest value of a set of data.

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Range = largest value – smallest value

$$R = L - S$$

- Range is a measure of dispersion. It gives an idea of the degree of variability in a set of values.
- The yield of a coconut tree on 5 occasions is 22, 8, 12, 5 and 13.
- The range is calculated as follows.

Largest value = 22

Smallest value = 5

Range = L – S

= 12 – 5

Range = 17

- When the range is divided by the sum of the extreme values, the resulting figure is called 'co-efficient of range'.
- Co-efficient of Range = large value – smallest value/largest value value +smallest value

$$\text{Coefficient of Range} = L - S/L + S$$

Merits of range

- It is easy to calculate.
- It helps in studying variation in a set of values.
- It helps to study the variability between data.
- It helps to study increase in price of commodities.

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Demerits of range

- It is crude method of calculation of range.
- It is based on only two extreme values of a data.
- If the extreme values are unusual, the range will be misleading.

Standard Deviation (S.D)

Introduction

- Karl Pearson introduced the concept of standard Deviation in 1893.
- It is the most important measure of dispersion and is widely used in Biology.
- It is also call as root mean square deviation or mean error or mean square error.
- It will give accurate results

Definition

- Standard deviation is defined as the square root of all the values from their mean.
- It is denoted by the Greek letter sigma.
- The formula for standard deviation is $\sim = h\sqrt{\frac{\sum fd^2}{N} - \left(\frac{\sum fd}{N}\right)^2}$

Where f= frequency
d=deviation
N= sum of frequencies
h=height of class interval

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- Steps involve in calculation of Standard Deviation value.

Step 1 : Find the fd

Step 2 : find fd^2.

Step 3 : Sum the fd and fd^2

Step 4 : substitute the values in the formula

Step 5 : Take the square root.

Uses of Standard Deviation

- It is a very good method of dispersion It is commonly used in Biological research
- It helps in calculation of Standard Error

Merits of Standard Deviation

- Standard Deviation helps to describe the normal curve i.e. the deviation of values from mean as a unit of Variation.
- Standard Deviation is based on all observations.
- It is the most important and widely used measures of dispersions.
- It is less affected by sampling fluctuations.
- It is possible for further algebraic treatment.
- It is useful for calculation of standard error.
- It also helps in calculating the sample size.
- It gives idea whether the observed difference of an individual from the mean is by Chance or is significant

Demerits of Standard Deviation

- It is difficult to understand.
- It is difficult to calculate.

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REPRESENTATION OF DATA

- Data representation is a method of analyzing numerical data is data representation.
- The given data is arranged in order, which may be ascending or descending order is called arrayed data.
- In a diagram, data representation depicts the relationship between facts, ideas, information and concepts.
- Presentation of data is the visual form of presenting the data.
- Presentation of data makes easy understanding.
- It facilitates comparision of data.
- Data is presented in two methods. They are Diagrammatic presentation & Graphic representation.
- In diagrammatic representation, data is represented in the form of diagrams such as circles, bars, maps ,cartograms.
- In the graphic representation data is represented in the form of graphs.

Diagramatic presentation

- One of the main functions of the statistics is to simplify complex data.
- A diagram is a drawing or a design to explain something.
- It is a visual form of presentation of statistical data.
- It is an acknowledged fact that visual aids are more useful and interesting than dry numbers.
- Diagram refers to the various types of device such as bars, circles, maps, cartograms, etc.
- An ordinary man can understand pictures and diagrams more easily than figures.

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- The use of diagrams is becoming more and more popular in the present time because they are more appealing, attractive and meaningful.

Significance and utility of a diagram

- Diagrams are more attractive.
- They create more effects on the minds of the readers.
- They simplify complexity.
- They save time and labour.
- They provide more information.
- They make comparison.

Limitation of a diagram

- A diagram shows only approximate values.
- It is a supplement to the tabular presentation but not an alternative to it.
- It cannot be analyzed further.
- It cannot represent all details.
- Small differences in large measurements cannot be studied. For example the difference between 1000 and 1005 shown in the diagram cannot be apparent.

Rules for drawing a diagram

- Each diagram should have a suitable title.
- A proper scale must be chosen for the diagram to make more attractive.
- Drawing should be neat and clean.
- Foot notes should be given at the bottom of the diagram.

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- Index must be given for identifying and understanding the diagram.
- The most important one is the selection of the proper diagram to represent the data.

Kinds of diagrams

- There are different types of diagrams by which statistical data can be presented. The common types are

1. Line diagram
2. Bar diagram
3. Pie diagram
4. Pictogram
5. Cartogram

1. LINE DIAGRAM

- A line diagram is a diagram where the statistical data are represented in the form of straight line.
- This is the simplest of all the diagrams.
- On the basis of the size of the figures, the heights of the lines are drawn.
- The distance between the lines is kept uniform.
- It makes comparision easy.
- This line diagram is not attractive so it is less important.

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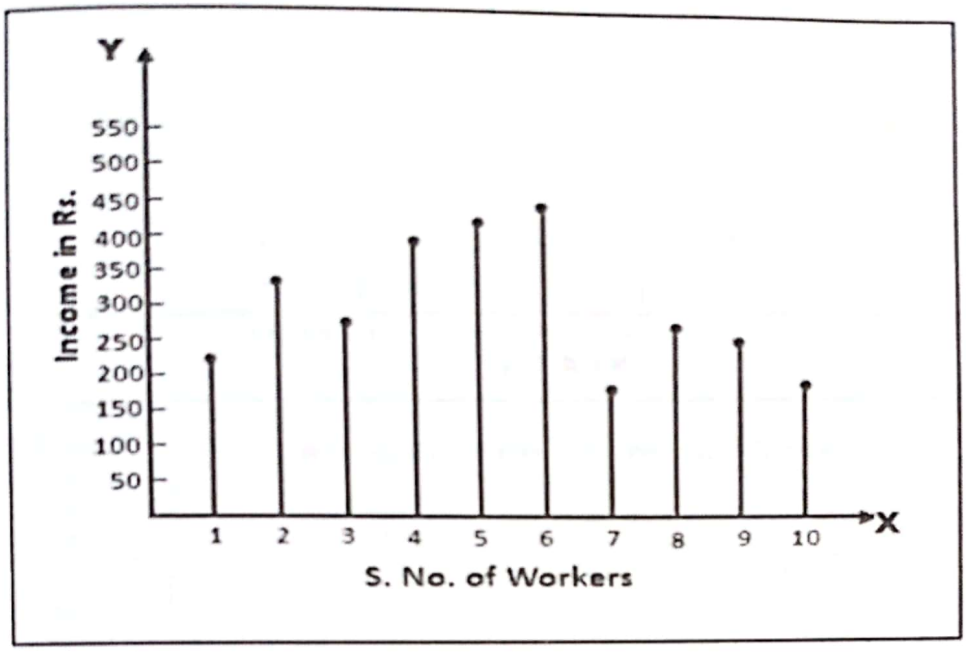
Line diagram



Line Diagram of Income of 10 workers

The length or height of each line represents the frequency.

2. BAR DIAGRAM

- In bar diagram, the data are drawn in the form of rectangles or bars.
- The Y - axis is marked with frequency on a scale.
- The X – axis is marked with variable on a scale.
- Vertical rectangles are drawn as per the height of frequency.
- Equal space is given between the rectangles.
- The width of the each rectangle should be also same.
- Colors or shades may be given for the rectangles.
- It is a one dimensional diagram as height alone is considered.
- The value may be written at the top of the rectangle.

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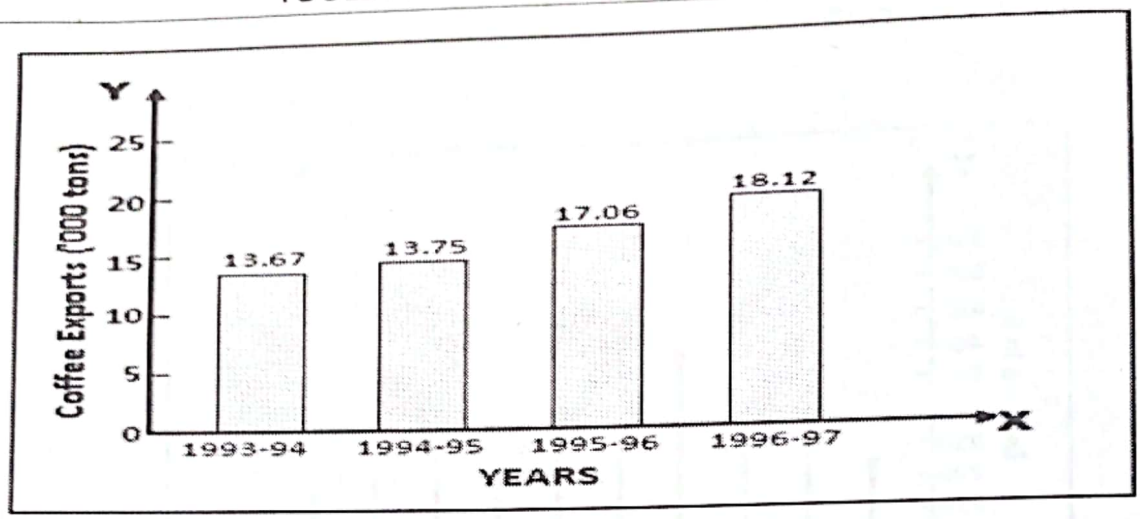

Bar Diagram of Coffee Exports

- The bar diagram is of following types.

1. Simple bar diagram
2. Multiple bar diagram
3. Component bar diagram
4. Percentage bar diagram

1. Simple bar diagram

- Simple bar diagram is very simple.
- It has vertical rectangles at regular intervals.
- The height of each rectangle is corresponding to the data.
- The width of each rectangle should be the same.
- The rectangles may be in given colors or shades.
- It is one – dimensional.

2. Multiple bar diagram

- The multiple bar diagrams contains two or more bars drawn side by side.

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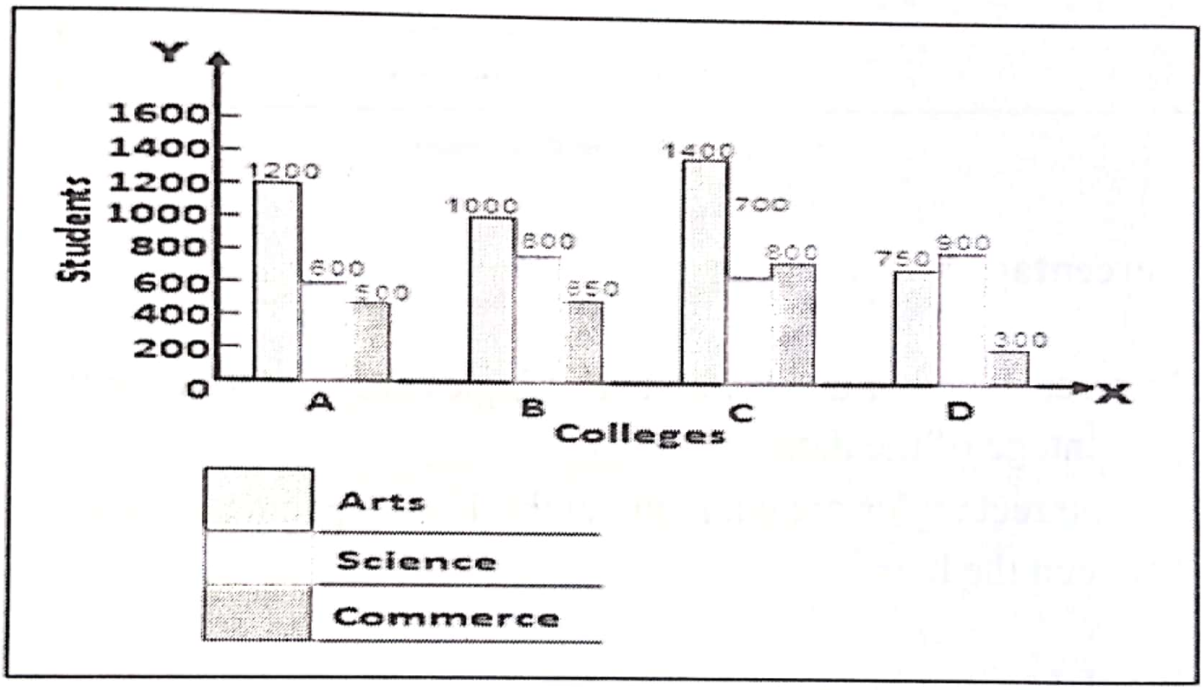
- It is also called compound bar diagram.
- Height is proportional to the data. Width should be same. Equal spacing should be given.
- It is a one - dimensional diagram.
- It helps comparision.

Multiple bar diagram



Multiple Bar Diagram

3. Component bar diagram

- In component bar diagram, each rectangle is subdivided into segments as per data.
- It is also called as subdivided bar diagrams.
- The height is proportional to the data. The width should be same. Equal space should be given between rectangles.
- The largest component should be given at the base of the data.
- It helps comparision.

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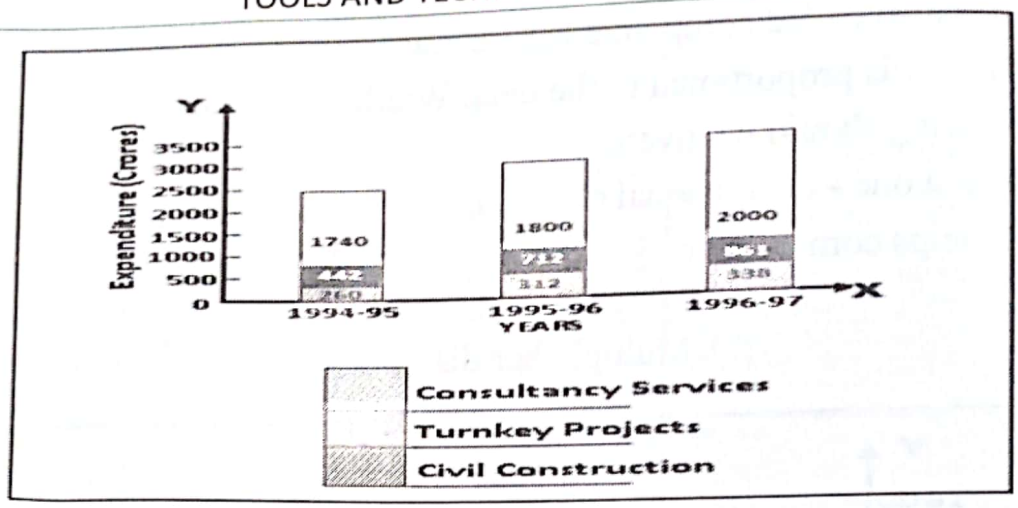

Component Bar Diagram

4. Percentage bar diagram

- In percentage bar diagram, the rectangles are drawn as per the percentage of the data.
- All the rectangles are equal in height. Equal spacing is given between the bars.

3. PIE DIAGRAM

- In pie diagram, the data are presented in the form of a circle.
- Pie diagram is also called as circle diagram.
- The total area of a circle id 360 °. So the data is converted into degrees.
- Accordingly the circle is partitioned. As the data is drawn as per angles (degrees), pie is also called angular diagram.
- The partitions are given different colours.
- It is an area diagram.
- It is a two – dimensional diagram.
- The values are added to get the total.
- Then each value is converted into degrees.

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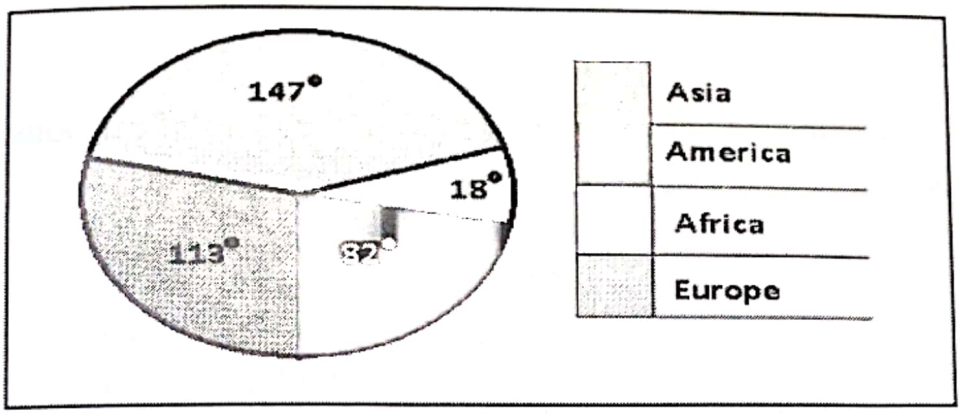

Pie-Chart

A pie diagram

4. PICTOGRAM

- In pictogram, data are drawn in the form of pictures.
- It is simple and it helps lay man.
- Eg. The data of fishes are represented in the form of fish pictures.

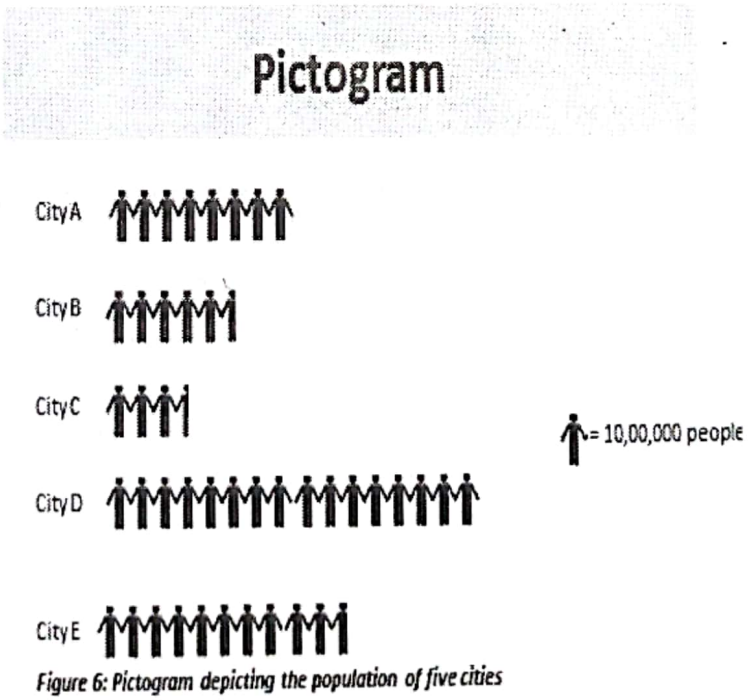

Figure 6: Pictogram depicting the population of five cities

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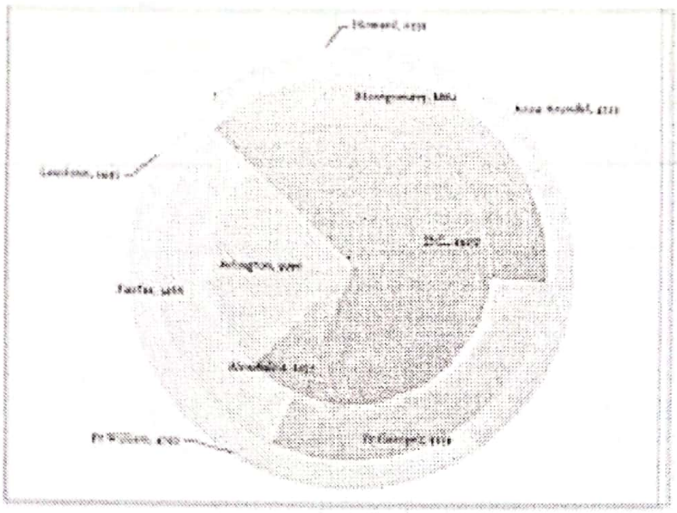
5. CARTOGRAM

- In cartogram, the data are presented in the form of cartoons.
- It is simple and it helps lay man.



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CLASSIFICATION AND TABULATION OF DATA

- The collected data after their scrutiny need to be classified in order to make the data fit for analysis and interpretation.
- The first step in analysis and interpretation of data is classification and tabulation.
- Classification is the first step in tabulation, even though the phrase classification and tabulation is used.
- Proper classification helps proper tabulation.

Classification

- It is the process of arranging the data on the basis of some common characteristics possessed by them.
- Eg. If sex is the basis of classification, the all the male population will be grouped together in one side.
- Likewise if age is the basis of classification, persons of same age will be grouped together and so on.

Objects of classification

- The chief objects of classification are

1. To condense the mass of data.
2. To present the facts in a simple form.
3. To bring out clearly the points of similarity and dissimilarity.
4. To facilitate the comparison.
5. To bring out the relationship.
6. To prepare data for tabulation.
7. To facilitate the statistical treatment of the data

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Types of classification

- There are numerous ways of classifying the data. The important types are

1. Geographical
2. Chronological
3. Qualitative
4. Quantitative

Geographical classification

- This type of classification is based on geographical region likes countries, states, districts, taluks, etc.
- For example, the yield of agricultural output per hectare for different countries is a particular year is given below.

Country	Average output (in kg per hectare)
U.S.A	600
China	300
Pakistan	250
India	150

Chronological classification

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- This type of classification is based on the time of occurrence such as years, months, weeks, days, hours, etc.
- Eg. The fish production in particular form of five years is given below.

Year	Fish production (in kg per hectare)
1987	1400
1988	1500
1989	1450
1990	1550
1991	1600

HISTOGRAM

- It is also called as bar graph or block diagram or stair case chart.
- Histogram is a graph that represents the class frequencies in a frequency distribution by vertical adjacent rectangles.
- It is basically an area diagram.
- The class intervals are marked in the X - axis and the corresponding frequencies on Y – axis. The upper ends of the vertical lines are joined together. This gives rectangles.
- The area of each rectangle is equal to the frequency of the class multiplied by their class intervals.

Merits of histogram

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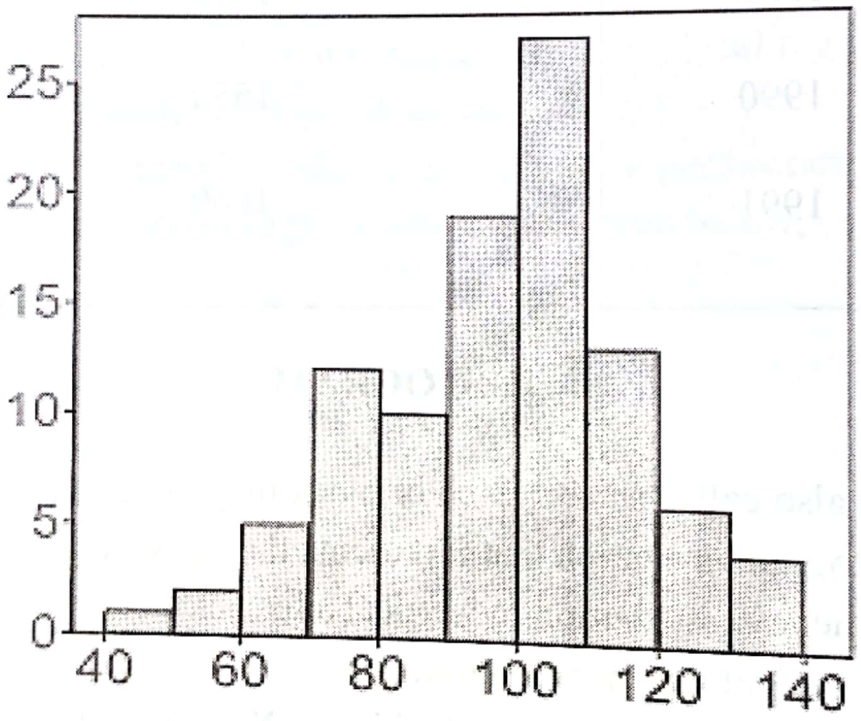
- It can be understood easily.
- The data can be presented in more attractive form.
- It shows the trend and tendency of values of the variable.
- It has the universal applicability.
- It is helpful In assimilating the data readily and quickly.

Disadvantages

- It does not show all the facts.
- It can reveal only the approximate position.
- It takes a lot of time to prepare graph.



Chi-square Test (χ^2 Test)

- It is a statistical test involves the calculation of a quantity which is used to compare an observed ratio with the expected or theoretical ratio and to determine how closely these two are related.
- It was first used by Karl Pearson in the year 1900.

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- Chi-square test is applied in biology to test the goodness of fit to verify the distribution of observed data with the theoretical data.
- It is a measure to study the difference of actual and expected frequencies.
- It has great use in biostatistics especially in sampling studies. It measures the difference between the expected frequencies and observed frequencies.

Features of Chi-square test

- The test is based on events or frequencies and not based on Mean or Standard Deviation.
- The test can be used between entire set of observed andexpected frequencies.
- To draw inferences, this test is applied especially testing hypothesis.
- It is a general test and is highly useful in biological research.
- **Formula for calculation of value**

$$\chi^2 = \frac{\sum (O-E)^2}{E}$$

O - Observed frequencies,

E - Expected frequencies

Degrees of freedom (df)

- When we compare the computed value of χ^2 with the table value the df is evident.

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- The df means the number of classes to which values can be assigned.
- If we have observed frequencies corresponding χ^2 distribution will have (n-1) degrees of freedom.
- For example in case of tossing a coin, there are two possibilities or classes namely head and tail.
- Here df = n-1 means df = 2 – 1 = 1.In such a way we fix df namely n-1.

Working procedure

- A hypothesis is established i.e. null hypothesis (Ho).
- Calculate the expected frequencies (E).
- Take difference between observed frequencies expected frequencies.(O-E)
- Square the difference for each value $(O-E)^2$.
- Divide the $(O-E)^2$ by its expected frequencies $(O-E)^2/E$.
- Add the values obtained the previous step.
- Find the chi-square from χ^2 table at certain level of significance.
- Compare the calculated χ^2 value with table χ^2 value and give the inference.
- **Inference**: If the calculated value of χ^2 is greater than the table value at certain degree of level of significance, we reject the null hypothesis.
- If the calculated χ^2 is zero , the observed values and expected values completely coincide. If the χ^2 is less than the table value at certain degree of level of significance, it is said to be non significant. i.e. we expect the Null hypotheis.

Conditions for using chi square test

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- The observations must be large.
- All the observations must be independent.
- All the events must be mutually exclusive.
- For comparison purposes, the data must be in original.

Uses of Chi-square test

- This test is very powerful test for testing the hypothesis of number.
- Test of goodness of fit: with the help of this test probabilities of association between two attributes are measured.
- Test of independent attributes in this test attributes are classified into two way table or contingency table.
- This discloses whether there is any or relationship.
- Test of homogeneity: this test may be used to test the homogeneity of the attributes in respect of particular
- It may also be used to test the population variance.

CORRELATION

- Correlation is a statistical measure that expresses the extent to which two variables are linearly related.
- Correlation was introduced Karl Pearson and Sir Francis Galton
- The change in one variable may cause positive or negative change another variable.
- Correlation analysis concerned with measuring the strength of degree of relationship between variables.
- The measure correlation is called correlation coefficient or coefficient.
- Correlation is denoted by '' γ.

Properties of correlation coefficient

- It is measure of closeness between the two variables.
- It lies between -1 and +1.

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- If $\gamma = +1$ and is perfect negative. If $\gamma = -1$, it is perfect negative. If $\gamma = 0$, there is there no correlation between two variables the two variables are said independent.

Types of correlation

- Correlation is divided into four important types. They are as follows

1. Positive and negative correlation
2. Partial and total correlation
3. Simple and multiple correlation
4. Linear and non linear correlation

1. Positive and negative correlation

- If two variables (X and Y) are directly proportional with each other and both the variables increases or decreases in the same proportion the correlation is said to be positive correlation.
- If we plot a graph between X and Y the shape of graph will be a straight line originating from the lower ends of X and Y axis
- Eg. Height and weight, rainfall and yield of crop.
- If two variables (X and Y) are inversely proportional to each other and if one variable increases and the other variable decreases the correlation is said to be negative correlation.
- If we plot a graph between X and Y the shape of the graph will be a straight line originating from the lower ends of and Y axis.
- Eg. Pressure and volume of a gas, dose of antibiotics and no. of bacterial colonies.

2. Partial and total correlation

- The study of only two variables by excluding all other variables is called as partial correlation.
- Eg. Age and weight of a baby eliminating the diet and health conditions of baby.
- The study of all variables is called as total correlation.

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3. **Simple and multiple correlations**

- If we study two variables at a time the relationship is called as simple correlation.
- Eg. Age and weight of a baby, height and weight of a Person.
- If we study more than two variables at a time then it is called as multiple correlations.
- Eg. Diet, age, height and weight of new born babies.

4. **Linear and non linear correlation**

- If the change between two variables is uniform then the correlation is called as linear correlation.
- If the change between two variables is not uniform then it is called as non linear correlation Degree of Correlation.

Degree of correlation

	Degree of correlation	Positive correlation	Negative correlation
1	**Perfect**	+1	-1
2	**Very high degree**	+0.9 or more	-0.9 or more
3	**Fairly high degree**	+0.75 to +0.9	-0.75 to -0.9
4	**Moderate degree**	+0.5 to +0.75	-0.5 to -0.75
5	**Low degree**	+0.25 to+0.5	-0.25 to -0.5
6	**Very low degree**	Less than 0.25	Less than 0.25
7	**No correlation**	0	0

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REGRESSION ANALYSIS

- Literally regression means stepping back or going back.
- It was introduced by Sir Francis Galton in the later half of nineteenth century.
- He studied the relationship between the heights of fathers and their sons.
- Galton studied the average relationship between two variables graphically and called the line describing the relationship as "the line of regression".
- Regression can be defined as the measure of average relationship between two or more variables.
- The above definition make it clear that regression analysis is done for estimating or predicting the unknown variable (dependent variable) from the known value of the other variable .(Independent variable)
- Regression analysis is very useful statistical tool which is used both in natural and social sciences.
- If we know that the yield of rice and rainfall related, we may find out the amount of rainfall required to achieve a certain production of rice.
- If we know that the weight of animal and the amount of nutrients supplied are related then we can find out the amount of nutrients required to achieve certain weight.

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- If we know the estriol level and birth weights are related, we may find out the birth weight from the estriol level.
- If we know that blood pressure and age in humans are related we may find out the blood pressure from the age.
- In the regression analysis the independent variable is also known as the "regressor" or "predictor" or "explanator" and the dependent variable is known as "regressed" or 'explained' variable.

Types of regression analysis

- Regression analysis is of three types. They are
  1. Simple and multiple
  2. Linear and non linear
  3. Total and partial

1. Simple and multiple regressions

- If we study only two variables at a time the relationship iscalled as simple regression of a person.
- Eg. Age and weight of a baby, height and weight • If we study more than two variables at a time then it is called as multiple regressions.
- Eg. Diet, age, height and weight of new born babies.

2. Linear and non linear regression

- In linear regression the regression is a straight .
- In non linear regression the regression is not a straight line.

3. Total and partial regression analysis

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- In total regression all the important variables are considered but in the case of partial regression one or more variables are considered but not all.

Methods of studying regression

- Regression can be studied by two methods.
  1. Graphic method
  2. Algebric method

Regression equations

- Regression equation is an algebric method of studying regression.
- It is an algebric method of expression of regression line.
- As there are two regression lines there are two regression equations. They are
  1. Regression equation of X on Y
  2. Regression equation of Y on X
- The regression equation of X on Y would be used to describe the variation in the values of X for given changes inthe values of Y.
- The regression equation of Y on X would be used to describe the variation in the values of Y for given changes in the values of X.
- Regression equation of Y on X

$$y - \bar{y} = byx\ (x - \bar{x})$$

- Regression equation of X on Y

$$(x - \bar{x}) = bxy\ (y - \bar{y})$$

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- Regression coefficient γ

$$\gamma = byx \cdot bxy$$

- Where byx and bxy can be calculated by the following formula.

$$byx = \frac{\sum (X - \bar{X})(y - \bar{y})}{(X - \bar{X})^2} = \frac{\sum dxdy}{\sum dx^2}$$

$$byx = \frac{\sum (X - \bar{X})(y - \bar{y})}{(y - \bar{y})} = \frac{\sum dxdy}{\sum dy^2}$$

Uses of regression analysis

- Regression analysis is used in all those fields where two or more relative variables are having the tendency to go back to the average. It is used in most of the biological studies.
- By using the regression analysis we can calculate the value of dependent variable from the value of independent variable.
- Regression equation helps to predict the value of dependent variable when the values of independent variables are used in the equation.
- We can calculate coefficient of correlation and coefficient of determination with the help of regression coefficient.

Student's t-test

- This test as used with small sample (n< 30) and was worked out by WS Gossette whose pen name was "Student".
- Hence this test is also called as Student's t-test.
- The 'T' may be defined as quantity representing the difference between the sample mean and true mean or population mean expressed in terms of the standard error.

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t= difference between sample mean

SE of the difference BW means

$$t = \frac{X - \overline{X}}{SE}$$

Conditions for applying t-test

- The sample must be smaller than 30.
- The samples must be randomly selected.
- The variable is assumed to follow normal distribution .
- The data must be quantitative

Application of t-test

- To test the significance of single mean when the population variance is unknown.
- To test the significance of difference between two sample means the population being equal or unknown.
- To test the significance of an observed sample correlation coefficient or difference between means of two samples.

Working procedure

- Calculate the means of two samples and find the observed difference between means of two samples.
- Calculate the SE of difference between the two means.
- Calculate the t valué Le the ratio between the observed difference and its standard error by substituting the above values in the formula.

  $t = | X - \bar{X} | \nearrow SE$
- Determine the pooled degrees of freedom from the formula.

  $df = (n-1) + (n_2+1) = n_1 + n_2 - 2$
- Compare the calculated value with the table value at particular degrees of freedom.

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TOOLS AND TECHNIQUES IN BIOLOGY

IMPORTANT QUESTIONS

1. Describe types of Electron Microscope with special reference to SEM& TEM.
2. Explain Basic principle and applications of histopathological techniques.
3. Write an essay on instrumentation and applications of UV/Visible/IR spectrophotometer.
4. Describe different types of chromatography techniques studied by you.
5. Write an essay on methodology and uses of ELISA.
6. Explain briefly Polymerase Chain Reaction (PCR) and its applications.
7. Explain Measures of central tendencies (Mean, Median & Mode).
8. Write an essay on presentation of Data.
9. Explain the working procedure of chi-square test. Mention its uses in Biology Research.
10. Write basic concepts of Correlation and Regression. Add a note on their comparision.

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TIPS TO GET HIGH MARKS IN EXAMINATIONS

- Write point by point one below the other
- Split paragraphs into points
- Under line the important terms in your answer paper
- Do not over write letters or words or scentences
- Leave two inches gap between answers.
- Draw horizontal lines between answers with gel pen.

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