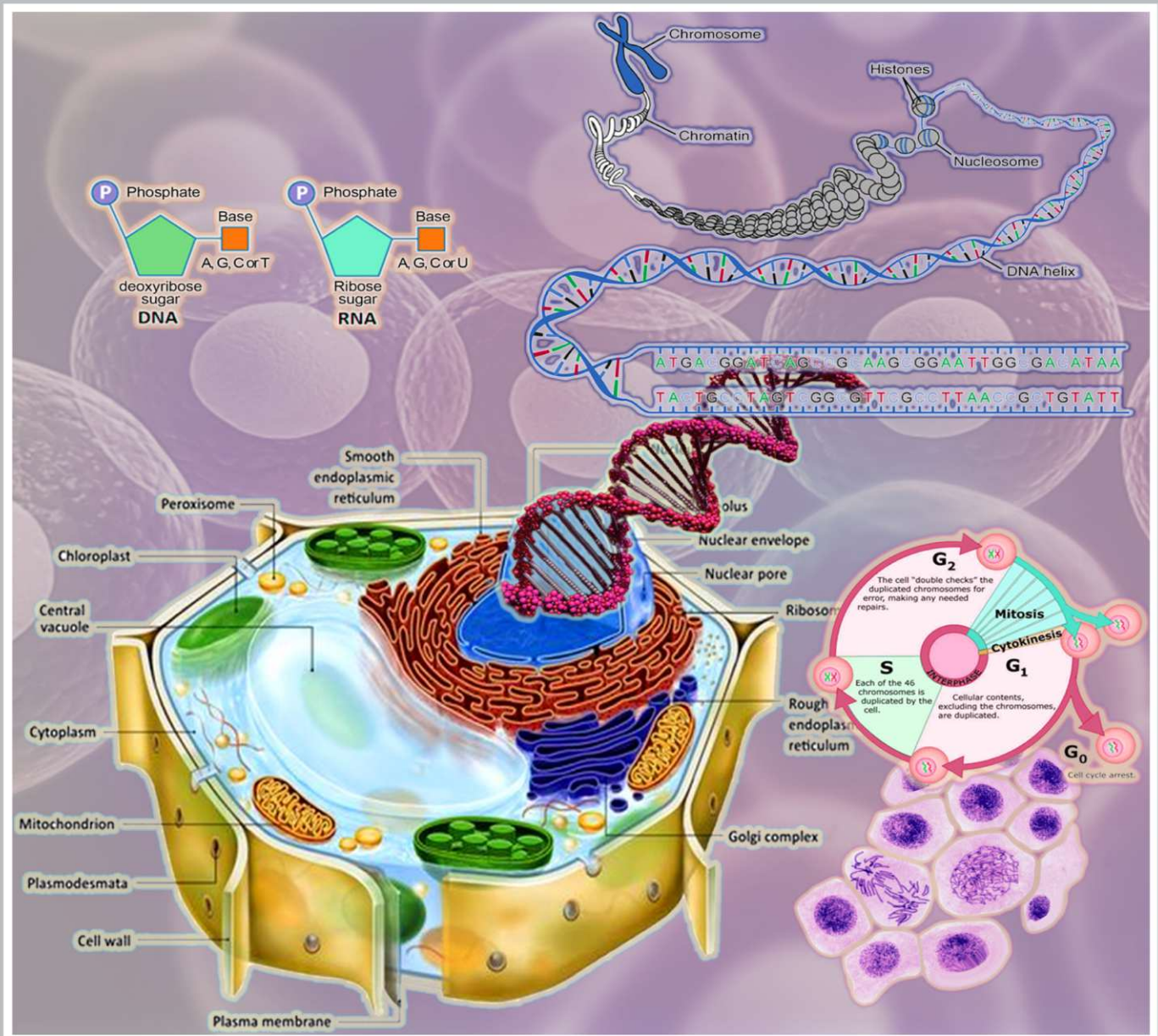




BBYET-141
CELL AND MOLECULAR
BIOLOGY

VOL

1

CELL AND MOLECULAR BIOLOGY

BLOCK 1

TECHNIQUES IN CELL BIOLOGY

5

BLOCK 2

CELL STRUCTURE AND FUNCTION

81

CELL AND MOLECULAR BIOLOGY

Cell and Molecular Biology is a Elective (BBYET-141) course of four credits and is divided into four blocks. In Blocks 1 and 2 we have dealt with subjects related to cell, as it is the fundamental unit of life. Here, we also introduce you to the techniques which have been responsible for elucidating the structural organization of cell. The detailed information related to techniques such as microscopy is also provided. In Blocks 3 and 4, we will study Molecular Biology which will make you familiar with the structure and functioning of the genetic material-DNA and RNA.

In this course, you will study about structure of cells, various cellular processes taking place in plants, and techniques involved in study of cellular organelles. In addition, you will also study about structures of various molecules along with the molecular techniques employed in the study of processes of growth and development in plants.

Cell biology deals with the study of complex details of cell structure, components of cell and various processes taking place in a cell. This course will provide information about the internal structure of cells, various organelles present in them, cellular processes and mechanisms related to it. It also describes structure of prokaryotic and eukaryotic cells, highlighting major differences between the two. The role of various organelles in cellular processes has been lucidly explained. A detailed account of various steps involved in cellular processes such as cell division and multiplication has also been included. Features of the hereditary material present in the nucleus and cell cycle will also be provided to you in detail. We have tried to incorporate recent findings in every unit.

Molecular Biology is the branch of science that deals with the study of various molecules, their structural organization and mechanism involved in various molecular processes. It is very interesting to study of structure of macromolecules such as DNA, RNA, and proteins. The information related to genetic makeup of the cell, expression of genes and their regulation has also been given in detail. Both Cell and Molecular Biology are integrated and form an important branch in botanical studies.

The present course comprises four different blocks which contain 14 units. The blocks on 'Cell Biology' provide you information about importance of cell, structure of cell, study of structural details, using various techniques and cell cycle.

Block 1 has 3 units dealing with various techniques that play an important role in the study of cells and molecules found in plants.

Block 2 has 5 units dealing with structure of cell and its organelles and their functions. It also mentions various steps involved in cell cycle.

Block 3 contains 3 units that provide information about hereditary material present in a cell. The information of DNA and steps involved in its replication has also been given.

Block 4 contains 3 units providing information about structure of RNA, and various steps involved in its synthesis (transcription). The important mechanism of synthesis of proteins (translation) has also been described. Regulation of gene expression is the last unit of this course. In this unit we are going to explore how the various steps in the gene expression are regulated so that each gene product is made at proper time and in proper amounts for each cell's need. Regulation is an important aspect of every process in nature. This is especially true for gene expression.

Objectives

After the study of this course you will be able to :

- ❖ appreciate various techniques used in study of cell and molecular biology;
- ❖ recognize the structure of cell and various organelles;
- ❖ acquainted with the function of cell organelles in various processes taking place in cell;
- ❖ appreciate details of cell cycle particularly the processes of mitosis and meiosis;
- ❖ describe the structure and function of DNA and RNA; and
- ❖ identify and appreciate processes such as transcription and translation.

Study Guide : General Study Guide

To get maximum benefit out of this study materials, please take note of the following points:

- i) Make a notebook with one side rule and the other side plain, keep a pen and some coloured pens/pencils with you.
- ii) Read the material slowly and attentively. Spend enough time on figures and flow charts. Try to draw the figures/flow charts and label them properly. This will help you in better understanding of the text.
- iii) While studying the text, underline the important points with a distinct colour (red, green, blue etc.) in the block itself. Write down salient points in the space provided on each page, or in your notebook, if necessary.
- iv) After finishing a section or subsection; ask yourself – what have I learnt? Try, to list the important points in you note book and compare them with the text and see if you have missed any.
- v) Attempt all the self-assessment questions (SAQs), wherever they appear. Don't skip any of them as they are designed to assess your understanding of the subject. If you cannot answer, read the text again.
- vi) The answer to the SAQs and Terminal Questions are given at the end of each unit. Don't get tempted to see the answers, before you try them.
- vii) If you don't understand any word in the text, consult a dictionary. For scientific and technical words consult the glossary given at the end of each block or a scientific dictionary, if necessary.
- viii) Some of the captions are lengthy because the explanations are avoided in the text; the captions explain the details of the figures. We advise you to spend some time for reading them carefully so as to have a complete understanding of the text as well as figures.

Block

1

TECHNIQUES IN CELL BIOLOGY

UNIT 1

Microscopy

9

UNIT 2

Chromatography

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UNIT 3

Techniques in Molecular Biology

58

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BLOCK 1 : Techniques in Cell Biology

First block of this course introduces you to various techniques involved in study of cell biology. These techniques have played a significant role in understanding the structure of cell and cellular processes taking place in it. The study of this Block will make you aware about the latest techniques used in the study of cellular structures, separation of molecules and quantification of compounds. The technique of microscopy has proved to be very useful in study of cell biology as it has helped us to study cell and its organelles. The technique of chromatography that helps in the separation of molecules is discussed in this block. Information related to major biological techniques such as Spectrophotometry, Centrifugation, X-ray diffraction analysis and Electrophoresis have also been included in this block.

The block contains three units which provide information about various techniques used in study of Cell and Molecular biology.

Unit 1 provides information about the technique of microscopy, its various types and applications. The basic principle of different types of microscopy has been described. A lucid account of Light microscopy techniques viz. optical, phase contrast, fluorescence, confocal and polarization microscopy have given. This is followed by a detailed discussion on electron microscopy techniques which encompass the principles of scanning and transmission electron microscopy, along with the preparatory techniques. Practical applications of these techniques have also been discussed.

Unit 2 discusses about the technique of chromatography, its various types and applications. Chromatographic separation techniques have played a pivotal role in Cell Biology in separation and purification of biomolecules. Techniques of paper, thin layer, adsorption, column and high pressure liquid chromatography have been described in detail.

Unit 3 provides detailed information about techniques such as spectrophotometry, centrifugation, electrophoresis and X ray diffraction. These techniques have helped us in the discovery and elucidation of various organelles in cell and molecular biology. Different centrifugation techniques have helped the researchers to separate sub cellular fractions, to isolate organelles, DNA, RNA and proteins. In addition, these techniques have helped us in determining the purity of macromolecules. The advent of electrophoresis has revolutionized the procedures for separation of charged molecules such as amino acids, proteins and nucleic acids. A description of various types of electrophoresis has been provided. Principles and techniques of spectrophotometry have also been dealt with in Unit 3. These techniques are an integral part of the observations in Cell biology which is related to the determination of absorbance or determining the course of the reaction.

Objectives

After studying this block, you should be able to:

- ❖ elucidate the principle of microscopy technique;
- ❖ describe the various types of microscopy and its applications;
- ❖ explain the chromatography technique;
- ❖ describe various types of chromatography; and
- ❖ explain the various techniques used in study of molecular biology.



UNIT 1

MICROSCOPY

Structure

1.1	Introduction		Scanning Transmission Electron Microscopy (STEM)
	Objectives		
1.2	Imaging Techniques (Microscopy)		Environmental Scanning Electron Microscopy (ESEM)
	Principles of Microscopy		Comparative account of different types of microscopes
	Light Microscopy	1.4	Techniques for preparing tissue for Electron Microscopy
	Phase Contrast Microscopy		Negative Staining
	Fluorescence Microscopy		Freeze Fracture
	Confocal Microscopy		Freeze-Etching
	Polarization Microscopy		Shadow Casting
1.3	Electron Microscopy (EM)		
	Transmission Electron Microscopy (TEM)	1.5	Summary
	Scanning Electron Microscopy (SEM)	1.6	Terminal Questions
		1.7	Answers

1.1 INTRODUCTION

Cell biology is the branch of biology which deals with the study of cell structure, their architectural details, major cellular as well as subcellular processes, and the interactive processes between the cells. Various techniques have been used to study the structural details and functioning of the cell. Imaging techniques such as microscopy have proven very useful in studying various aspects related to the architecture of cells, tissues and various organelles present in them. The minute structural details of the cell, cellular processes and other characteristics can be easily studied with the help of microscopy techniques. The information procured via these techniques provides a better understanding of the various cellular processes taking place in a living being. Our knowledge about cellular structures and their functions has improved manifold by using microscopy and biochemical techniques. In this unit you will study about the various aspects of the microscopy techniques and how microscopy helped to understand the structure of cell. An update about various types of microscopy will also be provided to you.

Objectives

After studying this unit, you would be able to:

- ❖ describe the structure, principle and functioning of a microscope in detail;
- ❖ enlist various types of microscopy techniques;
- ❖ provide details about electron microscopy techniques;
- ❖ enlist various types of electron microscopy;
- ❖ describe the preparation of tissue for electron microscopy; and
- ❖ describe the applications of microscopy techniques.

1.2 IMAGING TECHNIQUES (MICROSCOPY)

Various techniques are used to study the structural details of the cells and the processes taking place in them. Microscopy is the most common technique used to study the structural details of cells, tissues and organelles present in plants and animals. A microscope is an instrument used to view small things that cannot be seen with the unaided eyes. The word microscope is derived from the word 'micro' that means small and 'scope' that means to look at. A microscope magnifies objects many fold so that specimens can be seen easily. The microscopy technique proves useful in visualization of cells and microorganisms as the images appear much larger after magnification.

The most commonly used microscope is the optical or light microscope. In this microscope the glass lenses are used to magnify the image.

History of Microscope

The concept of magnification came into existence long back in 1267 A.D. An English philosopher **R. Bacon** invented eyeglasses in 1235 A.D. In his book *Perspectiva*, he mentioned about the small size of dust and sand particles. In 1538, an Italian physician **Girolamo Fracastoro** observed that an object appears much larger when seen with two spectacle glasses one superimposed on the other. He mentioned these facts in his book *Homocentrica*. The first simple microscope was invented by two Dutch scientists, **Zaccharias Janssen** and his father, Hans in 1590. They were the first to combine two concave lenses within a tube. They noted that nearby objects appeared closer and larger. They developed a microscope made up of wood and cardboard with an eyepiece and an objective lens. **Antonie van Leeuwenhoek**, a Dutch microbiologist made first simple microscope in the year 1668. He was able to see the presence of tiny living organisms present in pond water. The organisms appeared like dots. His microscope was made up of a double convex glass lens that was held between two silver plates (Fig. 1.1). Leeuwenhoek was the one who made microbiology popular throughout the world.

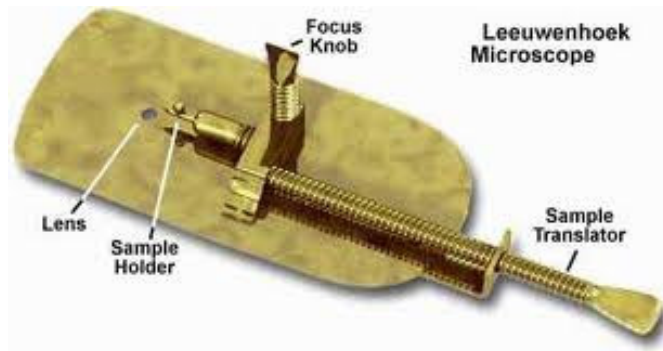
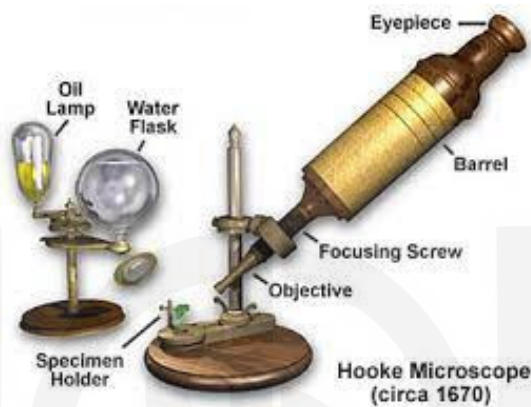
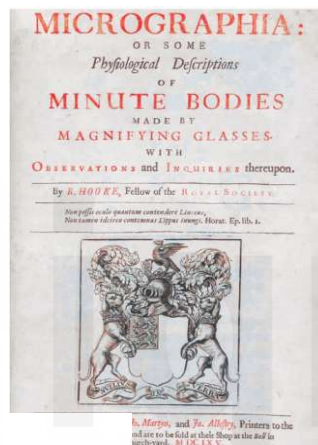


Fig 1.1: An image of microscope used by Leeuwenhoek.

Single lens microscopes were first used by English natural philosopher **Robert Hooke** in 1665 (Fig. 1.2).



(a)



(b)

Fig. 1.2: a) The microscopes used in earlier times by Robert Hooke; b) image of cover page of *Micrographia*.

Robert Hooke provided information about various microscopic objects (such as fleas, lice, and nettles) in his book *Micrographia*. He also coined the term 'cell' for small, microscopic structures each living being is composed of. Hence, invention of microscope resulted in the discovery of cell and ultimately the formulation of **Cell Theory**. Dutch optician **Francois Beeldsnijder**, developed an achromatic (non-colour-distorting) microscope objective in 1791.

In 1827, Scottish botanist **Robert Brown** demonstrated the presence of nucleus. He coined the term 'nucleus' in 1831. English scientist **Joseph Jackson Lister** designed microscope lenses free from achromatic aberrations in 1830. Two single lens microscopes were commonly used in earlier times (Fig.1.3).

In 1868, German physicist **Ernst Abbe** invented an apochromatic system of lenses. He published lens theory in 1873. A British microscopist **Julius Rheinberg** used colour filters in his microscope. The effect came to be known as **Rheinberg illumination** in the 19th century. The largest optical microscope was Burch reflector made in 1947. The weight of this instrument was about 200 kg (440 pounds). The smallest microscope was made by British optician **Horace Edward Dall** in 1950 that weighed no more than 24 grams (0.8 ounce). British doctor **John McArthur** designed commercially available small microscope in 1958.



Fig. 1.3: Images of two single lens microscopes used in older times.

1.2.1 Principles of Microscopy

A microscope is an instrument which contains two-lenses, a light source, and mechanical adjustable parts. We need to be familiar with some terms such as magnification, resolution, numerical aperture, illumination, and focusing before studying in detail about the instrument. Magnification and resolution affect the functioning of microscope to a great extent. The working of a microscope is based upon the basic principle of magnification.

Magnification (enlargement)

It can be calculated by multiplying the magnification of the objective used.
Example- 10X eyepiece, 40X objective = 400X total magnification.

Magnification - Magnification gives a measure of how much bigger an object appears under the microscope. In other words, it is the number of times the object is enlarged when observed under the microscope. You should know that light microscope generally magnifies the object about 400 times the actual size. Magnification of the object depends upon the ocular lens and the objective lens.

Resolution - A lens possesses the ability to show two adjacent objects as distinct units. The smallest distance by which two points can be separated and distinguished as individual objects is called resolution. The study of the smallest detail of the object depends on the resolution. Resolution is expressed in micrometres (μm), which is one millionth of a metre. Factors such as wavelength of light used and the numerical aperture regulate the resolving power of a lens. The diameter of the objective lens in relation to its focal length is known as numerical aperture. The resolution of the microscope is determined by the **numerical aperture** (N.A.) of the objective lens. Numerical aperture is also defined as the sine of half the angle of the cone of light taken by the objective multiplied by the refractive index (R.I.) of the medium in which the object is immersed. Numerical aperture increases as R.I. increases. The values of N.A. of microscope having low-magnification objectives range from 0.1 to 0.95 in case of dry objectives and 1.4 in case of oil-immersion objectives.

Numerical aperture (NA)

$$NA = n \sin \alpha$$

where

n = refractive index of the medium

α = half of the cone angle of light emitted from the condenser.

Abbe gave the following formula for calculating resolving power of the microscope.

$$d = \lambda / 2 NA$$

Where λ is the wavelength of light used in visualizing a specimen

NA is the numerical aperture. If using a green light of 514 nm and an oil immersion objective with an NA of 1.45, the (theoretical) limit of resolution will be 177 nm.

It has been noted that resolving power of the lens is higher if the wavelength is short. Hence smaller the value of resolving power, higher the resolution and more the clarity.

Box 1.1: Resolution

The resolution of an optical system is calculated using the formula

$$r = 0.61 \lambda / n \sin \alpha$$

where r is the resolving power (It is the minimum distance between two points that can be separated), λ is the wavelength of light used to illuminate an object, n is the refractive index of the medium in which the object is placed, and $\sin \alpha$ is the sine of half the angle between the specimen and the objective lens. The term **$n \sin \alpha$** is often referred to as the numerical aperture.

1.2.2 Light Microscopy

You might have used microscopes in the laboratories. These are called as **light microscopes** or optical microscopes. In this type of microscope, visible light passes through the specimen (the biological sample) and is bent to form a magnified image. Lenses focus light on the specimen, magnifying it, thus producing an image. The magnification varies depending on the types and number of lenses used in the microscope. Optical microscopes are of two types-simple or compound.

- **Simple light microscope** - A microscope having one lens is called a simple microscope. This type of microscope can magnify an image up to 300X. It has low magnification as it uses a single lens. The resolution power of a simple microscope is below 1 μm .
- **Compound light microscope** - A microscope having more than one lens is called compound microscope. This type of microscope can magnify images up to 2000X. It has a higher magnification as it uses two sets of lenses, an objective lens, and an eyepiece. The lenses are aligned in such a way that they can bend light for getting effective magnification of the image (Fig 1.4). The resolution of compound microscope is about 0.2 μm . The limitations of resolution (magnifying power) found in a simple microscope can be overcome using this microscope.

The magnification of the objective lens and eyepiece manage the magnifying power of the compound microscope.



Fig.1.4: A photograph showing a modern compound microscope.

Parts of a Microscope

A microscope mainly consists of many parts. These include an objective lens, ocular lens, lens tube, stage, and reflector. An object is magnified through the objective lens. When the target is focused, a magnified image can be observed through the ocular lens.

Objective

The objective lens gathers light from the specimen, magnifies the image of the specimen and projects this magnified image. Objective lenses show magnifications in the range of 2 to 100X. Objective is designed to minimize aberrations.

Objective can be of two types: dry or immersion. An objective which works with the air between the specimen and the objective lens is called as **dry**. In contrast, an objective that requires liquid and is usually transparent oil which occupies the space between the object and the front element is called as an **immersion** objective. In the first type of objective, refractive index (R.I.) of oil matches that of the glass

The focal length regulates the optics of the objective. The focal length is inversely proportional to the magnification. This means that focal length of an objective decreases with the increase in magnifying power. .

In some objectives special glasses are used. These are called apochromats and were first produced commercially by Abbe in the 1870s.

Aberration is noted in high-magnification objectives (magnifications greater than 25X). Aberration influences the sharpness or quality of the image. The aberrations of a lens increase as the relative aperture of the lens is increased.

Eyepiece (Ocular Lens)

Eyepiece is the lens which is used to observe the image. These lenses are usually of 10X or 15X magnification. The eyepiece helps in forming enlarged virtual image that can be seen by the observer.

Body Tube

A hollow tube through which light travels from the objective to the ocular lens is called as body tube (Fig. 1.5). It connects eyepiece to the objective. It contains a prism that bends the light rays so they can enter the tube. The upper end of body tube possesses ocular or eyepiece lens, while the lower portion consists of a movable nosepiece containing the objective lenses.

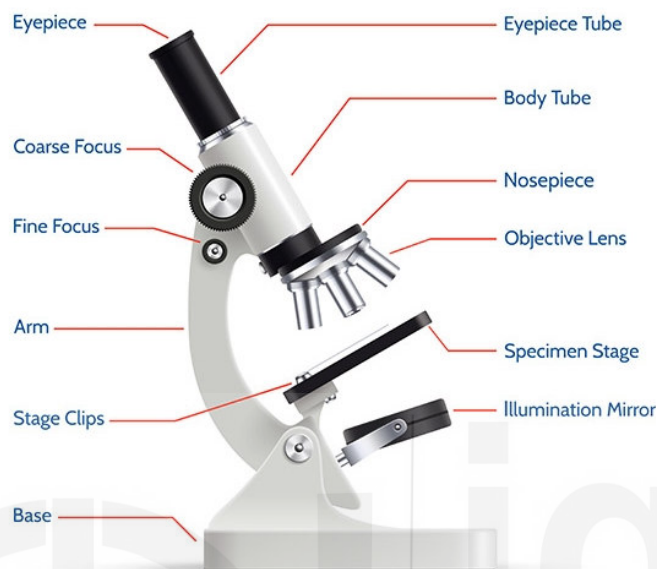


Fig. 1.5: Diagram showing parts of a microscope.

Arm

It is a structure that connects head of the microscope to the base.

Base

The bottom of the microscope is called base.

Stage

It is a flat platform having clips that supports the slides (Fig. 1.4). In case of mechanical stage, the slide kept in position with the help of two knobs. One knob moves the slide left and right, the other moves it forward and backward.

Nosepiece

The circular structure that screws the objective lenses is called nose piece. Objective lenses of varying magnification and numerical aperture are mounted on the rotating nosepiece.

Condenser Lens

The condenser is mounted below the stage (where the specimen is placed) and focuses beam of light onto the specimen. When it provides uniform illumination in the region of the object under observation, it is referred to as critical illumination. Alternatively, when the image is focused on the condenser followed by objective of the microscope, it is referred to as Köhler illumination. It is useful in getting sharp images of magnification 400X and above. For a microscope with a power of 1000X or above, condenser lens with an N.A. of 1.25 or greater is needed. Such microscopes are generally equipped with an Abbe condenser.

Diaphragm or Iris

The diaphragm or iris regulates the amount of light entering the condenser i.e. helps in adjusting the intensity of light. It is located under the stage or between the light source and condenser.

Illuminator

A steady light source is used for illumination in the microscope. Mirrors were used as a source of light in early microscopes. Mirror reflected light up from an external light source located at the bottom of the stage. Generally, a tungsten lamp is used as a light source. Effective illumination is required for getting good magnification and resolving power. The amount of light entering the microscope needs to be regulated as excessive illumination may obscure the specimen.

Box 1.2: Refractive index

The resolving power of microscope depends on the refractive index (R.I.). Refractive index is the capacity/power of light to bend while passing through air from the glass slide to the objective lens. The refractive index of air is lower than that of glass. When the light rays pass from the glass into the air, they bent or refract so that they do not pass into the objective lens. Loss of refracted light can be compensated by mineral oil which has the same refractive index as glass. The loss of light reduces the numerical aperture and resolving power of the objective lens. The decrease in light refraction produces more light rays which enter directly into the objective lens, producing a clear image with high resolution.

Electronic detectors such as a complementary metal-oxide semiconductor (CMOS) or charge-coupled device (CCD) chip is used to capture the magnified image in modern day microscopes. The digital signal obtained is transmitted to a computer and translated into an image on the monitor. The pictures as well as moving video sequences are captured using software.

The specimen is mounted on a glass slide and immersed in a material which has the same R.I. as that of the slide. The tissues are cut into thin sections after treatment with fixatives. The preparations of this type preserve the natural structural appearance of the cells. The acids and aldehydes such as formaldehyde, acetic acid, and picric acid are widely used as fixatives. The thin sections of the tissues are cut after getting them embedded in paraffin wax or quick-frozen. The sections are cut using an instrument called microtome. The thin sections are spread on the slides and stained. The sample preparation is such that contrasts between the sub-cellular structures as dark or light or coloured areas are produced. Contrast is produced by applying specific stains (which give colour to different structures) or by bringing alteration in light transmitting properties of cell components.

Box 1.3: Abbe theory of image formation in a microscope

The modern theory of image formation in the microscope was given by German physicist Ernst Abbe in 1873. According to this theory, convergent light obtained from a condenser helps in visualization of objects or specimens in the focal plane of the microscope. A collection of plane waves propagating in a specified set of directions is called as convergent light. The superimposition of waves produces incident illumination. The diffracted waves are collected by the objective and directed to the focal plane. Interference between the diffracted waves produces an image of the object.

Abbe showed that more is the number of diffracted waves reaching the objective, finer is the details of the image. The objective's ability to collect diffracted light was considered as the N.A. Greater is the magnification of the objective, greater is N.A. of the objective.

Specialized optical microscope

The modifications and a range of special adaptations have been made in basic optical microscope. In some specialized microscopes, the illuminator and lens systems are incorporated into a single unit.

Inverted microscope

The light source and condenser situated in the upper part of the microscope direct light down to the stage. The eyepiece in this instrument is angled upward while the objective is set with its front element. The specimens present in the liquid medium can be studied with the help of this microscope. The cell cultures have been studied using this microscope. Hence the microscope has proved useful in studies related to biology and medical research.

Stereoscopic microscopes (Dissecting microscope)

The microscope possesses two objectives and eyepieces. They provide separate optical paths and different viewing angles to eyes. This arrangement produces a three-dimensional visualization of the sample. Each eye can independently image the object. The magnifying power ranges between 5 and 250X. This microscope is generally used for viewing the dissected subjects and microsurgical procedures. They are also used in electronics manufacturing units to monitor the bonding of leads to integrated circuits.

Metallographic microscope

In this type of microscope, vertical source of light (illumination) is used. The light source is generally present below the eyepiece in the tube. Light is focused through the objective onto the specimen. The reflected light or scattered light gets back to the objective which is imaged back at the eyepiece. This type of microscope is used to identify defects in metal surfaces, determine crystal grain boundaries in metal alloys, and study rocks and minerals. They also have applications in forensic science and diagnostics.

Reflecting microscope

Microscopes of this type possess reflecting objectives. An objective usually consists of two components: a relatively large, primary concave mirror and a smaller, secondary convex mirror which is located between the primary mirror and the object. The objective lens of this type does not produce chromatic aberration and needs no correction. The objects can be observed over a wide range of visible light and also in the ultraviolet or infrared regions.

Brightfield microscope

Two lens systems are used for magnifying specimens. This includes ocular lens located in the eyepiece and objective lens located in the nosepiece. The beam of light obtained from tungsten lamp helps in illumination of the specimen. The light is focused on sub-stage lens called a condenser. This microscope provides a high-resolution image. These microscopes are used for observing non-viable, stained samples. We cannot observe living cells because of the absence of contrast between the specimen and the surrounding medium.

Darkfield microscope

In this type of microscope, the condenser is modified which directs light obliquely on the specimen. This results in deflection or scattering of light, hence making object appear bright against a dark background. This type of microscope is suitable for observing living specimens.

Interference microscope

Two beams of light are used in this microscope. One beam of light passes through the specimen while the other bypasses it. The combination of two beams results in interference between them, thus revealing the structure of the specimen. The difference between interfering beams of light is used to create the image. This type of microscopy was developed by British microscopist **Francis Smith** and French physicist **Maurice Françon** in 1947. Quartz lenses were used by them to produce two beams of light that were perpendicularly polarized.

1.2.3 Phase Contrast Microscopy

Phase-contrast microscope was invented by Dutch physicist **Frits Zernike** in 1934. He was awarded Nobel Prize in Physics in 1953. In this type of microscope, a cone of light is passed through the specimen, which gets diffracted. The light rays pass obliquely through the specimen, causing refracted waves to deviate away from the unrefracted rays (Fig. 1.6). The unrefracted and refracted rays are focused back into the image plane. Two or more light waves combine to produce interference. This happens because wave equal to the sum of the two combining waves is produced. The direct and undeflected light creates a difference in phase producing contrast. The phase difference of about one-fourth wavelength between two waves produces only minimal interference.

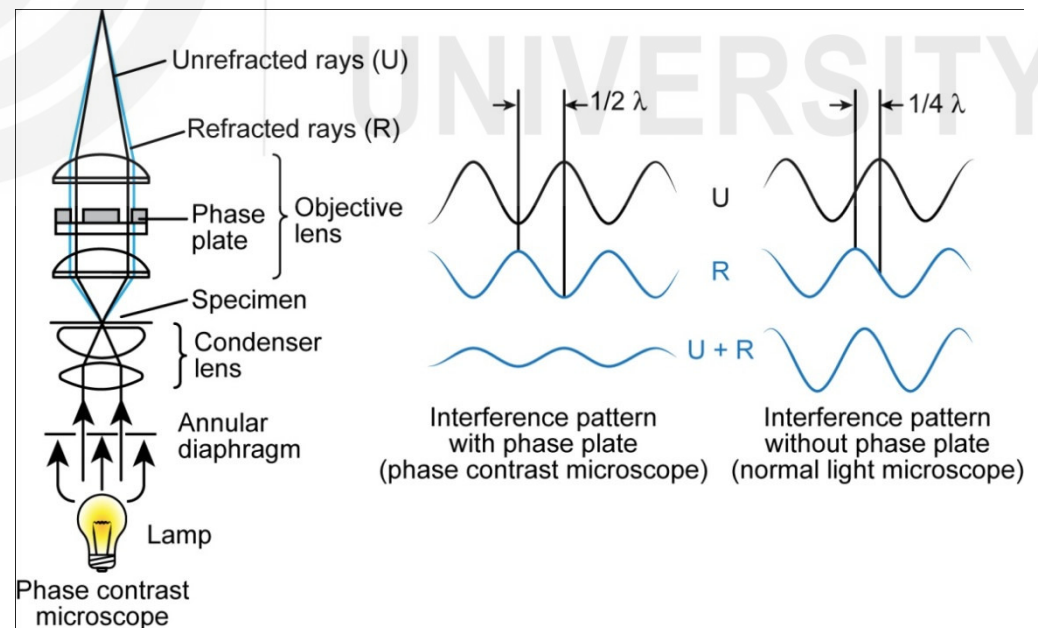


Fig.1.6: Graphic explanation of principle of the phase contrast microscope. In this microscope, delay in refracted rays by about one – fourth wavelength than normal light microscope has been noted. A comparison of the diagrams (on the right) illustrates that extra delay enhances the interference obtained when the unrefracted and refracted rays are focused back together (U+R).

The problem of staining and alterations in brightness can be overcome by this microscopy. The technique proves useful for observing colourless specimens. The transparent structure such as nuclei transmits a light when present in the mounting medium that surrounds them. The propagation of light through object produces a change in the optical path. The shift in the phase of light passing through the structure and that passing around the structure forms the visible image.

1.2.4 Fluorescence Microscopy

Fluorescence microscope is used to see tissues that fluoresce (absorb one wavelength of light and emit another). Light of one wavelength excites the fluorescent molecules while the light of other wavelength collects the emitted light. Some cellular components fluoresce naturally and appear in various colours, while non fluorescing structures fluoresce by staining with fluorescent dyes (fluorochromes). Fluorescence dyes are used in preparation of antibodies that bind to specific cellular proteins. The antibodies are first tagged with fluorescein (a fluorescent dye) and then fluorescein – labelled antibody is applied to cells (Fig. 1.7).

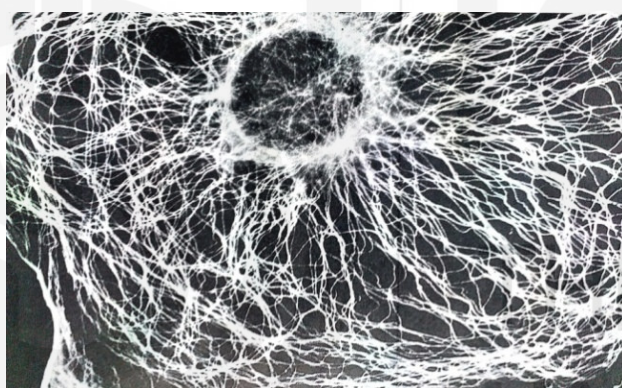


Fig.1.7: The network of microtubules present in this cell revealed using fluorescent antibody labelling and fluorescence microscopy.

1.2.5 Confocal Microscopy

Confocal microscopy is an advanced microscopy technique which uses a 'pinhole' to eliminate out of focus light. The discrete optical sections can be collected after eliminating out of focus light above and below the plane. High power lasers illuminate the sample. They help in collecting light from the desired focal plane. The beam of laser light gets scanned across the sample and light only from the desired focal plane can enter the detector. This microscope uses fluorescence optics. Fluorophores i.e. a fluorescent chemical compound that can re-emit light upon excitation is used in the sample. Fluorophores contain aromatic groups, planar or cyclic molecules with several π bonds. When the laser light gets focused on a defined spot at a specific depth located within the sample, fluorescent light gets emitted exactly at that point. A pinhole cuts off out of focus signals from the optical path, thus allowing the fluorescence signals from the illuminated spot to enter the light detector and form image at different depths in the same sample (Fig. 1.8). The technique is suitable for both live as well for fixed cells and tissues.

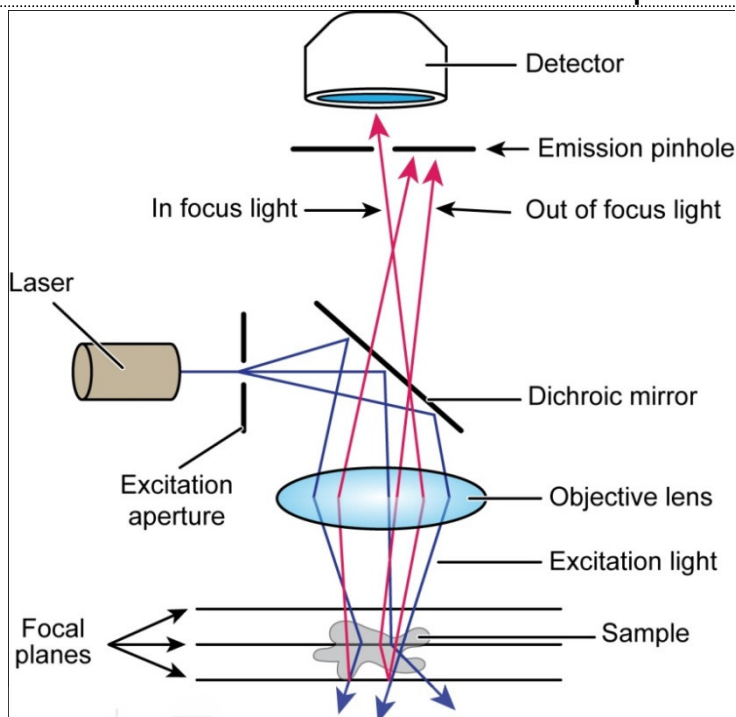


Fig. 1.8: An image showing the working of a confocal microscope.

1.2.6 Polarization Microscopy

In polarization microscope, light source is equipped with a polarizing filter. The linearly polarized light (whose wave travels in a single plane) passes through the object. It either remains unaffected or splits into two beams with different polarizations. The polarized light improves the contrast. **Polarizer** is a device that transmits polarized light through the specimen. **Analyzer** is a device placed above the objective. An analyzer fitted to the eyepiece blocks polarization of the light. The analyzer is rotated to get maximum contrast in the image and a difference in refractive index between planes is determined. Polarization in the beam of light is removed by using objectives with low numerical aperture. The differences in refractive index provide useful information about the molecular substructure of cell organelles.

This microscopy is useful for visualising birefringent structures which differ in refractive index in different planes. The crystalline substances, geologic samples and fibrous materials such as the mitotic spindle and muscle fibers can be easily studied using this microscope.

SAQ 1

- a) Fill in the blanks with the appropriate word :
- provides measure of how much larger a microscope causes an object to appear.
 - Phase-contrast microscopy was invented by.....
 - is a chemical compound that can re-emit fluorescent light upon excitation.
 - Illumination source in a phase microscope is a cone of light whose rays pass through the specimen.

- v) is the instrument used to cut sections of tissues.
 - vi) In a microscope, visible light passes through the specimen, gets bent through the lens system and forms the magnified image.
- b) State whether the following statements are 'true' or 'false' :
- i) The confocal microscope uses fluorescence optics. Laser light is focused onto a defined spot at a specific depth within the sample.
 - ii) In fluorescence microscopy, the light of one wavelength is used to excite the fluorescent molecules, and the light of a different wavelength is used for refraction.
 - iii) The advantage of a confocal microscope is that discrete optical sections can be collected, eliminating the out of focus light above and below the current plane of focus.
 - iv) Different organic compounds are used to preserve different tissues.
 - v) Fluorescein (a fluorescent dye) labels antibody when applied to the cells.
- c) Write the functions of the following:
- i) Eyepiece
 - ii) Diaphragm
 - iii) Body tube
- d) Draw a neat and labelled diagram of a compound microscope showing its different parts.

1.3 ELECTRON MICROSCOPY (EM)

Electron microscopy (EM) is a technique that helps in getting high resolution images of specimens (biological and non-biological). A beam of electrons is used as the source of illumination. The electrostatic and electromagnetic lenses are used to control the beam of electron and focus it to form an image.

Basic Components of an Electron Microscope

1. The vacuum system creates strong vacuum in the entire column i.e., along the path of electron beam. This is required for making the electrons travel as they do not travel easily in air.
2. Electron gun is used for emission of electron beam. Electron gun consists of:
 - a) cathode which is a filament made of tungsten that emits electrons at 50-100kV.

- b) anode that produces a powerful electric field. The hole in anode provides an electron beam which makes the electrons travel at a high speed (several hundred thousand kilometres per second). The voltage applied is the range of 3-5 kilovolts (voltage is required to draw electrons from the source).
3. Electromagnetic lenses are arranged together. They help in illumination, focus, and magnification. The condenser lens controls the electron beam, objective lens, intermediate lens and projector lens work together to produce a final image (Fig. 1.9).
4. The photographic system is used to record image as an electron micrograph.
5. A cooling system attached to the column prevents heating up of system (since high voltage is used for emission of electrons).

This microscopy is widely used in biomedical research and study of detailed structure of tissues, cells, organelles, and macromolecular complexes.

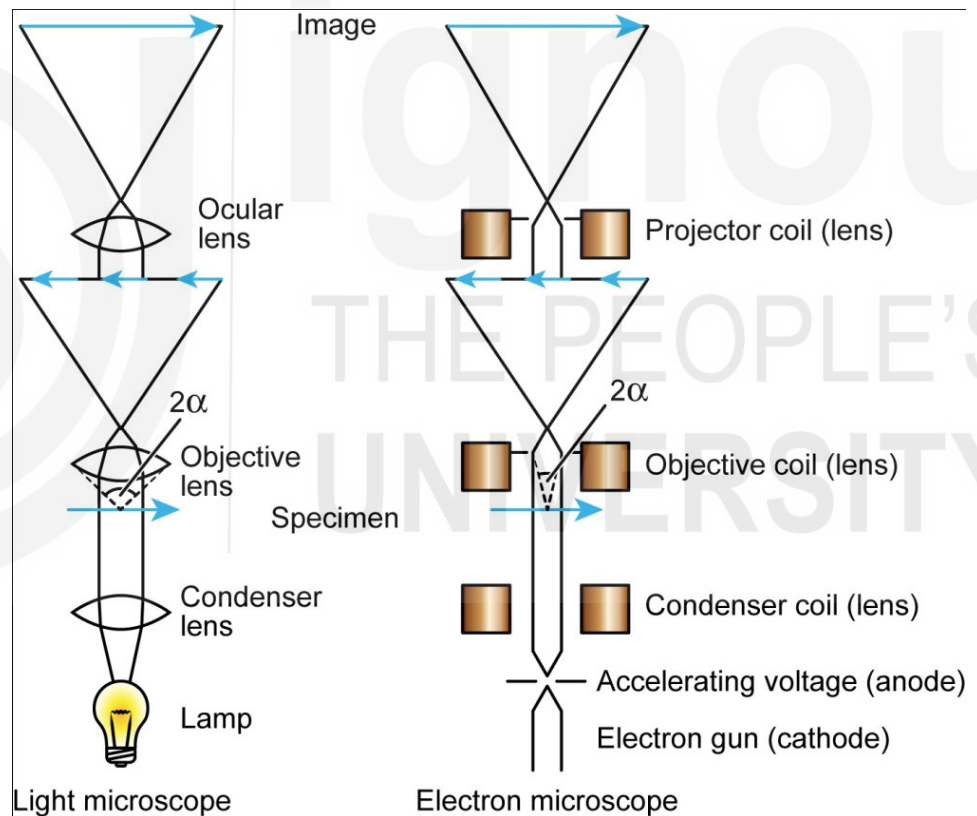


Fig. 1.9: Schematic diagrams of Light and Electron Microscopes showing difference in the principle of working.

Electron microscopy is of two types- **Transmission Electron Microscopy (TEM)** and **Scanning Electron Microscopy (SEM)**. In TEM, the electrons are transmitted through an object, focused by the lenses to form the image while in SEM, electrons are reflected by the object to form an image.

1.3.1 Transmission Electron Microscopy (TEM)

The transmission electron microscope possesses high resolution. The concept of electron microscopy was given by German physicist **Ernst Ruska** and the

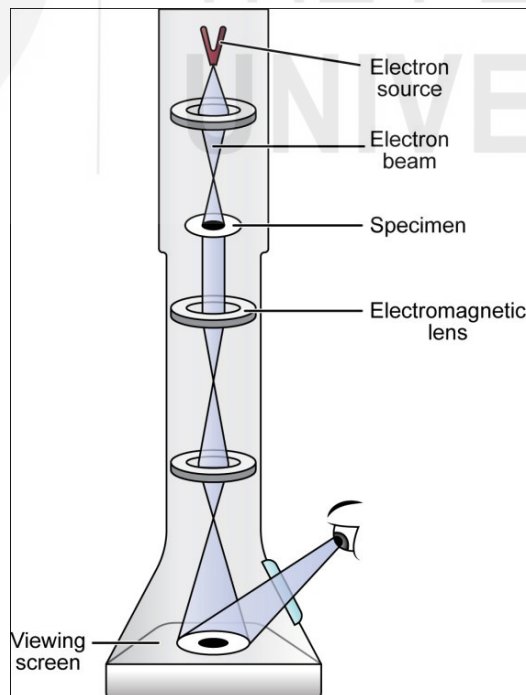
electrical engineer **Max Knoll** in 1931. The first functional electron microscope was made by **E.F. Burton** and his students **C. Hall, J. Hillier** and **A. Prebus** at the University of Toronto in 1938. Later in 1939, the first commercial TEM was developed by Siemens.

In this type of microscope, a beam of highly focused electrons is directed towards a thin section of the specimen (<200 nm). The highly energetic incident electrons interact with the atoms in the sample and produce characteristic radiation and particles which form image. A high voltage electron beam is emitted by electron gun which is made up of a tungsten filament. The cathode is used as the electron source while acceleration of electron beam is produced by anode (Fig. 1.10). The electron beam is focused by electrostatic and electromagnetic lenses. The transmission of electron beam through the specimen results in magnification of the structure of the specimen through objective lens. The transmitted electrons hit a fluorescent screen at the bottom of the microscope and give rise to a "shadow image" of the specimen. The image is projected onto a fluorescent viewing screen coated with a phosphorescent or scintillator material. The photographic film or plate is exposed directly to the electron beam or a fibre optic light-guide to the sensor of a CCD camera. The image detected by the CCD may be visualized on a monitor or computer.

The sample/specimen needs to be processed before viewing under an electron microscope. The material/sample is prepared by different ways. This is done either by cutting very thin sections of the specimen from a piece of tissue after fixing it in resin or freezing it or isolating a specimen and studying it after negative staining.



(a)



(b)

Fig.1.10: a) Photograph of a TEM; b) Schematic representation of TEM showing different electromagnetic lenses.

Sample Preparation

As the TEM works under conditions of vacuum, water present in biological materials needs to be removed. The tissue is preserved using different fixatives to avoid tissue disruption caused by this loss of water. These fixatives stabilize the macromolecular structure of specimen by cross linking of proteins with aldehydes such as formaldehyde and glutaraldehyde, and lipids with osmium tetroxide. The tissue is dehydrated using alcohol or acetone and embedded before sectioning. The tissue is passed through a 'transition solvent' such as propylene oxide and then infiltrated with an epoxy resin such as Araldite, Epon or Durcupan. The resin gets polymerized (hardened) and the hardened sample is cut into very thin sections using a diamond or glass knife present in the instrument called ultramicrotome. The thin sections are picked up using a boat filled with water made present around the glass knife. The cut sections float on the surface of water which are picked up and placed on the surface of copper grid (Fig. 1.11). The staining is done with heavy metals such as lead, uranium, or tungsten. The scattering of electrons produces contrast between different structures. The specimens can be stained "*en bloc*" before embedding or later after sectioning. In '*en bloc*' staining, a fixed biological specimen (block tissue) is immersed in an aqueous solution of uranyl acetate for several minutes and/or followed by aqueous lead citrate and/or lead aspartate. This process enhances the image contrast of membrane structures or fibrous structures in cells.

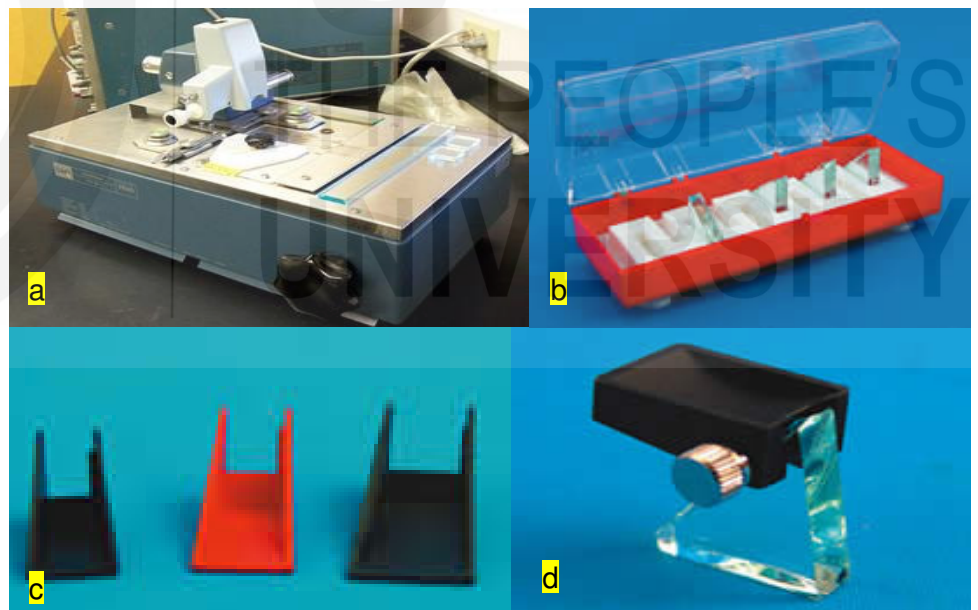


Fig.1.11: Image showing a) Knife Maker; b) Glass knife; c) Boats; d) Glass knife with boat.

An Ultramicrotome

A microtome is a tool used to cut extremely thin sections of specimen/sample. The word microtome is derived from Greek '*micros*' meaning "small" and '*temnein*' meaning "to cut". It is also known as an ultratome or ultra-microtome. The instrument is used in the preparation of ultrathin sections (50-100 Å) that can be observed under TEM (Fig. 1.12). Glass and diamond knives present in the instrument help in cutting very thin sections.



Fig. 1.12: Photograph of an Ultra-microtome.

TEM shows enhanced resolution but the electron beam used is very weak and pass an appreciable distance through air, therefore high vacuum is required inside the internal chamber of electron microscope. The photographs of cells taken using an electron microscope are called **electron micrographs**. You can see that the picture below shows the minute structural details of the cellular entities (Fig. 1.13).

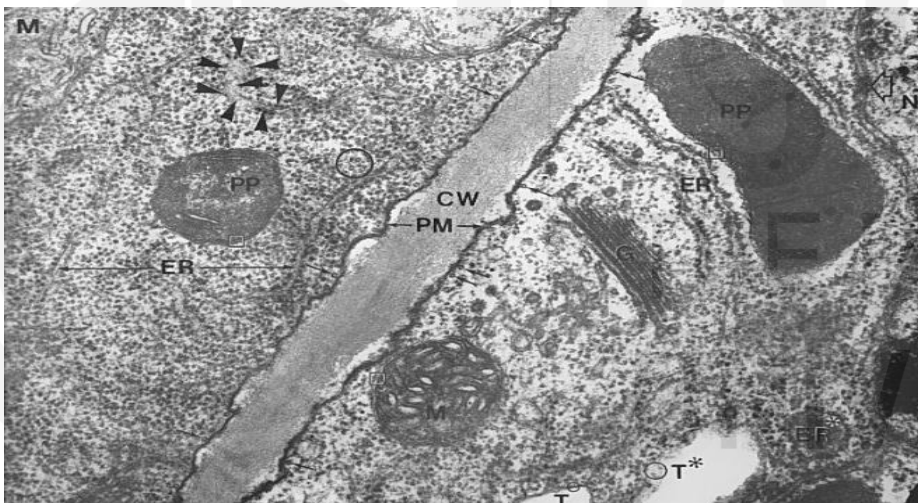


Fig. 1.13: Electron Micrograph showing two plant cells (X 45, 000). CW - Cell wall; ER- Endoplasmic, M-Mitochondria, PM- Plasma membrane, PP- Proplastids, T-Tonoplast.

1.3.2 Scanning Electron Microscopy (SEM)

In scanning electron microscope, electron beam moves over the specimen, the molecules get excited and move to high energy level and emit secondary electrons. These electrons are used to form image of the specimen. Image is produced when electron beam is focused into an intense spot on the surface of specimen. The spot is moved back and forth across the specimen by charged plates called beam deflectors. These deflectors are located between the condenser lens and the specimen. The signals sent by the deflector attract or repel the beam. Secondary electrons are captured by a detector. The scintillator emits photons of light on excitation when electrons are incident upon it (Fig. 1.14). The deflected and emitted electrons are detected by a photomultiplier tube. This tube forms a 3-D image of surface features of the specimen. The electronic signal generated is transferred to the video screen.



Fig. 1.14: Scanning Electron Microscope.

Generally, the image resolution of SEM is less than that of TEM. The SEM image depends upon the surface processes. SEM can produce images of bulk samples up to many centimeters in size. It produces images of three-dimensional shape of the sample (Fig 1.15). This microscope has proved very useful in studying the minute structural details of the cell surface (Fig. 1.16). The increasing popularity of SEM among cell biologists is because of its remarkable ability to provide details about surface topography, show 3D structure and improved resolution (30-100 Å).

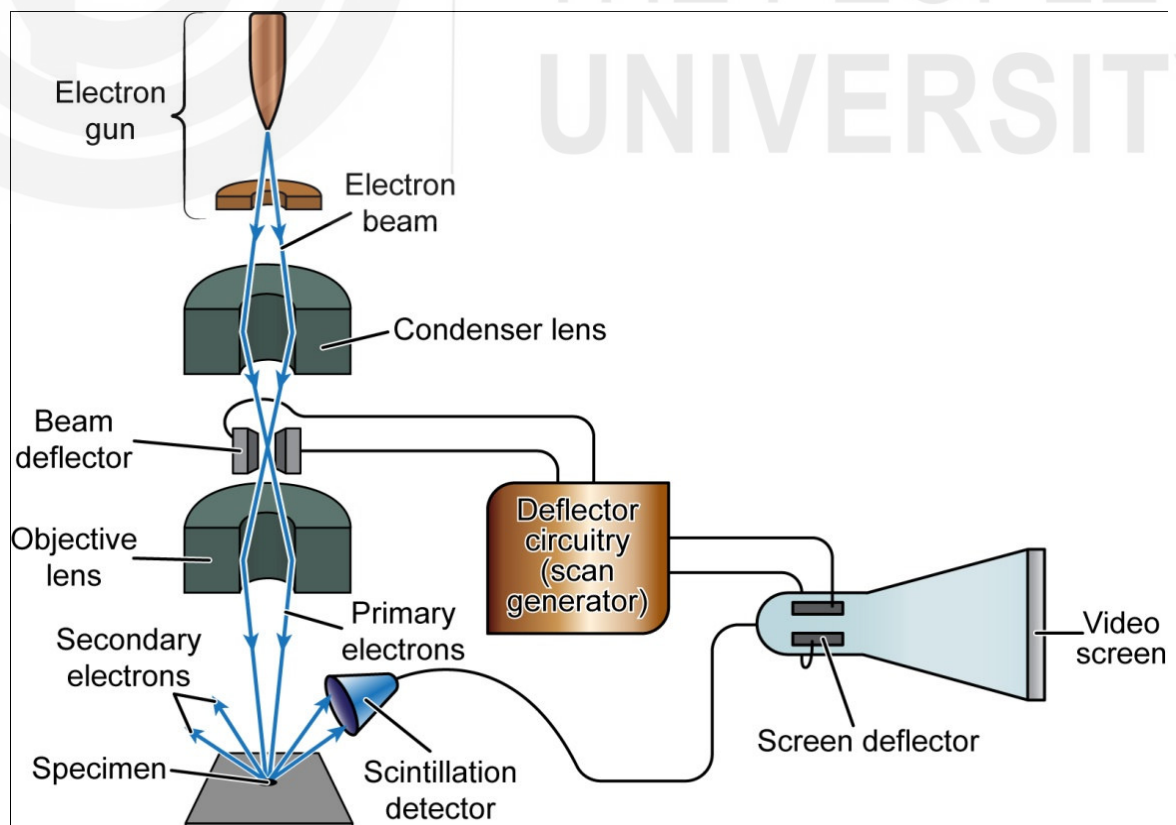


Fig. 1.15: Schematic diagram of a SEM.

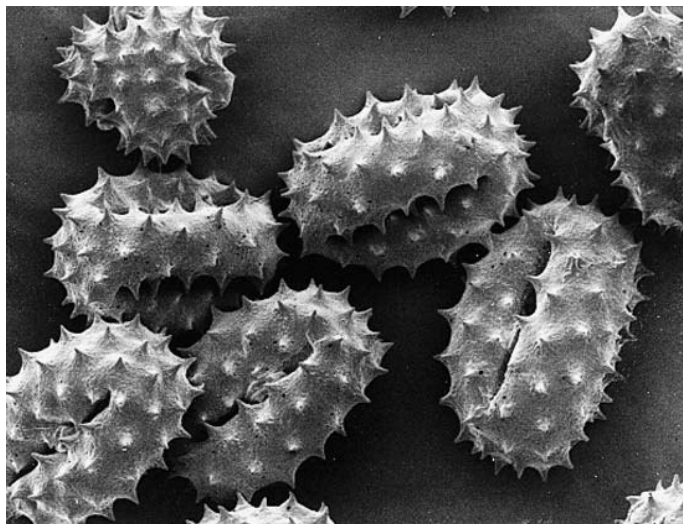


Fig. 1.16: Pollen structure of *Cineraria* plant showing spines as observed under scanning electron microscope (image magnified 1,500X).

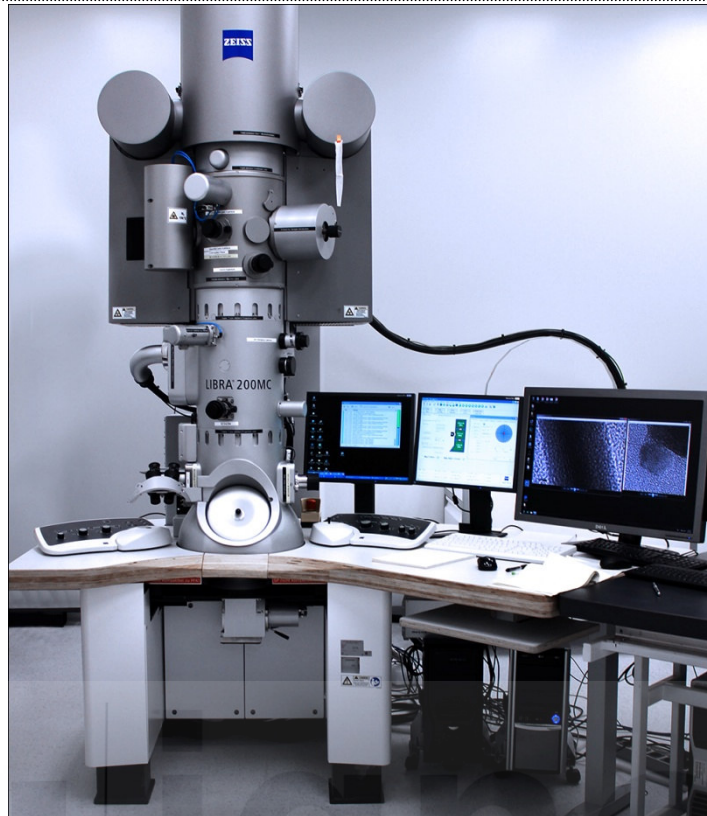
Sample preparation

The sample preparation for SEM involves various steps:

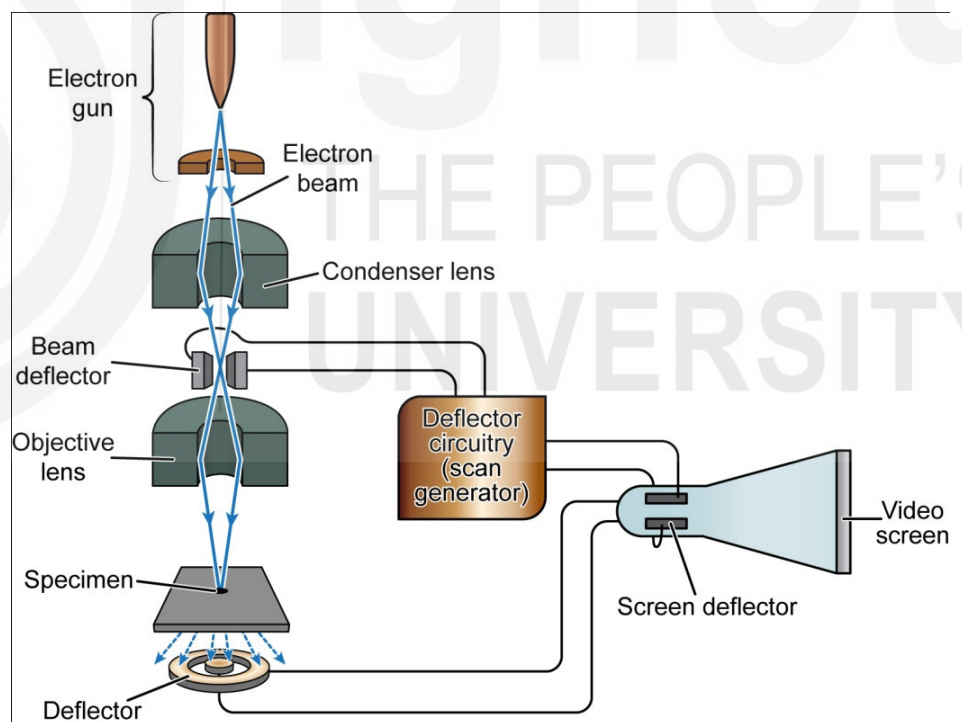
1. The material is fixed by immersing specimen in 2.8% glutaraldehyde [prepared in 0.1M Hepes buffer (pH 7.2, having 0.02% Triton X-100)] for several hours at room temperature or overnight at 4° C.
2. The fixed specimen is washed thrice (for 5 to 10 min) in 0.1 M Hepes buffer (pH 7.2).
3. The specimen is dehydrated by treating it with 25% ethanol for 10 min, 50% ethanol for 10 min, 70% ethanol for 10 min, 85% ethanol for 10 min, 95% ethanol for 10 min, and 100% ethanol for 10 min.
4. The automated process of Critical Point Drying is carried for 40 min.
5. The sample is mounted on metal stub with double sided carbon tape.
6. The sample is coated by applying a thin layer of metals (gold and palladium) using an automated sputter coater.

1.3.3 Scanning Transmission Electron Microscopy (STEM)

Scanning Transmission Electron Microscope combines elements of both TEM and SEM. STEM is a sophisticated technique and requires high vacuum conditions. It is electronically more complex than TEM or a SEM (Fig. 1.17 a). STEM is equipped with scanning coils, detectors and necessary circuitry. The image is formed by the secondary electrons since the primary electrons transmit light across the specimen. The electron beam is focused to a fine spot (size 0.05 – 0.2 nm) which is then scanned over the sample. The magnetic lenses focus beam of light that seeps over the surface of the specimen (Fig. 1.17 b). The sample is illuminated at each point with the beam parallel to the optical axis. The technique of STEM helps in distinguishing specific characteristics of the electron transmitted by the specimen, thus providing unique information about the specimen.



(a)



(b)

Fig. 1.17: a) Scanning Transmission Electron Microscope (STEM); b) Schematic diagram of STEM.

1.3.4 Environmental Scanning Electron Microscopy (ESEM)

Environmental Scanning Electron Microscope (ESEM) is a specialized SEM in which uncoated specimens are analysed. The first commercial ESEM was produced by the Electro Scan Corporation in USA in 1988. The instrument

produces high quality and resolution images in wet samples and those contained in low vacuum or gas (Fig. 1.18). The samples present even in low-pressure gaseous and high humidity (up to 100 %) conditions could be observed clearly. The technique enables the imaging of biological samples unstable in high vacuum conditions (conventional electron microscopes). This is because this microscope possesses a secondary-electron detector which is capable of operating in the presence of water vapour. The pressure-limiting apertures separate the vacuum region from the sample chamber. The specimen is placed in a relatively high-pressure chamber and the electron optical column is differentially pumped to keep vacuum adequately low at the site where the electron beam is exposed.



Fig. 1.18: Environmental Scanning Electron Microscope (ESEM).

ESEM is useful for studying non-metallic and biological materials (Fig. 1.19).

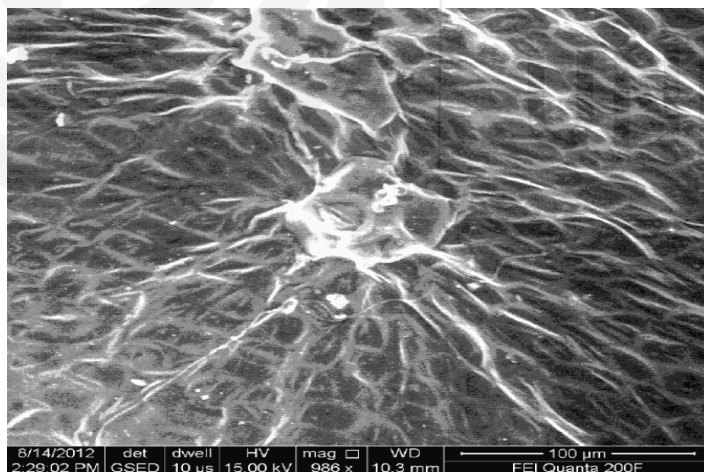


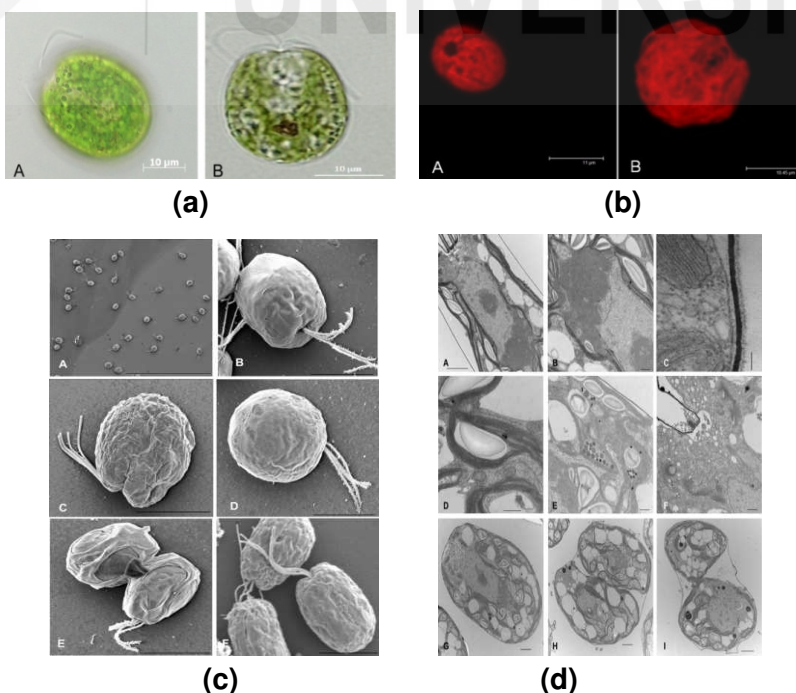
Fig. 1.19: Structure of epidermis with stomata as seen in ESEM.

1.3.5 Comparative Account of Different Types of Microscopes

Each microscope shows variations in its structure, technical details and produces images of characteristic type. The details about various microscopic techniques have been provided to you in the sections above, a summary of comparative account of the characteristics of different microscopes has also been given to you for more clarity (Table 1.1 and Fig. 1.20).

Table 1.1: Comparative account of different types of microscopes.

Characteristics	Compound Microscope	Confocal Microscope	Scanning Electron microscope (SEM)	Transmission Electron Microscope (TEM)
Source of illumination for image formation	Light	Laser light	Electrons	Electrons
Type of Lenses	Glass	Glass with dichromatic mirrors	One electrostatic and few electromagnetic lenses	One electrostatic and few electromagnetic lenses
Medium of image formation	Air	Air	Vacuum	Vacuum
Sample preparation	Sample preparation is not required	Sample preparation is not required	The specimen is coated with gold	Thin sections of the specimen are used.
Specimen mounting	Glass slides	Glass slides with dyed samples	Aluminum stubs	Coated or uncoated copper grids
Focusing, Magnification and Adjustment	Changing objectives	Digitally enhanced	Electrical	Electrical i.e., changing current of the projector lens coil
Means for obtaining specimen Contrast	Absorption of light	Laser light with dichromatic mirror concentrated at pinhole	Electron scattering	Scattering of electrons
Type of Image obtained	Two-dimensional image	Three-dimensional image.	3-D image is seen	2-D view image is formed.

**Fig. 1.20: Image micrographs of an algal cell as seen under different microscopes. a) Compound; b) Confocal; c) SEM; d) TEM.**

SAQ 2

a) Match the items in column I correctly with those of Column II.

Column I	Column II
i) Confocal microscope	1. invented by Manfred von Ardenne.
ii) SEM	2. uses electron as the source of illumination and 3-D images is formed
iii) TEM	3. uses laser light as the source of illumination
iv) Ultra-microtome	4. useful for non-metallic and biological materials
v) ESEM	5. used for the preparation of ultrathin sections

b) Fill in the blanks with appropriate words

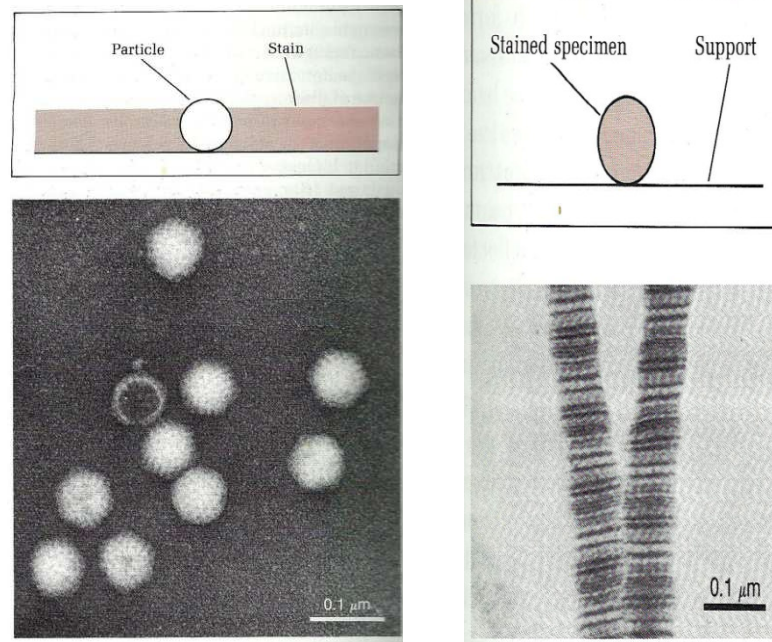
- i) In an electron microscope, a strong is required for the travel of path of electrons.
- ii) A system is attached to the column in an electron microscope to prevent heating of system due to high voltage of electrons.
- iii) In a transmission electron microscope (TEM), electron gun made up of filament cathode acts as the electron source.
- iv) is a more sophisticated and electronically more complex technique than TEM or SEM.
- v) A specialized SEM in which microanalysis can be performed on uncoated specimens is

1.4 TECHNIQUES FOR PREPARING TISSUES FOR ELECTRON MICROSCOPY

Technique of electron microscopy helps in characterizing the microstructural features of a sample ranging from 100 nm to 100 μm . Various methods pertaining to processing/preparation of samples have been developed over the years.

1.4.1 Negative Staining

Negative staining method is used for examining very small objects. It is generally used in TEM. The shape and surface characteristics of intact organelles or viruses can be examined using this technique. In the method, thin sections are not cut but particles are suspended in a small drop of liquid applied to copper grid and allowed to dry in air. The dried surface of sample is treated with stain such as phosphotungstic acid or uranyl acetate (Fig. 1.21). The specimen is visualized against the stained dark background.



NEGATIVE STAINING

POSITIVE STAINING

Fig 1.21: Electron micrograph of negatively stained wound-tumor virus (left) and positively stained collagen fibers (right).

1.4.2 Freeze-fracture

The technique involves physical breaking (fracturing) of a frozen biological sample along the planes of natural weakness that run through each cell. These planes generally occur between the two layers of lipid molecules which forms part of membrane and around various organelles of the cell. The main steps involved in formation of a freeze-fracture replica are; (i) rapid freezing, (ii) fracturing, (iii) formation of replica, and (iv) replica cleaning. The pretreatment carried out before freezing comprises fixation of material in glutaraldehyde followed by cryoprotection with glycerol. Cryoprotection is done to reduce the formation of ice crystals during freezing. This kind of sample preparation provides planar views of the internal organization of membranes. Images provided by these samples have proved useful understanding the functional morphology of the cell. This technique has been used to study membranes and reveal the pattern of integral membrane proteins.

General outline of technique:

- i) Cells are frozen in liquid Freon (-130°C). This results in instant immobilization of cell components.
- ii) The block of frozen cells is fractured in an evacuated chamber. The fracture is irregular and occurs along lines of weakness like the plasma membrane or surfaces of organelles.
- iii) A replica of the fractured specimen is made by shadowing with platinum (Fig. 1.22).
- iv) A carbon replica is produced when a thin layer of carbon is evaporated vertically onto the surface.
- v) Replica is left behind after digestion of organic material by acid.
- vi) The replica is studied under EM.

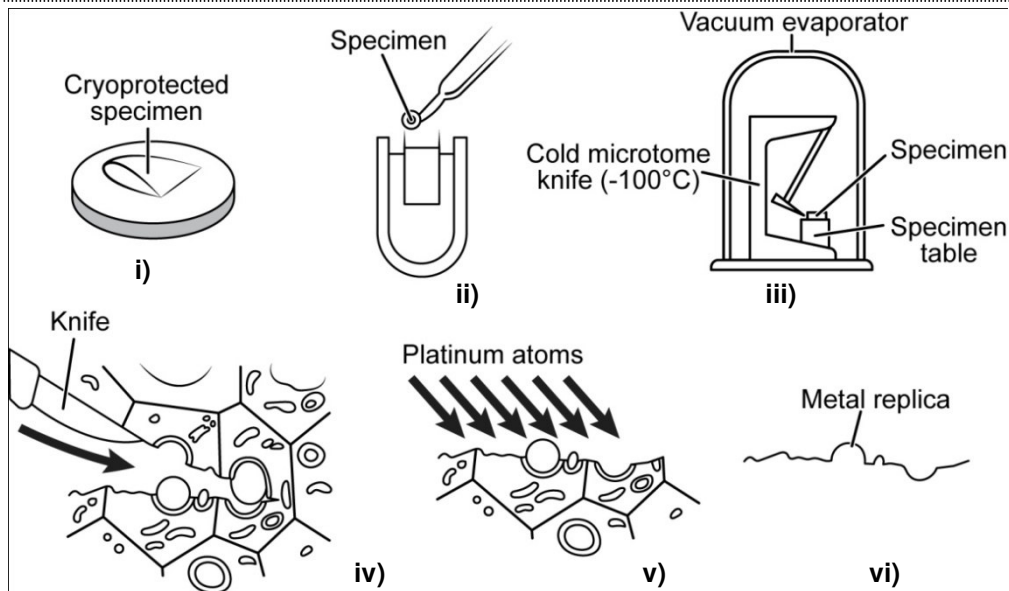


Fig. 1.22: Diagrammatic representation of various steps of freeze fracture technique (i-vi).

1.4.3 Freeze-Etching

The freeze-etching technique of sample preparation is related to freeze fracture. One more step is added to freeze fracture procedure. In this method, the specimens are rapidly frozen in liquid Freon under vacuum and struck with a sharp knife edge as in freeze fracture method (Fig. 1.23).

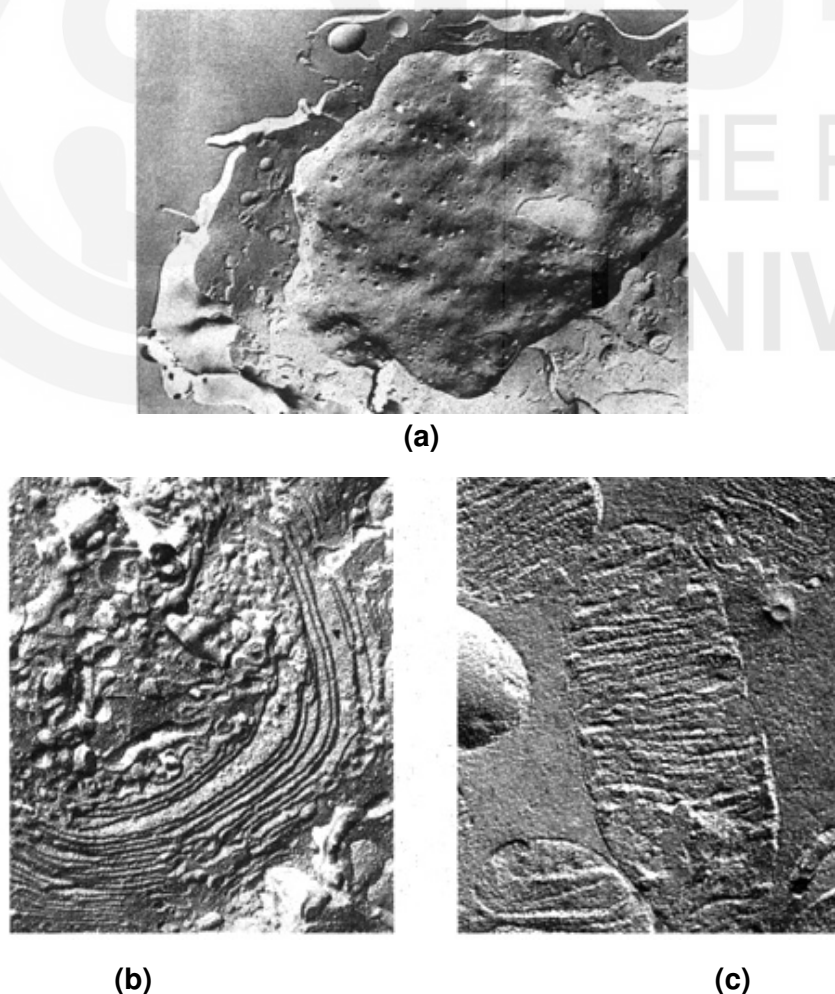


Fig. 1.23: Freeze fracture views of organelles a) entire cell showing nucleus and nuclear pores; b) Golgi apparatus; c) Mitochondria.

Biological samples fracture along lines of natural weakness at low temperature. The tissue is subjected to vacuum which results in sublimation of water from the fractured surfaces. This removal of water produces an “etching” effect. This etching will cause small areas of the cell surface around the periphery of the fracture face to stand out against the background. A replica of the specimen is made by heavy metal such as platinum, followed by backing it with a carbon film. After dissolving the tissue in strong acid, the remaining metal replica can be viewed with the electron microscope. Such preparations provide a unique picture of cells. This procedure is useful because it avoids exposure to fixatives, embedding agents, and stains which can deform ultrastructure of cell (Fig. 1.23). This type of specimen treatment causes minimal tissue distortion and permits immediate arrest of cell function

1.4.4 Shadow Casting

Shadow-casting is a technique which helps in studying surface texture of the specimen/material. The method involves in vacuum deposition of a metallic film on dried specimens. A three-dimensional electron beam image is produced using this technique. This process involves deposition of an electron dense substance upon the specimen at oblique angle so that only a part of the specimen is coated (Fig. 1.24). The deposition of metal on one side creates a “shadow” resulting in three-dimensional appearance.

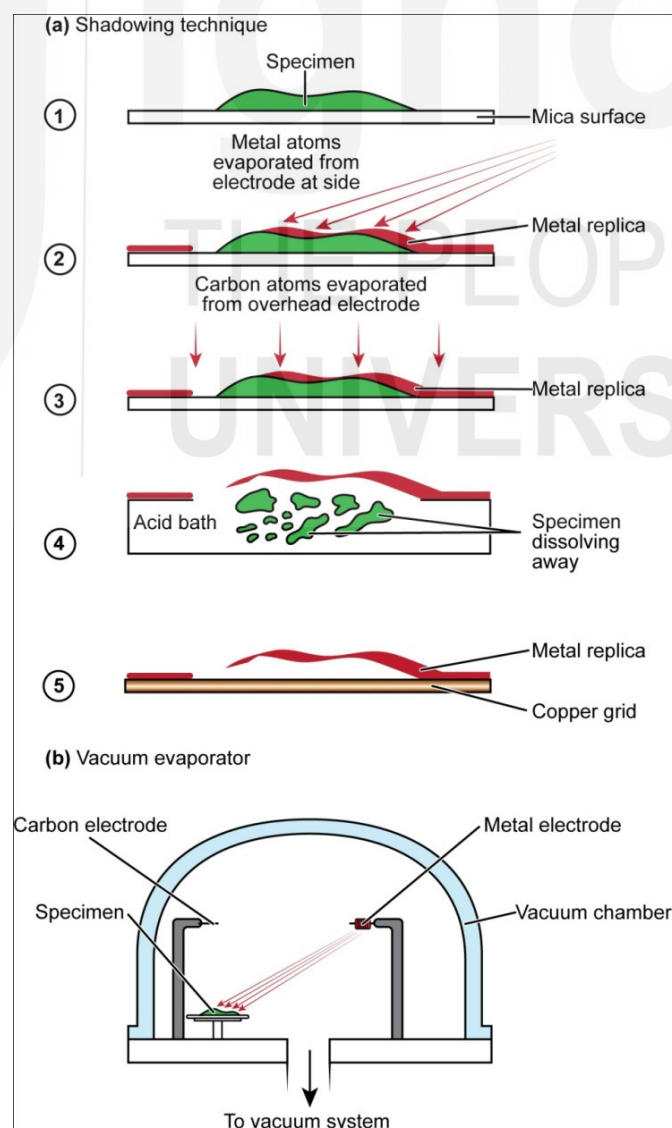


Fig. 1.24: a) Technique of shadow casting; b) Vacuum evaporator.

The thin metal film is formed on the specimen by condensation after vaporization. The metals with the high vaporization temperature condense quickly after vaporization (Fig. 1.25). The isolated particles can be visualized by placing them in an evacuated chamber and spraying heavy metal across their surfaces. The particle size of a film of evaporated gold will be larger than that of platinum or palladium. The "grain" size of evaporated tungsten is exceedingly fine, but its deposition time is long. The concurrent evaporation of two or more elements results in smaller aggregate size by increasing the distance over which any atom diffuses within a crystal lattice.

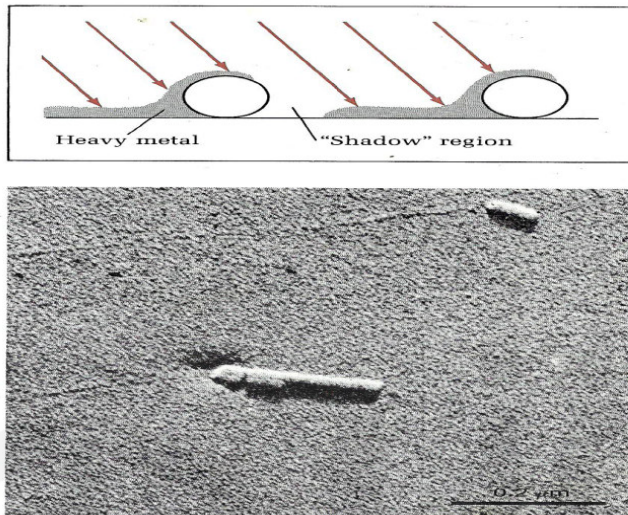


Fig. 1.25: Electron micrograph of Tobacco mosaic virus shadowed with uranium.

SAQ 3

a) Answer in one word:

- i) The process in which two or more two or more light waves combine to reinforce or cancel one another, producing a wave equal to the sum of the two combining waves.
- ii) The other name for Bright field microscope.
- iii) A fluorescent dye used for staining the microscopic samples.
- iv) Focused beams of laser light are scanned across the sample and light only from the desired focal plane can enter the detector.
- v) This type of microscopy is useful for visualising birefringent structure, which differ in refractive index in different planes.
- vi) In TEM technique, these fixatives stabilize the specimen's mobile macromolecular structure by chemical cross linking of protein.
- vii) The photographs of cells taken using an electron microscope.
- viii) The technique consists of physically breaking apart (fracturing) a frozen biological sample along the planes of natural weakness that run through each cell.

b) Enlist major differences between Freeze fracture and Freeze etching techniques.

1.4 SUMMARY

- Cell biology is the branch of biology that provides information about cell structure and function. A combination of microscopic and biochemical techniques has enhanced our knowledge and understanding of cell structure and function.
- The discovery of microscope has proved as a milestone in the study of cell biology as minute structural details and major cellular mechanism could be revealed by this technique.
- Initially, light microscope proved very useful in studying the structural details of cells, though lower resolution (200 nm) restricted the study of detailed cell architecture.
- Various other versions such as phase-contrast, confocal and fluorescence microscopes developed over the time provided a medium of studying structural details of the cell/sample/specimen present in any medium or form.
- The change of source of illumination from light to electrons significantly improved the resolving power of the microscope. The invention of the transmission electron microscope improved our understanding of cell biology but the sample to be viewed required preparation before use.
- Various advancements such as Transmission Electron Microscope (TEM) and Scanning Electron Microscope (SEM) proved very useful as they showed remarkable ability to study surface topography and 3D structure, along with improved resolution (30-100 Å). The electrons are transmitted through an object and then focused by the lenses to form the image in TEM while, the electrons are reflected by the object in a scanned pattern which forms the image in SEM.
- Scanning Transmission Electron Microscope (STEM) contains elements of both TEM and SEM. It provides unique information about the specimen. STEM requires high vacuum condition and electronically more complex than a TEM or a SEM. This microscope is equipped with additional scanning coils, detectors and necessary circuitry.
- A specialized SEM, Environmental Scanning Electron Microscope (ESEM) performs analysis using uncoated specimens. The samples are observed in low-pressure gaseous and high relative humidity (up to 100 %) environments. It produces quality images and gives good resolution even for wet samples or those contained in low vacuum or gas.
- Diverse set of procedures for specimen preparation, such as thin sectioning, negative staining, positive staining, shadow casting, whole mounting, and freeze-fracture has been developed so that minute architectural details, three-dimensional view of the cell surface can be captured, and details of the cellular processes can also be revealed.

1.5 TERMINAL QUESTIONS

1. Define the following terms
 - a) magnification
 - b) resolution of microscope
 - c) ESEM
 - d) STEM
 - e) negative Staining
 - f) shadow Casting
2. Discuss the factors which determine the resolving power of a microscope.
3. Describe various types of electron microscopes.
4. Differentiate between
 - a) simple and compound microscope
 - b) TEM and SEM
5. Enlist the differences between confocal and fluorescence microscopy.

1.5 ANSWERS

Self-Assessment Questions

1.
 - a)
 - i) magnification
 - ii) Frits Zernike
 - iii) fluorophore
 - iv) obliquely
 - v) microtome
 - vi) light
 - b) i) True; ii) False; c) True; d) False; e) True
 - c)
 - i) Refer to Subsection 1.2.1
 - ii) Refer to Subsection 1.2.1
 - iii) Refer to Subsection 1.2.1
 - d) Refer to Fig. 1.4
2.
 - a)
 - i) uses a laser light as the source of illumination
 - ii) invented by Manfred von Ardenne
 - iii) uses electron as the source of illumination and 3-D image is formed
 - iv) used for the preparation of ultrathin sections
 - v) useful for non-metallic and biological materials

- b)
 - i) vacuum
 - ii) cooling
 - iii) tungsten
 - iv) Scanning Transmission Electron Microscopy (STEM)
 - v) Environmental Scanning Electron Microscope (ESEM)
- 3. a)
 - i) interference
 - ii) compound light microscope
 - iii) fluorescein
 - iv) confocal microscopy
 - v) polarization microscopy
 - vi) formaldehyde and glutaraldehyde
 - vii) electron micrographs
 - viii) freeze-fracture
- b) Refer to Subsection 1.4.2 and 1.4.3

Terminal Questions

1. a) Magnification is the measure of the ability of a lens or other optical instruments to magnify. It is expressed as the ratio of the size of the image to that of the object.
- b) The smallest distance by which two points can be separated and distinguished as individual objects is called resolution. It is expressed in micrometres (μm).
- c) Environmental scanning electron microscopy (ESEM) is a unique system in which uncoated biological and industrial materials can be examined with an electron beam in a high pressure atmosphere. The specimens can be analyzed using ESEM without destruction/distortion and additional specimen preparation procedures. The surface of a solid sample is excited with a highly focused energetic beam of electrons which induces X-ray fluorescence from the elements within the sample.
- d) Scanning transmission electron microscopy (STEM) combines the principles of transmission electron microscopy and scanning electron microscopy and can be performed on either type of instrument. STEM requires very thin samples and looks primarily at beam electrons transmitted by the sample. STEM is a technically sophisticated microscope which is electronically complex than a TEM or a SEM. The STEM technique scans a very finely focused beam of electrons across the sample.

- e) Negative staining method is the simplest technique used in TEM for examining very small objects. Particles are suspended in a small drop of liquid applied to copper grid and allowed to dry in air. After drying, a drop of stain such as phosphotungstic acid or uranyl acetate is applied to the surface.
 - f) Shadow-casting is a technique which shows the surface texture of microscopic material. Sections or smears may be studied throughout the whole range of microscopic magnification. The method involves vacuum deposition of a metallic film on dried specimens. Metal is deposited from an oblique angle so that it coats some surfaces of specimen more than others.
2. The resolution is defined as the shortest distance between two points on a specimen that can still be distinguished by the observer or camera system as separate entities. The resolving power of a compound microscope depends on (a) wavelength of light used to illuminate the object, (b) refractive index of the medium between the object and the objective, and (c) the semi vertical angle of the cone of light from the object to the objective. The primary factor in determining resolution is the objective numerical aperture.
3. Refer to Section 1.3.
4. a) A microscope is a combination of magnifying glasses. If one lens is used in the microscope, it is called as simple microscope but when multiple lenses are used, they are called compound microscopes.
- b) Scanning Electron Microscope (SEM) uses electrons as the source of illumination. The image is seen in 3-D. with high magnification and high resolution. The specimen is coated with gold and the electrons are reflected and give the details of surface topography of the specimen.
- Transmission Electron Microscope (TEM) also uses electrons as the source of illumination and gives a 2-D view. Thin sections of the specimen are obtained. The electron beams pass through the sections and form an image with high magnification and high resolution.
5. Refer to Subsections 1.2.4 and 1.2.5.

Acknowledgement

Fig 1.1 : Source:
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- Fig. 1.3** : a) Source:
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b) <https://www.google.co.in/imgres?imgurl=https%3A%2F%2Fthumbs-prod.si-cdn.com>)
- Fig. 1.4** : Source: OpenStax Biology. Modification of work by "GcG"/Wikimedia Commons
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- Fig. 1.5** : Source:
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- Fig.1.19** : Source:
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You tube videos

<https://www.youtube.com/watch?v=icRQE73AUlI>

<https://www.youtube.com/watch?v=CVusz4wHaic>

<https://www.youtube.com/watch?v=gyOIFWhvUn0>

<https://www.youtube.com/watch?v=NG44AEWHtRQ>

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UNIT 2

CHROMATOGRAPHY |

Structure

- | | |
|-------------------------------|----------------------------|
| 2.1 Introduction | 2.3 Comparative Account of |
| Objectives | Different Types of |
| | Chromatography |
| 2.2 Chromatography | 2.4 Summary |
| Paper Chromatography | 2.5 Terminal questions |
| Thin Layer Chromatography | 2.6 Answers |
| Adsorption Chromatography | |
| Column Chromatography | |
| Ion Exchange Chromatography | |
| Gel Filtration Chromatography | |
| High Pressure Liquid | |
| Chromatography | |

2.1 INTRODUCTION

In the previous unit 1 you have studied about microscopes, their structure and function. The architectural and structural details of cells, cellular processes along with growth and developmental events in plants can be studied with the help of other various techniques. The present unit provides detailed information about various chromatographic techniques used in the study of cell biology of plants. Chromatography is a method used for separating different organic and inorganic components present in a compound. The chromatographic analysis provides detailed information about the various components present in test sample.

The technique of chromatography was invented by a Russian botanist **M.S. Tswett** in 1903 while studying the coloring materials i.e. pigments in plants. The word chromatography is derived from two words '**chroma**' means colour and '**graphy**' means writing. The technique became important because it could help in the separation of molecules present in biological samples. In this unit, you will study about the basic principles of chromatography, types of chromatography and their practical applications.

Objectives

After studying this unit, you would be able to :

- ❖ define chromatography;
- ❖ enlist the different types of chromatography;
- ❖ describe the principle of various chromatographic techniques such as ion exchange chromatography, column chromatography, adsorption chromatography and high-performance liquid chromatography;
- ❖ enumerate the differences between the different chromatographic techniques; and
- ❖ describe the practical applications of each of these chromatographic techniques.

2.2 CHROMATOGRAPHY

Chromatography is a technique used for separating the components from a mixture. The separation is based on various factors such as rate of mobility, relative amount, ionic composition, and binding affinity of molecules/compounds. Two major components in the chromatography technique are:

- Stationary phase- this represents a material or a liquid coated on the surface of a solid phase.
- Mobile phase- this represents a gas or liquid flowing over the stationary phase.

The mobile phase is a moving fluid i.e. solvent that moves through the stationary phase. The solid is called solute and the liquid or fluid is called solvent. When the mixture of molecules is applied onto the surface of solid, the molecules in the stationary phase get carried away with the fluid mobile phase. Kinetic motion helps in the exchange of solute molecules between the two phases. The properties such as adsorption (liquid-solid), partition (liquid-solid), affinity or differences in molecular weights regulate the separation process. Some molecules travel at a faster rate while others are retained for long by the stationary phase. The components having high molecular weight stay for long in the stationary phase while those having less molecular weight move rapidly through the chromatogram.

When mobile phase is liquid, the chromatography is termed as liquid chromatography (LC) and if the mobile phase is a gas, the chromatography is known as gas chromatography (GC). Liquid chromatography is generally used for thermo-unstable and non-volatile samples, while gas chromatography is applied for gases, and mixtures of volatile liquids and solid material.

Chromatography is an effective technique for separation and identification of small molecules such as amino acids, carbohydrates, and fatty acids. The technique has numerous applications in the field of biology, chemistry and

biochemical research. It has also found its application in separation and identification of chemical compounds present in a sample and petroleum industry for analyzing the hydrocarbon components.

Depending upon the stationary and mobile phase, chromatography has been categorized into different types- paper chromatography, thin-layer chromatography, column chromatography, ion-exchange chromatography, gel-permeation (molecular sieve) chromatography, affinity chromatography, gas chromatography, and high-pressure liquid chromatography (HPLC).

2.2.1 Paper Chromatography

In this type of chromatography paper is used as the stationary phase. The solvent runs on a piece of specialized paper mainly made up of cellulose. The movement of solvent carries the compound along with itself thereby separating the compound. The separation of the chemical substances depends upon the rate of migration across the paper. The substance gets distributed or partitioned between liquid phases. One mobile phase is water, which is held in the pores of the filter paper and other is the solvent which moves/runs over the paper. The movement of solvent occurs due to capillary action of pores present in the paper (Fig. 2.1). The compounds present in the sample get separated due to differences in their affinity towards water.

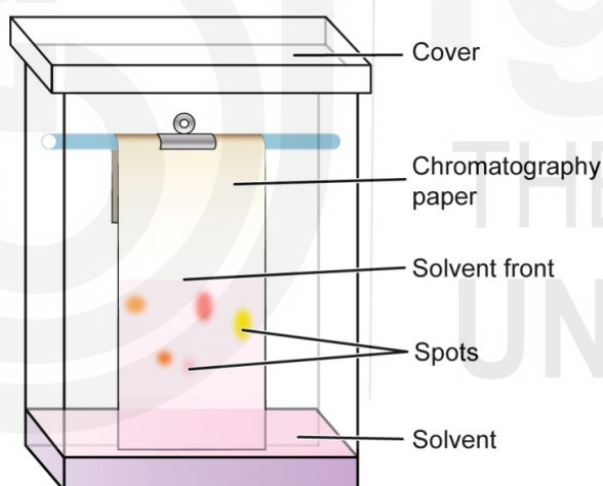


Fig. 2.1: Figure showing set up for ascending paper chromatography.

The sample is placed or applied as a spot near one corner of the paper. The edge of the paper near the spot is immersed in another solvent (in which components of the mixture are soluble). The solvent moves through the paper, travels across the sample and separates the various components of the sample in the process. The differences in relative solubilities of two solvents results in separation of the components. The separated components appear as spots which are later identified by applying reagents that form coloured compounds (Fig. 2.2).

Paper chromatography is an inexpensive method that requires small quantities of sample. It is commonly used for the separation of polar and non-polar compounds such as amino acids, peptides, carbohydrates, steroids, purines, and simple organic compounds.

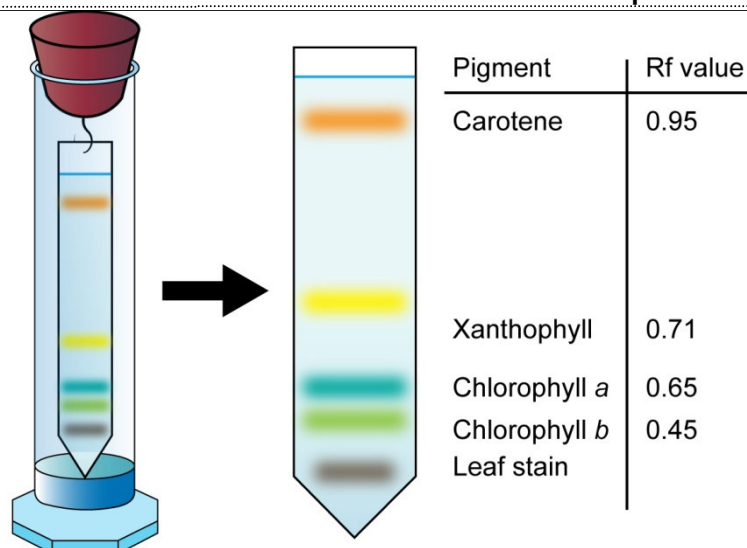


Fig. 2.2: Diagrammatic representation of a chromatogram showing separation of compounds using ascending paper chromatography.

Paper chromatography is of two types:

Ascending chromatography - In this type of chromatography, the solvent moves up i.e. in upward direction on the paper, while the solvent reservoir remains at the bottom. You have already performed this experiment (see Exp. 8, BBYCL-138)

Descending chromatography - In this case, solvent travels down the paper. The movement of solvent is assisted by gravity. The solvent reservoir remains at the top.

Besides these two types, other forms such as radial and two-dimensional modes in chromatography are also used.

2.2.2 Thin Layer Chromatography

Thin-layer chromatography (TLC) is a technique that separates the chemical compounds of a sample on the basis of properties of stationary and mobile phase. You will appreciate to know that this type of chromatography is similar to paper chromatography but the stationary phase used here is thin layer of adsorbent like silica gel, alumina, cellulose or an inert substrate. The solid adsorbent substance is coated on the glass plate and the mobile phase (solvent) moves upward through the stationary phase by means of capillary action (Fig. 2.3).

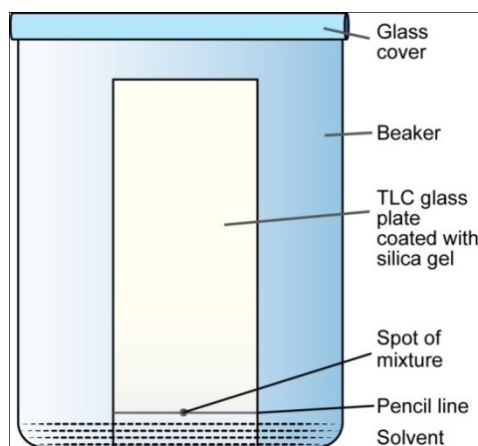


Fig. 2.3: Diagram showing set up of thin layer chromatography (TLC).

The components of a sample get separated depending upon the difference in the flow rates (Fig. 2.4). The flow rate of solvent depends on the polarity of the material, solid phase and solvent. A coloured (visible) product is formed using dye which can be observed under normal room light or UV light. In case of colourless samples/compound, radioactive labelling or fluorescent dye binding is done for visualization. The position of each molecule present in the mixture can be measured by calculating the ratio between the distances travelled by the molecule and the solvent. This measurement value is called **retention factor** and expressed by a symbol **R_f**. R_f value is used for quantitative description of the movement of molecules.

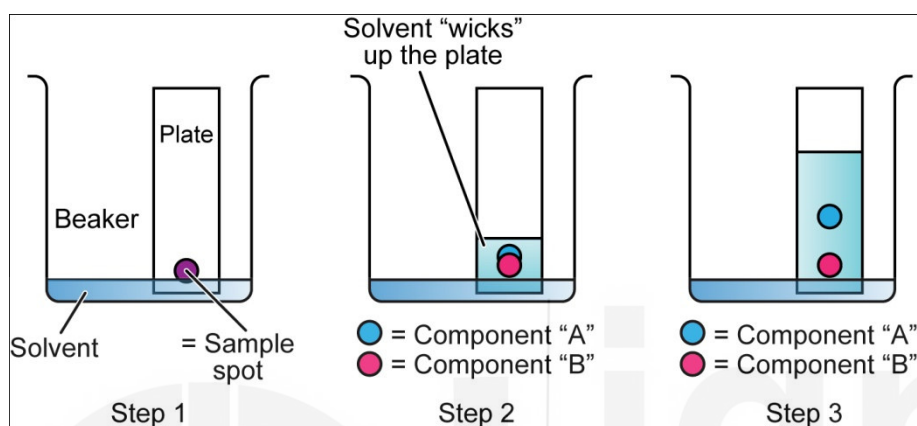


Fig. 2.4: Diagrammatic representation of various steps involved in TLC.

This technique helps in separation of multiple samples present on the same stationary phase. The technique provides fast and better separation of compounds which can be quantified. This type of chromatography helps in screening and testing of drugs.

2.2.3 Adsorption Chromatography

It is a type of chromatography where separation of a chemical mixture is based on the interaction of the adsorbate with the adsorbent. The adsorbent is the surface that adsorbs the substance and adsorbate comprises the molecules which get adsorbed on the adsorbent. The stationary phase (adsorbent) is made up of alumina or silica or calcium phosphate. Adsorbent is porous in nature and possesses high surface area that can adsorb substances with the help of intermolecular forces. The specific sites on the adsorbent help in interacting with the adsorbate. Efficiency of separation depends on the solubility of molecules in the mobile phase and binding strength of adsorbate (molecule/compound) to the stationary phase (adsorbent). Higher is the binding strength, slower is the movement of the adsorbate.

The sample consisting of molecules of varying porosity is passed through a column of polar adsorbent packed in a glass or steel tube. The polar molecules present in the sample interact with the stationary phase and retain in the column. The non-polar molecules move faster and elute out first because they do not interact with the adsorbent (Fig. 2.5). Some compounds get adsorbed strongly to form a zone at the top of the column while those showing weaker adsorption form a zone at bottom of the column. Compounds that do not get adsorbed simply run through the entire column and are collected first. The polarity of the mobile phase is increased for eluting highly polar molecules.

RETENTION FACTOR (R_f)

R_f is a quantitative indication of how far any compound travels in a particular solvent. R_f is the distance moved by the solute divided by the distance travelled by the solvent.

$$R_f = D_1/D_2$$

Where

D₁ is distance that a compound has travelled, and

D₂ is total distance travelled by the solvent.

Adsorption chromatography is used for determining the concentration or purity of a compound, or separation of individual components from a mixture. This technique is generally used for the separation of molecules such as proteins. The protein fractions (1 to 2 mL) can be collected after passing the sample through the column. The protein content in each fraction is estimated by measuring absorption at 280 nm using a UV spectrophotometer.

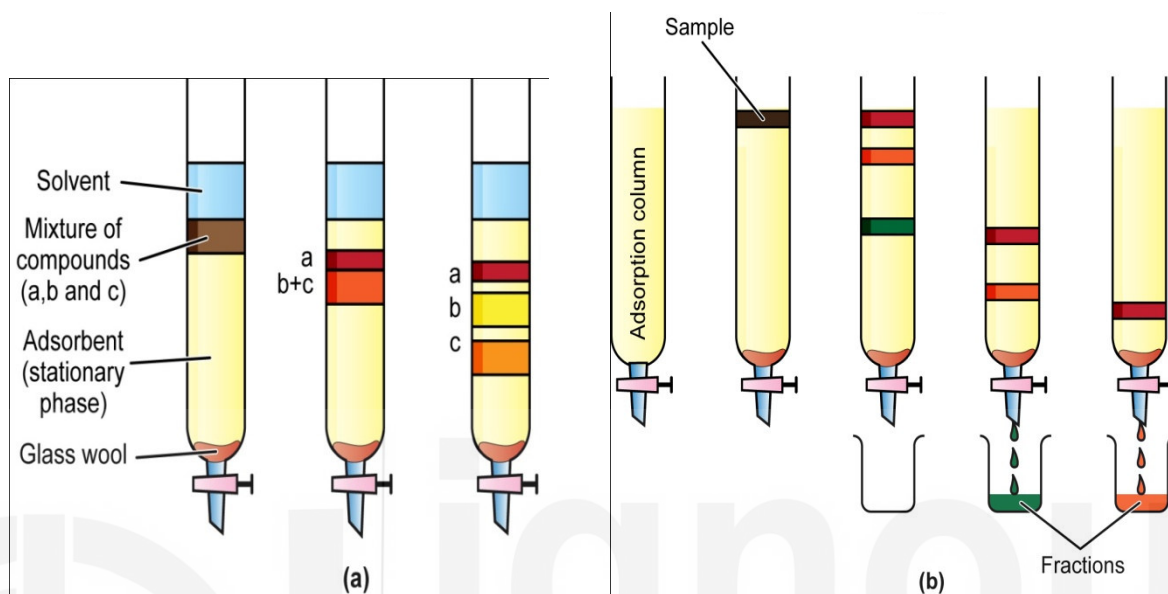


Fig. 2.5: a) The set up for adsorption chromatography (Diagrammatic representation); b) Various steps involved in separation of molecules.

2.2.4 Column Chromatography

In this type of chromatography, the sample is loaded on a column. The column acts as the stationary phase while a liquid or gas acts as the mobile phase. If the mobile phase used is a liquid, technique is called liquid column chromatography. When the mobile phase used is a gas, the technique is termed as gas chromatography. The sample moves through the column and the components of the sample get accumulated at the bottom of the column in a time and volume-dependent manner. The technique is generally used for separation of biomolecules.

Liquid column chromatography

In this technique the mixture is separated using two phases, a mobile phase which is a solvent, and an immobile phase which consists of a matrix or material packed in a column. The solvent moves through the column and reaches bottom and series of fractions get collected in tubes (Fig. 2.6). If we have proteins to be fractionated they are dissolved in the solvent and passed through the column. The protein molecules interact with the materials of the matrix, bind to sites present in the immobile phase and their mobility gets reduced. High affinity of a particular protein towards matrix lowers the speed of its passage through the column. Hence, more time is required for elution. The movement of proteins in the mixture varies according to their affinity for the matrix. The proteins with the least affinity for the matrix come out in the first fraction from the column.

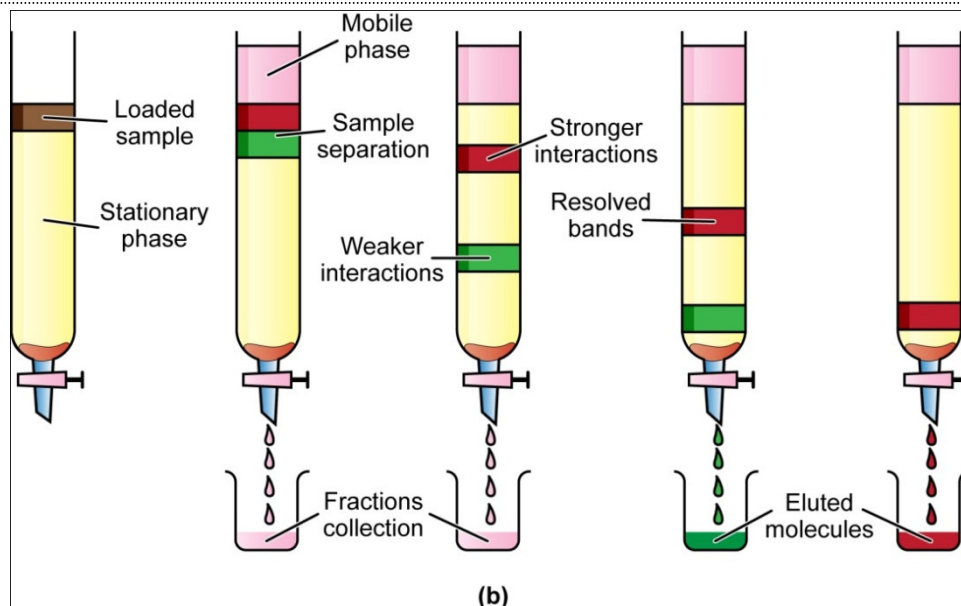


Fig. 2.6: Different steps of column chromatography.

Gas chromatography

In gas chromatography mobile phase is an inert gas which is passed through a column (stationary phase) under high pressure. Gases such as helium or hydrogen are generally used as a mobile carrier (Fig 2.7). The sample gets heated up, vaporizes and enters a gaseous mobile phase. The components contained in the sample are dispersed between mobile phase and stationary phase. This method is a simple, highly sensitive way of separating small molecules. Small number of analytes can be separated by using this technique.

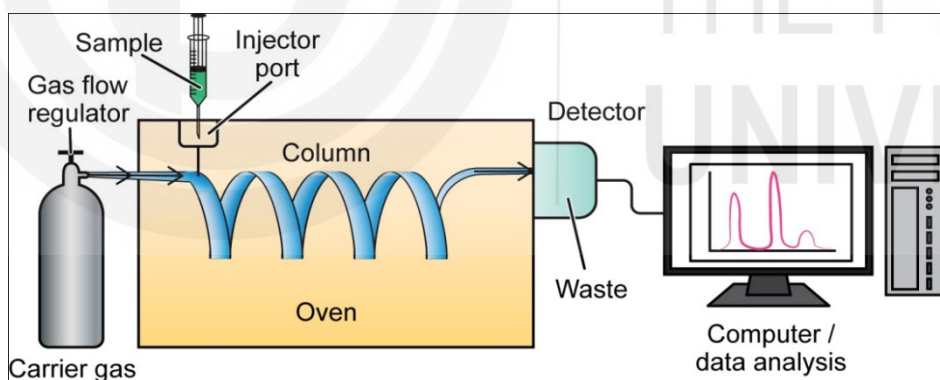


Fig. 2.7: Gas chromatography (Diagrammatic).

SAQ 1

- a) Fill in the blanks with the appropriate word.
 - i) Two important components of chromatography are and phase.
 - ii) In paper chromatography, solvent moves upwards on the paper.
 - iii) In adsorption chromatography separation is based on the interaction of with

- iv) In liquid column chromatography, the proteins with least for the matrix come out in the first fraction from the column.
- v) In paper chromatography solvent moves through the paper due to the of pores present in the paper.
- b) Differentiate between:
 - i) Solute and solvent
 - ii) Mobile and stationary phase
 - iii) Adsorbate and adsorbent
 - iv) Ion exchange and adsorption chromatography
 - v) Ascending and descending chromatography
- c) Match the following:

i) Thin-layer chromatography	1. sample is loaded on a column
ii) Adsorption chromatography	2. carrier gases such as helium or hydrogen
iii) Column chromatography	3. silica gel, alumina, cellulose
iv) Gas chromatography	4. adsorbate-adsorbent interaction

2.2.5 Ion Exchange Chromatography

Ion exchange chromatography or affinity chromatography is based on the principle of ionic bonding of the molecules (such as proteins) to an inert matrix material. The resin/inert material is packed in the column and the solution is allowed to percolate through it. Ion exchangers contain charged groups covalently linked to the surface of an insoluble matrix. Attraction occurs between sample and the oppositely charged stationary phase (ion exchanger).

The basic principle behind the separation is the exchange of ions present in the sample solution with those present on ion exchangers. Ions and polar molecules get separated based on their affinity with ion exchangers. Two types of exchangers viz. cationic and anionic are used in the process.

1. Cationic exchangers- They possess negatively charged groups and attract positively charged cations. Also known as “acidic ion exchange materials”. e.g., - carboxymethyl (CM) cellulose.
2. Anionic exchangers – They have positively charged groups that attract negatively charged anions. Also known as “Basic ion exchange materials”. e.g., - diethylaminoethyl (DEAE), cellulose.

The substrate, ligand, and antibody get covalently linked to an inert immobilized material or matrix present in the column. The charged groups of

the matrix get surrounded by ions of the opposite charge. The molecules get adsorbed to the solid support and this is carried with the help of ionic interaction between the oppositely charged ionic groups in the sample molecule (Fig. 2.8). In cation exchange chromatography positively charged molecules are attracted to negatively charged ions found on the matrix. On the contrary, in anion exchange chromatography, negatively charged molecules are attracted to positively charged ions on the matrix. The affinity of binding to the column depends upon the ionic strength of the matrix. The weakly associated ions get eluted out first.

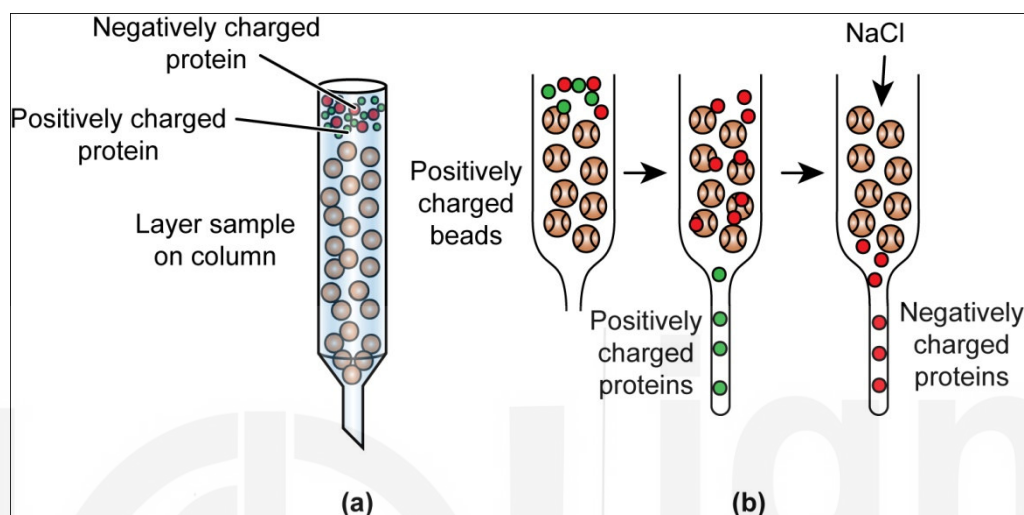


Fig. 2.8: Diagram showing a) set up for ion-exchange chromatography; b) Various steps in separation of molecules.

Ion exchange chromatography is the most popular method for the purification of proteins and other charged molecules. The specific proteins or molecules bind reversibly to the resin and specific type of protein gets removed from solution after passing through a column. The proteins binding to the matrix remain in the column (attached to the matrix) while free proteins leave the column. The bound proteins are then eluted by changing the ionic strength of the medium/buffer solution, altering pH or concentration of ion salts.

All proteins do not possess the same charge. The charge of a protein depends upon the amino acids. While charge of each amino acid depends on the pH of the medium. As the pH is lowered, the negatively charged groups become neutralized and opposite occurs if the pH is increased. The pH at which total number of negative charges of amino acids equals to total number of positive charges is called as **isoelectric point**. At this pH, the protein becomes neutral. The isoelectric point of most proteins is below 7.0.

The technique proves useful in studying interaction of enzymes with substrates, binding of receptors with ligands and binding of antigens with antibodies. The technique has been extensively used for the purification of enzymes, hormones, antibodies, nucleic acids, and specific proteins.

2.2.6 Gel Filtration Chromatography

In this technique (also known as molecular sieve technique) separation of molecules occurs with the help of tiny beads packed into a column. The gel used is Sephadex, which is a cross-linked dextran (produced by bacteria).

KDa (kilodalton) is non-SI unit of mass which is generally used to estimate the molecular mass of large molecules like proteins and polysaccharides.

1KDa = 1000 daltons

1 dalton = $1.6.660.33 \times 10^{-23}$ kg (one twelfth of the mass of an atom of carbon -12)

Agarose and polyacrylamide are also used as column material. The materials are composed of cross-linked polysaccharides of different porosity. These allow proteins to diffuse in and out of the beads. Sephadex G type is the most frequently used column material. Sephadex types such as G-30, G-50, G-100, G150, G-200 differ according to their pore size. Sephadex G-150 is used to separate proteins of size less than 200kDa.

The solution having molecules of different dimensions is passed through the column at a constant flow rate. The proteins move down through the column according to their molecular size. Larger molecules pass easily through spaces between porous particles. They show rapid movement through the column and get eluted out first (Fig. 2.9). The small molecules because of long retention time, leave the column later. The volume of the protein that is eluted out first is proportional to its molecular size.

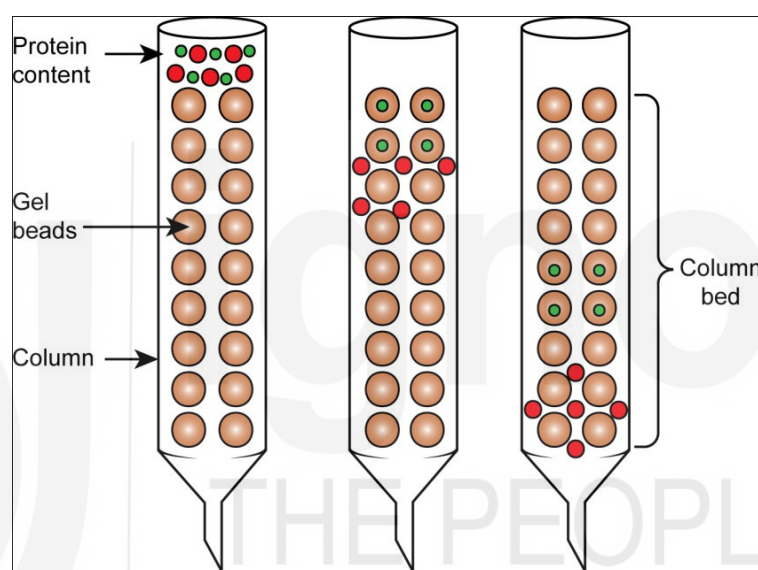


Fig. 2.9: Diagrammatic representation of set up for gel or molecular sieve chromatography.

The technique of gel filtration helps in separation of components based on difference in molecular weight or size. The molecules with larger size cannot enter the pore and move out of the column. If the mobile phase used is an aqueous solution, the process is called **gel filtration chromatography** and if the mobile phase used is an organic solvent, it is termed as **gel permeation chromatography**.

Gel filtration technique plays a key role in the purification of enzymes, polysaccharides, nucleic acids, proteins, and other biological macromolecules. The technique is used in protein fractionation experiments. The molecular weight of the compounds such as proteins can be determined using this technique. Columns calibrated with proteins of known molecular weight are used. This technique separates molecules primarily on the basis of their molecular size; hence molecular weights of molecules can be determined using this technique.

2.2.7 High Pressure Liquid Chromatography

It is an analytical technique that helps in the separation, identification and quantification of components present in a mixture. The separation occurs on

the basis of distribution of the analyte (sample) between a mobile phase (eluent/solvent) and a stationary phase (packing material of the column). The purification takes place in a column. The stationary phase is a granular material with very small porous particles, while the mobile phase is a solvent which is forced at high pressure into the column.

Analyte (a substance which is being analyzed for its constituents) is carried with a mobile phase at high pressure through a column having packing material (stationary phase). The carrier gas such as helium or nitrogen is used as the mobile phase. The molecules are held while passing the stationary phase depending on the chemical structure of the analyte. The interaction between the stationary phase, molecules analyzed, and solvent determine the retention time of the sample.

Long narrow columns packed with non-compressible matrix are used (Fig. 2.10). The smaller particle size of the column material provides greater surface area for interactions between the stationary phase and the molecules flowing through it. The mobile phase passes through columns under pressure of 10–400 atm and flow rate of 0.1–5 cm/s. Application of high pressure increases the rate of solvent flow. As the sample passes through the column it interacts with two phases at different rates, primarily due to different polarities in the analytes. Analytes that have less interaction with the stationary phase exit the column faster. After leaving the column, substances are identified by a suitable detector and signal is passed to computer. The computer provides image of chromatogram.

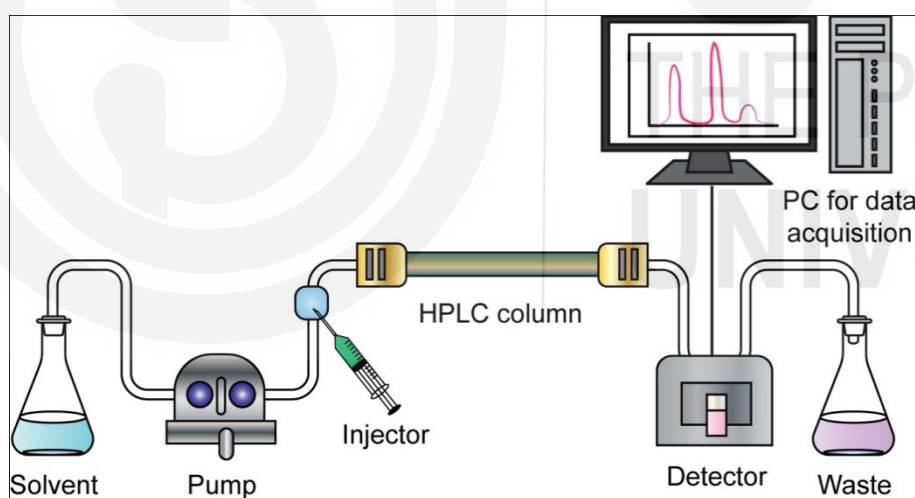


Fig. 2.10: Diagrammatic representation of set up for High pressure liquid chromatography.

It is a highly automated and sensitive technique used to separate compounds that are dissolved in a liquid and present at very low concentrations even in **trace amounts** (parts per million). Identification and quantification of various components can be done using this technique. The technique proves very useful for separation, identification, structural and functional analysis and purification of molecules such as amino acids, carbohydrates, lipids, nucleic acids, proteins, steroids, and many other biologically active molecules. The technique finds its practical use in analysis of drugs, synthetic polymers, pollutants in environment, water purification, and detection of trace components.

SAQ 2

- a) State whether these statements are 'True' or 'False':
- i) In gel filtration chromatography the proteins are separated primarily based on their molecular weight.
 - ii) HPLC can be used to detect compounds present in trace amounts.
 - iii) All proteins possess same charge.
 - iv) In ion exchange chromatography, proteins bind to an inert matrix material containing covalently linked charged groups.
 - v) The specific protein makes a complex with the ligand attached to the matrix.
 - vi) The protein with the greater affinity for the matrix material shows slower velocity while passing through the column.
- b) Answer in one word:
- i) Stationary phase used in thin layer chromatography.
 - ii) This paper is used as the stationary phase in paper chromatography.
 - iii) Mobile phase is an inert gas in this type of chromatography.
 - iv) The measurement of relative mobility of compound is given by this value.
- c) Fill in the blanks:
- i) and are the ion exchange resins commonly used in affinity chromatography.
 - ii) The pH at which total number of negative charges equal total number of positive charges is called
 - iii) Dextran, agarose and polyacrylamide, used as column materials are composed of
 - iv) In gel filtration chromatography the separation of compounds occurs because of difference in their and
- d) What is the principle of gel filtration chromatography?
- e) What detailed note on high pressure liquid chromatography.

2.3 COMPARATIVE ACCOUNT OF DIFFERENT TYPES OF CHROMATOGRAPHY

As you have studied about different types of chromatography in the previous sections, a comparative account of all these types is provided to you in a tabular form for quick review (Table 2.1)

Table 2.1 : Comparison between different types of chromatography

Type of chromatography	Principle	Uses
Paper chromatography	Paper is the stationary phase. capillary action pulls the solutes up through the paper and separate them	This chromatography is used to separate amino acids, anions, histamines and antibiotics.
Thin layer chromatography (TLC)	Thin layer adsorbent such as silica is the stationary phase. The mobile phase (solvent) moves upward by means of capillary action	This chromatography is used to check purity of organic compounds and to detect the presence of pesticides residues.
Gas chromatography (GC)	Helium or Nitrogen is used as the carrier gas to move compound through the column.	This chromatography is used to calculate the concentration of chemicals found in biological samples. Even small (trace) concentration of compounds can be detected using this technique.
Gel filtration chromatography	The molecules get partitioned between solvent (mobile phase) and gel matrix which acts as the stationary phase.	This chromatography is used to separate molecules such as proteins, RNA, DNA particles, and viruses
Adsorption chromatography	Separation of chemical mixture is based on the interaction of adsorbate with the adsorbent.	This technique is used for determining purity of compound, find its concentration and separation of individual components.
Column chromatography	The solvent is the mobile phase which carries along with it the material which is fixed (stationary phase)	This technique is used for quality control in food industry for analyzing preservatives and additives.
Ion exchange chromatography	The ions and polar molecules are separated on the basis of their affinity towards the matrix.	This technique is used for purification of proteins and other charged molecules.
High Pressure liquid chromatography (HPLC)	The separation of compound occurs on the basis of distribution of analyte (sample) between mobile phase (eluent/solvent) and the stationary phase.	This technique is used for separation of compounds present in trace amounts (ppm). the technique is useful for separation of molecules such as proteins, lipids, carbohydrates, steroids and other biologically active molecules.

SAQ 3

- a) Give a comparative account of different types of chromatography in a tabular form.
- b) Draw a neat, well labelled diagram to show the set-up of gel filtration chromatography.

2.4 SUMMARY

- Chromatography technique is used for the separation of mixture of molecules. The two major components in chromatography are stationary and mobile phase. Stationary phase is a solid phase, or it is a liquid phase coated on the surface of a solid phase. Mobile phase is a gaseous or liquid phase. The molecules in the stationary phase move along with mobile phase. If mobile phase is liquid, it is termed as liquid chromatography (LC), and when gas is used as a mobile phase, it is called gas chromatography (GC).
- The column chromatography is used for the purification of biomolecules. The mixture is separated using two phases, a mobile phase consisting of moving solvent and an immobile phase consisting of matrix. The sample is loaded on a column (stationary phase) followed by buffer (mobile phase). The samples get accumulated at the bottom of the column in a time and volume-dependent manner.
- Ion exchange chromatography is based on the principle of ionic bonding of the proteins to an inert matrix material. The resin is packed in the column and the solution is allowed to percolate through the column. The weakly associated ions get eluted out first, followed by strongly bound ions.
- In gel filtration or molecular sieve chromatography, the separation is carried out with the help of column packed with material such as sephadex, dextran, agarose or polyacrylamide. The proteins or nucleic acids get separated primarily based on their molecular size. The stationary phase consists of inert molecules with small pores. Larger molecules pass through spaces between porous particles, move rapidly and are eluted out first. The movement of molecules through the column varies according to their molecular size.
- HPLC technique separates and quantifies components present in a mixture. The separation is based on the distribution of the analyte (sample) between a mobile phase (eluent/solvent) and a stationary phase (packing material of the column). The sample is carried by a moving carrier gas stream of helium or nitrogen. A sample is pumped in a solvent (mobile phase) at high pressure through a column with stationary phase.

2.5 TERMINAL QUESTIONS

1. Enumerate the applications of column chromatography.
2. List the advantages of High-pressure liquid chromatography (HPLC).
3. What is the principle of ion exchange chromatography?
4. Write the full form of these abbreviations
 - a) TLC
 - b) DEAE
 - c) HPLC
 - d) CM
5. Differentiate between:
 - a) Paper and column chromatography
 - b) Adsorption and Ion exchange chromatography

2.6 ANSWERS

Self-Assessment Questions

1.
 - a)
 - i) stationary and mobile
 - ii) ascending
 - iii) adsorbate, adsorbent
 - iv) affinity
 - v) capillary action
 - b)
 - i) Solute- the solid or liquid compounds that need to be separated by chromatography

Solvent- the liquid or gas phase that carries the solute during chromatography
 - ii) Mobile phase- moving fluid or gas that carries the solute or compound to be separated by chromatography

Stationary phase - the solid or compound that gets separated by chromatography
 - iii) Adsorbate- the molecules which get adsorbed on the adsorbent.

Adsorbent- the surface on which adsorbate gets attached. This possesses specific site where interaction with the adsorbate occurs.
 - iv) Ion exchange chromatography-It is based on the principle of ionic bonding of the proteins to an inert matrix material such

as cellulose, containing covalently linked charged groups. Ion exchange resins used in the process are diethylamino ethyl (DEAE) cellulose and carboxymethyl (CM) cellulose. The resin is packed in the column and the protein solution is allowed to percolate through the column. Proteins bind to the resin reversibly. DEAE cellulose is positively charged and binds negatively charged molecules, while CM cellulose is negatively charged and acts as a cation exchanger. Proteins are eluted from the column based on their binding. The weakly associated ions get eluted out first followed by strongly bound ions.

Adsorption chromatography- In this technique, separation is based on the interaction of the adsorbate with the adsorbent. The adsorbent possess specific site where its interaction with the adsorbate occurs. The efficiency of the separation depends on the solubility of the molecule in the mobile phase and the binding strength to the stationary phase. Greater is the binding strength slow is the movement of the adsorbate. The sample interacts with adsorbent packed in the column. The polar molecules interact with the stationary phase and are retained in the column. The non-polar molecules move faster and elute out first.

- v) **Ascending chromatography-** In this type of chromatography, the chromatogram ascends i.e., solvent movement occurs towards upward on the paper. The solvent reservoir is at the bottom.

Descending chromatography- In this case, solvent travel downwards on the paper i.e., movement of solvent is assisted by gravity. The solvent reservoir is at the top.

- c) i) silica gel, alumina, cellulose
 ii) adsorbate -adsorbent interaction
 iii) sample is loaded on a column
 iv) carrier gases such as helium or hydrogen
2. a) i) False; ii) True; iii) False; iv) True; v) True; vi) True
 b) i) silica gel/alumina/cellulose or an inert substrate
 ii) cellulose filter paper
 iii) gas chromatography
 iv) R_f value
 c) i) diethylaminoethyl (DEAE) cellulose and carboxymethyl (CM) cellulose
 ii) isoelectric point
 iii) cross linked polysaccharide
 iv) molecular weight, size

- d) Refer to Subsection 2.2.6
- e) Refer to Subsection 2.2.7
- 3. a) Refer to Section 2.3
- b) Refer to Subsection 2.2.6 and Fig. 2.9.

Terminal Questions

- 1. Refer to Subsection 2.2.4
- 2. Refer to Subsection 2.2.7
- 3. Ion exchange chromatography is based on the principle of ionic bonding of the proteins to an inert matrix material such as cellulose, containing covalently linked charged groups. This chromatography is based on electrostatic interactions between charged protein groups and solid support material (matrix). The affinity of the protein binding to the column depends upon ionic strength of the matrix.
- 4. a) Thin layer chromatography
- b) Diethyl aminoethyl
- c) High performance liquid chromatography
- d) Carboxymethyl
- 5. a) Refer to Subsections 2.2.1 and 2.2.4
- b) Refer to Subsections 2.2.3 and 2.2.5

Acknowledgement

You tube videos

https://www.youtube.com/watch?v=vum3y2_5XaE

<https://www.youtube.com/watch?v=iHrKsfw827c>

TECHNIQUES IN MOLECULAR BIOLOGY

Structure

3.1	Introduction	Differential centrifugation
	Objectives	Ultracentrifugation
3.2	Spectrophotometry	3.4 X-ray Diffraction
	Principle of spectrophotometer	3.5 Electrophoresis
	Working and applications of spectrophotometer	3.6 Summary
3.3	Centrifugation	3.7 Terminal questions
	Density gradient centrifugation	3.8 Answers

3.1 INTRODUCTION

In the previous unit you have studied about the chromatography techniques used for the detection and separation of some biological compounds. This unit will introduce you to some other techniques used in cell and molecular biology. These techniques also help in the separation of various molecules, compounds and cellular structures such as cell organelles. The components separated using these techniques increase our knowledge about structural details and various cellular processes associated with them. The present unit provides detailed information about spectrophotometry, centrifugation, X-ray diffraction analysis and electrophoresis techniques.

Objectives

After studying this unit, you would be able to :

- ❖ describe the principles and application of spectrophotometry;
- ❖ describe the technique of centrifugation;
- ❖ enlist different types of centrifugation;
- ❖ describe the technique of X-ray diffraction analysis; and
- ❖ provide a detailed information about electrophoresis technique.

3.2 SPECTROPHOTOMETRY

Spectrophotometry is a technique that measures the quantity of compounds present in any solution. It is the branch of spectroscopy that measures electromagnetic radiation absorbed, transmitted or reflected by any chemical substance over a certain range of wavelength. The technique of spectrophotometry is used for quantitative analysis of various chemical compounds. Spectrophotometer is the instrument used for this purpose. The concentration of a substance present in a solution can be quantified by measuring light absorbed by it at a particular wavelength. Spectrophotometers have been classified into two types on the basis of range of wavelength of light source used in the instrument:

- 1) **UV- Visible Spectrophotometer:** In this type of spectrophotometer absorbance can be measured in the UV (ultraviolet) range (185 to 400nm) and visible region of the electromagnetic spectrum (400 to 700nm).
- 2) **IR –Spectrophotometer:** This type of spectrophotometer measures absorbance in the infrared range (700 to 1500nm) of electromagnetic spectrum.

3.2.1 Principle of Spectrophotometer

Working of a spectrophotometer is based on the photometric technique. When a beam of light is passed through the coloured solution, some amount of light gets absorbed and rest gets transmitted. A coloured solution absorbs all colours of white light but transmits its own colour. Spectrophotometer measures the amount of light that a sample absorbs when a beam of light is passed through it. A beam of incident light of intensity (I_0) passes through a solution, a part of the incident light is reflected (I_r), a part is absorbed (I_a) and rest of the light is transmitted (I_t). The spectrophotometer works on the principle of Beer-Lambert's law. This law states that the amount of light absorbed by a colored solution is directly proportional to the concentration of the solution. It is represented by the equation

$A \propto cl$ where,

A = Absorbance / Optical density of solution

c = Concentration of solution

l = Path length

or $A = \epsilon cl$

ϵ = Absorption coefficient

Spectrophotometer helps in the quantitative measurement of coloured compound present in the sample.

3.2.2 Working and Applications of Spectrophotometer

A spectrophotometer consists of a light source, a monochromator which is a wavelength selection device, filter that produces rays of single wavelength, a

sample chamber, a light detector, a display device, and computer storage set up (Fig. 3.1). Spectrophotometer can be single beam or double beam. Single beam spectrophotometer uses a single beam of light and operates between 325 nm to 1000 nm wavelength. In this type of instrument, light travels in one direction and same light source is used for reading blank and samples. Double beam spectrophotometer operates between 185 nm to 1000 nm wavelength. It has two photocells. This instrument splits the light from the monochromator into two beams. One beam is used for reference and the other for sample reading. It gives more accurate and precise results because the errors occurring due to fluctuations in the light output and the sensitivity of the detector get eliminated in the process.

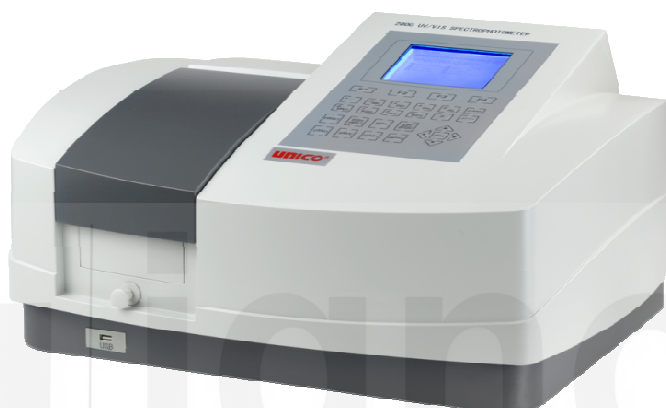


Fig. 3.1: Photograph of a spectrophotometer.

The instrument is calibrated using the standard solutions. The calibration is done with the known concentration of the solute whose quantity needs to be determined in the test solution. The standard solution is filled in the cuvette and placed in the cuvette holder present in the spectrophotometer. A ray of light of certain wavelength (specific for the assay) is directed towards the solution. The ray of light passes through a prism. The prism splits the beam of light into different wavelengths. When the monochromatic light (light of one wavelength) reaches the cuvette containing the standard or test solution, some amount of light is absorbed by the solution and the remaining is transmitted through the solution. The photodetector system measures the intensity of transmitted light and converts it into the electrical signals. The electrical signals are displayed in the digital form. The digits represent the absorbance or optical density of the solution analyzed. If the absorption of the solution is higher than more light will be absorbed by the solution (Fig. 3.2).

The absorption spectrum varies from UV (ultra-violet) or visible region of the electromagnetic spectrum depending upon the compound quantified.

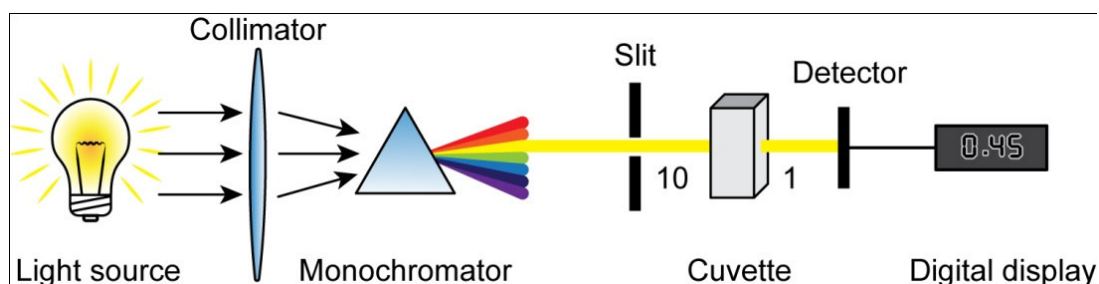


Fig. 3.2: Diagram showing basic set up of a spectrophotometer.

Applications

The spectrophotometer is commonly used for determining the concentration of coloured as well as colourless compounds via measurement of optical density or absorbance. It can also be used for determining the course of the reaction by measuring rate of formation and disappearance of the compound in the visible and UV region of electromagnetic spectrum. Spectrophotometer is also used in various industries like paints, plastics, paper, metals and fabrics.

SAQ 1

- State the law on the basis of which spectrophotometer works.
- Define spectrophotometry. Draw a neat and labelled diagram showing the basic setup of a spectrophotometer.

3.3 CENTRIFUGATION

Centrifugation is a technique that is based on the use of the centrifugal force to separate particles from a solution according to their size, shape, and density. The principle behind the separation of components from suspension is the centrifugal force/acceleration. The acceleration/force causes the denser molecules to move towards the periphery while the less dense particles move to the centre.

This technique involves various steps. The samples are homogenized and the suspension is placed in a centrifuge tube. The tube is then placed in a rotor and spun at a defined speed (Fig. 3.3). The rotation at a fixed point (axis), result in strong force perpendicular to the axis. The perpendicular force created during the rotation helps in the separation of components. The rate of centrifugation depends on size and density of the particles present in the solution. The components move with different speeds depending upon their size and form series of bands. In a homogenate suspension, the cell components are separated on the basis of their size.



Fig. 3.3: Photographs of benchtop centrifuge and refrigerated high speed centrifuge.

Relative centrifugal force (RCF or g) is the force acting perpendicular on the sample relative to the gravity of the earth. It is the force exerted on the contents of the rotor as a result of the rotation. The formula to calculate the relative centrifugal force (RCF/g) is given as:

$$\text{RCF (g)} = 1.118 \times 10^{-5} \times r \times (\text{rpm})^2$$

where r is the radius of the rotor (in centimeters), and rpm (revolution per minute) is the speed of the rotor.

The technique helps in separation of various cell components.

3.3.1 Density Gradient Centrifugation

rpm/g can be converted into RCF by the following formula.

$$\text{rpm} = \sqrt{\left[\frac{\text{RCF}}{r} \times 1.118 \right]} \times 1 \times 10^5$$

In this type of centrifugation, the molecules present in a heterogeneous mixture settle down or get sedimented under a centrifugal force or under the influence of gravity. The cell components or contents of sample get separated as distinct bands. The distribution of contents in different layers depends upon the density. Larger and denser particles sediment faster in comparison to small, less dense particles. A sucrose solution is used in the process. The centrifuge tube is filled with the gradient of sucrose and sample solution is layered on the top of the column. The density of the solution increases towards the bottom of the centrifuge tube (Fig. 3.4). The rate of sedimentation depends upon the size of components. This is known as sedimentation coefficient (Svedberg units). The **Svedberg unit (S)** is a measure of the sedimentation rate of a particle when centrifuged. It is a measure of time and is equal to the **value** of 100 femto seconds (10^{-13} s).

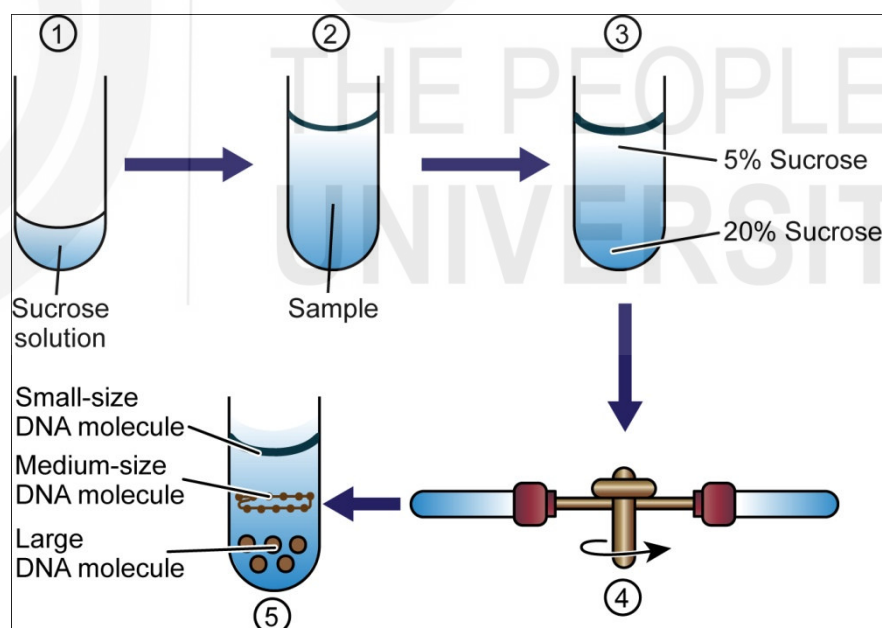


Fig. 3.4: Diagrammatic representation of various steps involved in density gradient centrifugation.

The molecules such as DNA and RNA get separated by this technique. The speed of 80,000 rpm produces a force equivalent to 500,000 times of gravity which helps in the separation of small molecules (such as tRNA). Various cellular activities such as synthesis of membrane bound proteins, formation of transport vesicles, DNA synthesis and transport of solutes etc can be studied using this technique. The technique can be applied for the purification of biomolecules and separation of cell organelles.

3.3.2 Differential Centrifugation

In this type of centrifugation, components get separated under the influence of increasing centrifugal force. The basic principle of the technique is that rate of sedimentation of biological particles differs due to difference in size and density. The sedimentation of the larger molecules occurs under the influence of high centrifugal force. The sedimentation of smaller molecules takes longer time and requires much higher force. The large particles form a pellet on the bottom of the centrifuge tube, while small particles remain in the supernatant. The settling of the particles depends upon the speed, time of centrifugation, density and relative size of the particles.

Centrifugal force

An outward force experienced by an object moving in a circular path directed away from the center of rotation.

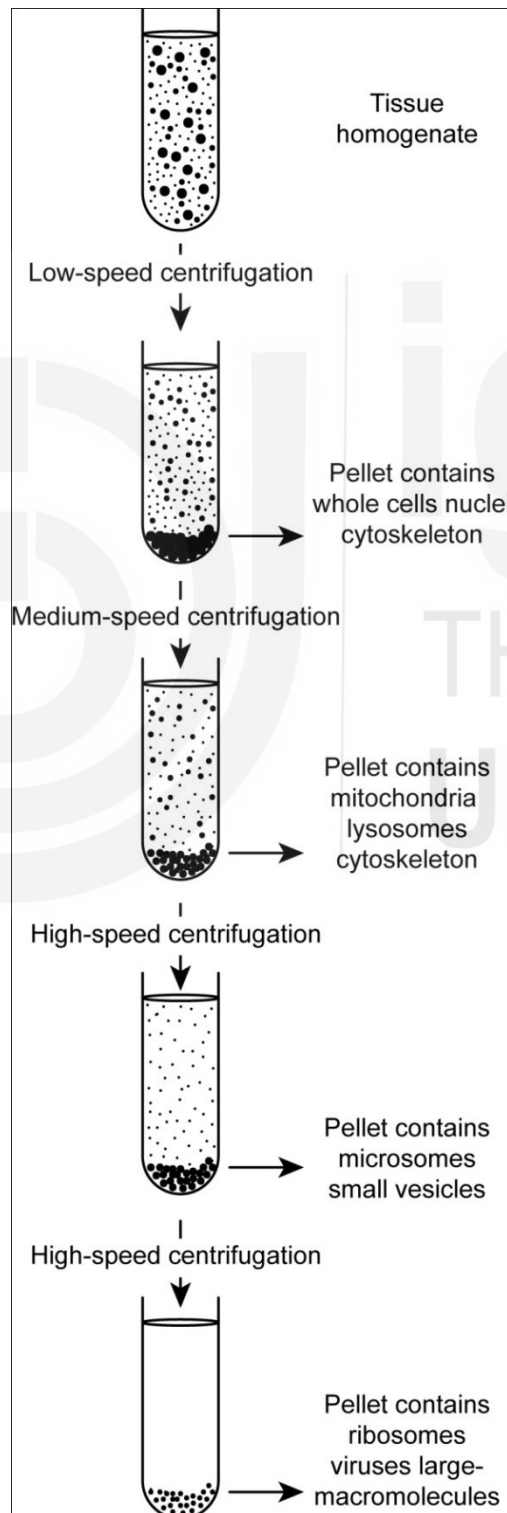


Fig. 3.5: Various steps in differential centrifugation.

The sample is homogenized in the medium containing buffer. The homogenate is placed in the centrifuge tube and subjected to a centrifugal force for a specific time at a particular temperature. The pellet formed at the bottom of the tube is separated from the supernatant. The supernatant is then added to a new centrifuge tube and subjected to another speed for a particular time and particular temperature. The supernatant is separated from the pellet formed again and again. The steps are repeated until all particles are separated from each other.

This technique proves very useful for separation of cell organelles. Cells are homogenized in an isotonic buffer solution (to prevent rupture of membrane vesicles). The homogenate is subjected to a series of centrifugations (sequential) with increase in centrifugal force. Initially the homogenate is subjected to low centrifugal force for a short period of time. This separates the larger cellular organelles such as nuclei. These organelles get sedimented as a pellet. At higher centrifugal forces, larger cytoplasmic organelles such as mitochondria, chloroplasts and lysosomes get extracted from the suspension. In subsequent steps, microsomes (fragments of vacuoles and membranes of the cytosol) and ribosomes are removed from suspension (Fig. 3.5). The organelles get separated in different fractions viz. nuclear fraction, mitochondrial fraction, microsomal fraction and soluble fraction. Ultra or high centrifugation normally occurs at the speed of 75,000 revolutions per min and produce forces equivalent to 500,000 times that of gravity.

3.3.3 Ultracentrifugation

Ultracentrifuges are the highly sophisticated and advanced type of centrifuges that operate at extremely high speeds. These centrifuges are large in size and separate samples in large batches and in a continuous flow system. They help in separation of smaller molecules like ribosomes, proteins, and viruses. They help in separation of molecules that cannot be separated with other centrifuges. The speed of these centrifuges ranges from 60,000 to 150,000 rpm. Most of the ultracentrifuges are refrigerated because this helps in balancing heat produced during intense spinning.



Fig. 3.6: An ultracentrifuge.

The working of this centrifuge is based on the principle of sedimentation. The denser particles settle down fast under the influence of gravity (Fig. 3.6). The molecules get separated on the basis of their buoyant density. The band of highest buoyant density is at the bottom while one with lowest buoyant density is at the top. The separation is independent of size and shape. A high concentration of sucrose or cesium chloride is used in this type of centrifugation. Each component moves down and reaches a position where the density of the solution is equal to its own buoyant density (Fig. 3.7). The denser molecules move outwards to the periphery of the tube whereas the less dense molecules get inclined towards the center of the tube.

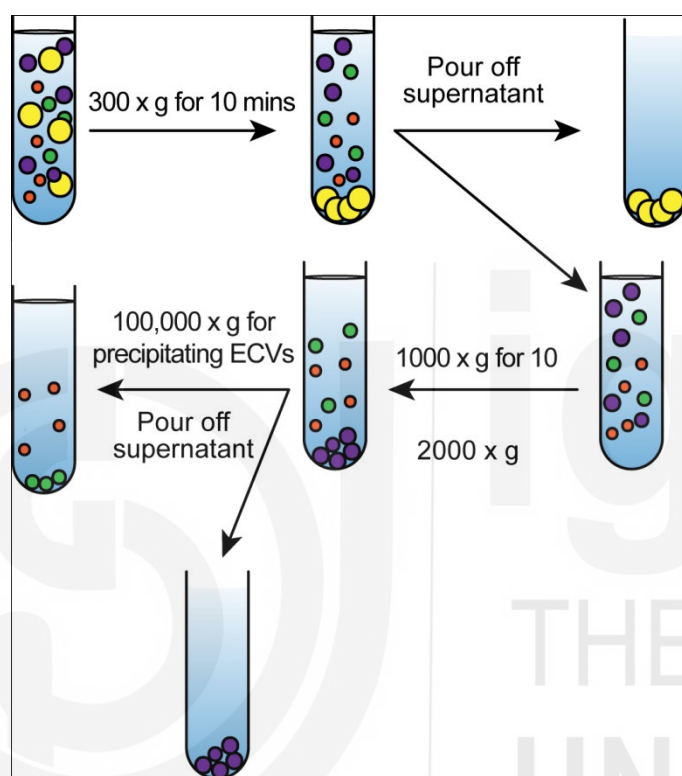


Fig. 3.7: Various steps involved in ultracentrifugation.

Ultracentrifuges can also be used for the determination of properties such as size, shape, and density of macromolecules. Ultracentrifuges are used for separation of cell organelles like mitochondria, ribosomes and viruses. The macromolecular conformational changes due to changes in pH, temperature, and other environmental factors can be detected using this instrument. The densities of various macromolecules can also be determined using this type of centrifuge. The purification of various biological crude extracts can also be made using this instrument.

Besides these, some centrifuges are also available that possess temperature control system required for certain special processes. Refrigerated centrifuges provide temperature control ranging from -20°C to -30°C . Microcentrifuges are the centrifuges used for the separation of samples with smaller volumes ranging from 0.5 to 2 μL . They usually operate at a speed of about 12,000-13,000 rpm and are used for the molecular separation of cell organelles like nuclei and DNA and phenol extraction. Microcentrifuges are also called microfuges.

SAQ 2

Fill in the blanks with appropriate words:

- The spectrophotometer works on the principle of
- In a spectrophotometer, the concentration of colored compounds is measured as
- gives the measure of the strength of rotors used during centrifugation.
- In differential centrifugation, components get separated by increasing
- The centrifuges used for the separation of samples with smaller volumes are known as

3.4 X RAY DIFFRACTION

X-Ray Diffraction (XRD) is a technique used to analyze the structure of crystalline materials. The technique is used for analyzing the atomic or molecular structure of materials. The chemical composition of the crystalline structures can also be known through this technique. The structure of crystalline materials depends on the dual wave/particle nature of X-rays. The atomic structures determined by X-ray crystallography indicate the role of individual molecules that make up a multisubunit complex.

Most of the materials are composed of many tiny crystallites called as polycrystalline aggregates or powder. The finely ground powder with crystallites is placed in an X-ray beam for analysis. XRD reveals the geometry or shape of a molecule. The scattering of X-rays from structures determine the shape or geometry of the molecule. The X-rays get diffracted by a crystal and provide the inter-atomic spacing in the crystals. When an incident beam of monochromatic X-rays hit the three-dimensional arrangement of atoms in a crystal, X-rays get scattered after striking the atoms within the target material (Fig. 3.8).

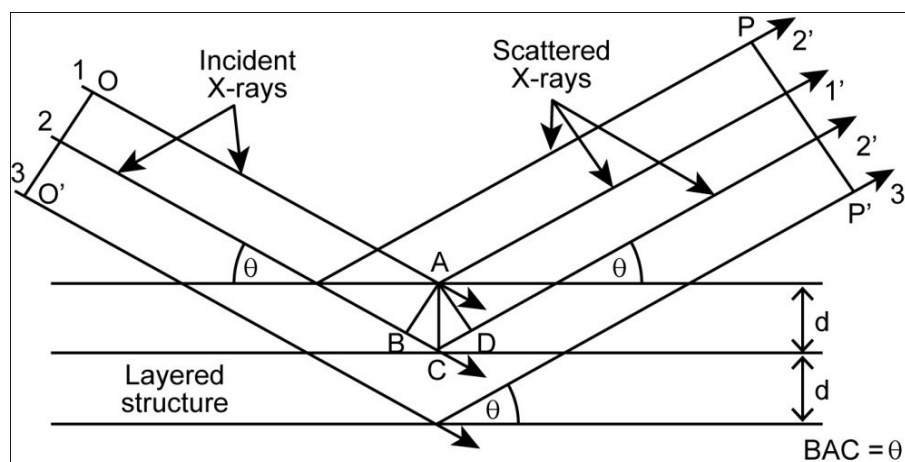


Fig. 3.8: Diagram showing principle of X-ray diffraction.

Most of the X-rays destructively interfere with each-other and cancel each-other out, but in some specific directions X-ray beams interfere constructively and reinforce one another. The reinforced X-rays produce the characteristic diffraction pattern which helps in determining the structure of crystal. Diffraction occurs when light bends slightly as it passes around the edge of an object or encounters an obstacle or aperture (Fig. 3.9). The degree of diffraction depends mainly on the size of the material. The direction of diffraction depends on the size and shape of the material, while the intensity of diffraction depends on the arrangement of atoms in the crystal structure.

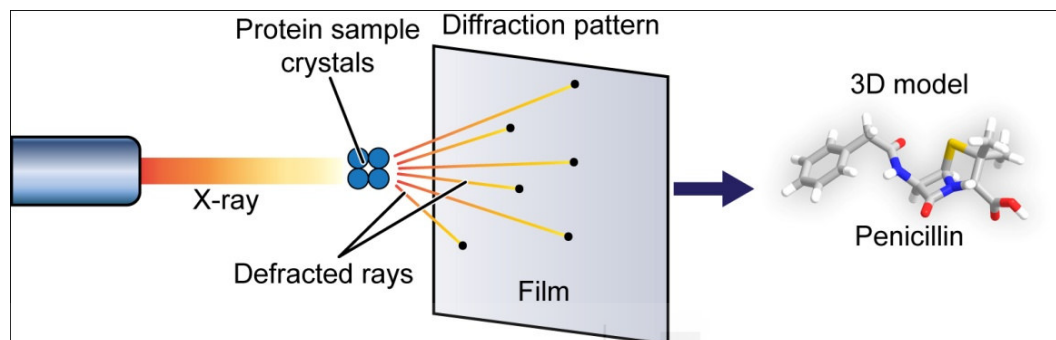


Fig. 3.9: Representation of the setup for XRD technique.

The technique is based on the principle of **W.L. Bragg** which states that diffracted X-rays act as rays reflected from various planes within the crystals. These planes were later named as Bragg's planes and mainly include rows of atoms that make up the crystal structure. The Bragg's equation is represented as follows:

$$n(\lambda) = 2d \sin\theta$$

where 'n' is an integer (1,2,3,...n),

λ (lambda) is the wavelength,

d is the distance between the atomic planes, and

θ (theta) is the angle of incidence of the X-ray beams.

The technique got advanced with the passage of time. Synchrotrons are being used to generate highly focused and intense X ray beams. The beams possess high energy particle accelerators. Highly sensitive electronic detectors (charged coupled devices, CCD) that provide a digital readout of the diffraction data are used.

The XRD technique is mainly used in the identification of unknown crystalline materials. The method proves useful for studying minerals, polymers and unknown materials. The tertiary structure of proteins and structure of large molecular structures can be determined using this technique. The technique has got applications in the field of Geology, Engineering, Biology, Material science, Environmental science, and Pharmaceuticals.

3.5 ELECTROPHORESIS

Electrophoresis is a technique by which biomolecules are separated based on their size and electrical charge. In this technique, the charged particles

migrate under the influence of an electric field. The separation of the molecules depends on their ability to move in an electric field. When placed in an electric field, molecules get separated on the basis of difference in their net charge. The technique was introduced by Swedish chemist **Arne Tiselius** in 1930.

Biomolecules are electrically charged particles and possess ionizable functional groups. They exist as either positively or negatively charged ions in a solution of a given pH. When subjected to an electric field, the ionized biomolecules migrate depending on the mass and the net charge of each particle. The negatively charged particles (anions) migrate towards a positively charged electrode (*cathode*), while the positively charged particles (*cations*) move towards a negatively charged electrode (*anode*). The differences in the speed and the direction of each charged particle result in a unique migration pattern leading to the isolation of components of the biomolecules that possess similar characteristics.

Types of Electrophoresis

The electrophoresis can be of different types depending on the type of buffer solution used and mobility of the charged particles. The major types of electrophoresis include:

Moving boundary electrophoresis - In this type of electrophoresis, the samples are present in solution with constant pH. The separation is low because mixing of samples occurs in the buffer solution which results in the overlapping of components or particles that possess similar characteristics. The mobilization of separating particles can be measured without intervening factors.

Zone electrophoresis (ZE) - Here the separation takes place in a homogenous buffer system. Samples are surrounded with electrophoretic buffer solution and separated in the matrix for a definite separation time. The matrix provides an additional **sieving effect** for the separation. The matrix or supporting medium controls diffusion of sample. The technique is widely used in biochemistry and molecular biology research. It is also useful in biochemical investigations.

Isotacho-electrophoresis or isotachopheresis (ITP) - It is a form of electrophoresis in which ions migrate at same velocity. The samples are placed between two non-homogeneous buffer solutions composed of leading electrolyte at one electrode and a terminating electrolyte at the other end (Fig. 3.10). The leading electrolyte shows the highest mobility. Thereafter follow the charged particles and terminating electrolytes under the influence of electric current. This type of electrophoresis is carried out in capillaries. The advantage of this method is that it does not require any sample preparation and is applicable to wide range of samples.

Isoelectric focusing (IEF) - Isoelectric focusing or IEF is the process of separating charged macromolecules like proteins or peptides. The separation depends on their isoelectric point or the pH at which a particular molecule

does not have any charge. This type of electrophoresis is performed in a pH gradient, which runs from low to high or from anode to cathode. This technique is applicable only to amphoteric molecules because they can donate and receive protons acting as acid and base.

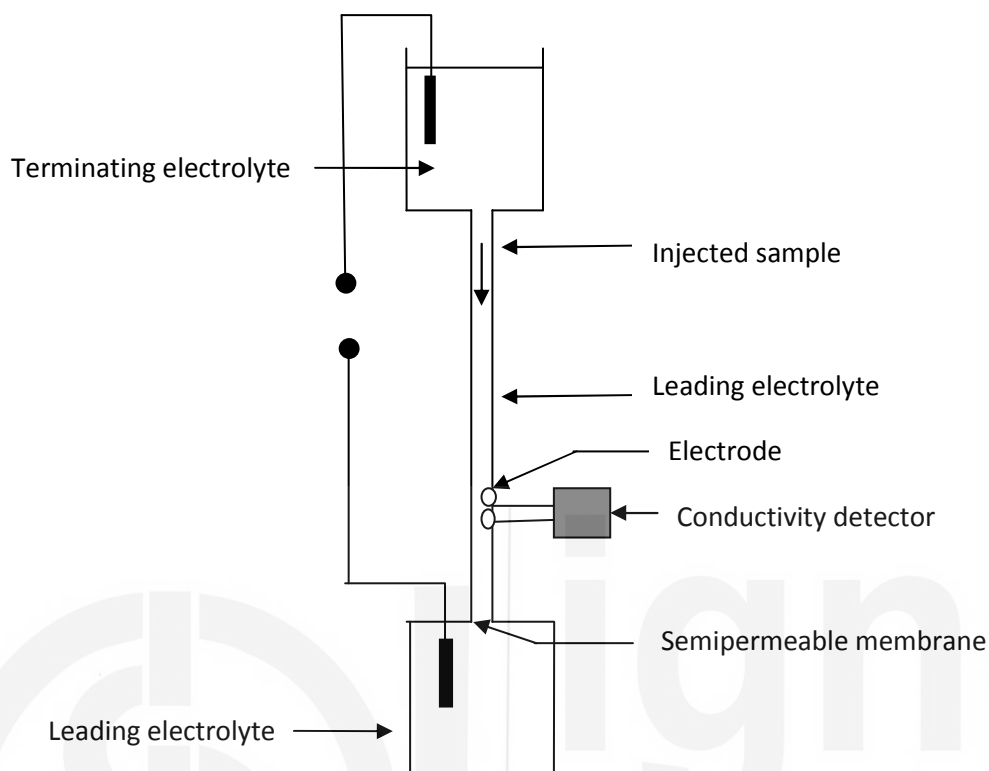


Fig. 3.10: Isotachopheresis (Diagrammatic representation).

Electrophoresis can be performed in both one- and two-dimensions (2D). In two-dimensional electrophoresis, second electrophoretic separation is carried out in a direction perpendicular to the first. 2D-electrophoresis has shown to provide better resolution and has found its use in analysis of clinical and field samples.

Gel Electrophoresis - In gel electrophoresis biological molecules get separated on the basis of their size. The molecules get separated by placing them in a gel with small pores and creating an electric field across the gel. The gel acts like a permeable matrix/sieve through which molecules can travel when electric current is applied. Smaller molecules migrate more quickly and travel far through the gel particles under the influence of electric current. The separation of molecules is based on their size and electric current.

The gel is cast into strips or slabs with slots or sample wells. It is immersed in an electrophoresis buffer solution before complete polymerization. Each well of the gel is loaded and electric current is applied. The components in the samples get separated on the basis of their mass under the influence of electric current. Gel electrophoresis is commonly used in research and routine diagnosis. The separation of various biomolecules can be achieved by changing the type of polymers used in casting a gel or altering the pore size of the gel.

There are two types of gel electrophoresis: **native and denaturing**.

Native gel electrophoresis - In this type of electrophoresis, RNA or protein is maintained in its native structure during electrophoresis. This type of electrophoresis is usually used to identify large protein complexes.

Denaturing gel electrophoresis – In this type of electrophoresis, RNA or protein is reduced i.e., present in its linear structure. The denaturation of the RNA or protein is done by adding a reducing agent to the sample, gel and/or buffer. The reducing agent separates bonds within the RNA or protein molecule thereby reducing its secondary structure. This type of electrophoresis helps in knowing the size of the molecule.

Both polyacrylamide and agarose gels are used in electrophoresis.

Polyacrylamide is compound which forms transparent gel after co-polymerization of acrylamide monomers. Along with it a cross linking agent such as N, N-methylene-bisacrylamide ('bis-acrylamide') is used. The polymerization reaction is catalyzed by ammonium persulphate (APS) and N,N,N',N'-tetramethylethylenediamine (TEMED). DNA and proteins are separated using a low percentage of acrylamide gel (3%-15%). Acrylamide gel of about 10%-20% is used in sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE) in which separation of proteins occurs in denatured condition (separation according to their size).

Agarose is a natural polysaccharide made of galactose and 3, 6-anhydrogalactose chains and is obtained from agar isolated from red seaweeds. To cast a gel, agarose powder is dissolved in a buffer solution, heated and allowed to cool to room temperature. The concentration of the agarose in the buffer solution determines the pore size of the gel. Agarose gel of about 0.8% (w/v) to 5% (w/v) is used to separate DNA and RNA molecules.

Pulsed-field Gel Electrophoresis (PFGE)

In pulsed-field gel electrophoresis (PFGE), two electrical fields are applied periodically in rotation at different angles. High-molecular-weight DNA molecules generally occur in a coiled state and get stretched after application of electric field in a gel. Alteration in the direction of the electric field helps in the reorientation of large DNA molecules. Two alternating electric fields from different angles help in revealing migration distance for small quantity of high-molecular-weight DNA molecule. Generally shorter migration distance has been noted in case of large high-molecular-weight DNA. This type of electrophoresis is used for the separation of chromosomes and high-molecular-weight DNA molecules (more than 20 kb).

Capillary Electrophoresis (CE)

Capillary electrophoresis (CE) or High-Performance Capillary Electrophoresis (HPCE) is performed in a narrow capillary immersed in an electrolyte buffer. The capillary is typically 20-30 cm long and have inner diameter of 25-75 μm . A sample is injected into the capillary which is now subjected to high voltage, pressure and electric field. The sample components get separated along the length of the capillary. The separated components are revealed by detector. The major advantage of this technique is that a small sample volume can be used for the separation.

Immuno-electrophoresis

In this type of electrophoresis, antigens including proteins and peptides get separated on the basis of their reaction and specificity to antibodies or immunoglobulins (Ig). The binding of antigen to its corresponding antibody at a specific point results in the precipitation of antigen-antibody complex. This type of electrophoresis can be performed as a one-dimensional or two-dimensional mode.

Affinity Electrophoresis

It is a type of electrophoresis in which separation of biomolecule is achieved after interaction or binding to another molecule having a similar affinity. The technique is based on the principle that electrical mobility (μ) changes when a biomolecule including nucleic acids, proteins, peptides, and polysaccharides bind to another molecule. The change in the electrical mobility gets reflected in the electrophoretic pattern.

Set up for Electrophoresis

An electrophoresis apparatus consists of two opposite charged electrodes (anode, cathode) connected by a medium called an electrolyte (conducting solvent). In electrophoresis chamber, a power supply and an electrophoresis unit are present together. The power supply provides the uninterrupted electric current to the chamber. The chamber is composed of two electrodes, cathode and anode, along with a reservoir containing buffer solution (Fig. 3.11).

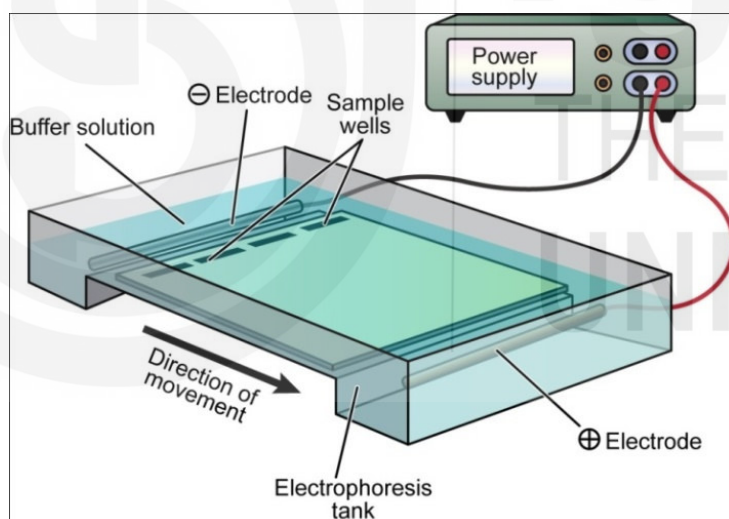


Fig. 3.11: Set up for electrophoresis.

Gel casting trays, glass plates, and combs are other requirements essential for gel casting. An internal cooling system is a prerequisite to provide thermo-stable condition during electrophoresis and prevent excessive heating. Components present in the sample separated after electrophoresis are not visible, therefore detection and analytical methods are required for the interpretation of results obtained.

The separated samples or components are generally not visible under normal light, staining of gel is done. The staining varies for each type of molecule. Coomassie Brilliant Blue or silver is used for staining proteins.

Ethidium bromide (dye) binds itself to nucleotide bases and fluoresces under short-wave ultraviolet (UV) light (Fig. 3.12). The stained gel is visualized using

a Gel Documentation System. The image of the gel can be procured through this system as it is equipped with a camera. The gel image tells us about the pattern of migration of bands (Fig. 3.13).

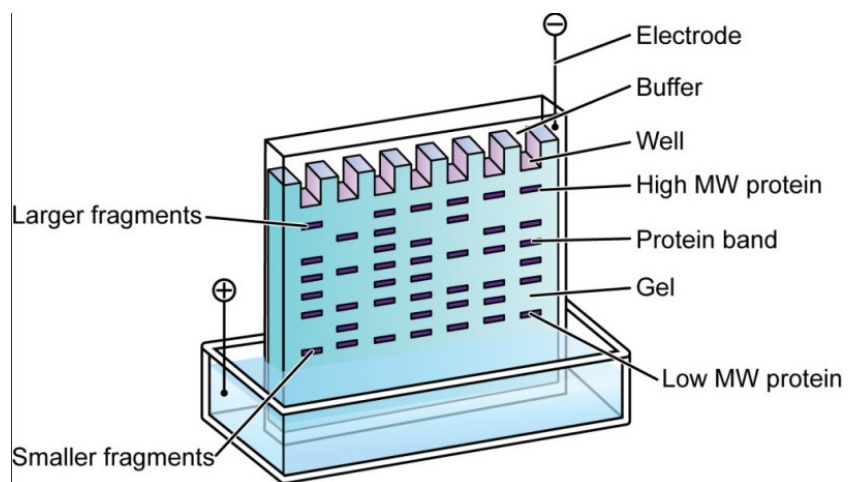


Fig. 3.12: An image showing gel in an electrophoresis chamber.

Besides staining, several other approaches such as autoradiography is also used. In autoradiography, the molecules of interest are labeled with radioisotopes.



Fig. 3.13: A photograph showing Gel Documentation System.

Principle of gel electrophoresis

The principle of gel electrophoresis is based on the fact that smaller molecules move faster when present in the gel matrix in comparison to large molecules that remain in the matrix. The small DNA molecules easily slip between the various components of the gel matrix. The rate of migration of molecules through the gel matrix depends upon the structure and charge of the proteins. When proteins are separated by electrophoresis through a gel matrix, smaller proteins migrate faster due to less resistance from the gel matrix. The use of sodium dodecyl sulfate (SDS) or sodium lauryl sulfate and polyacrylamide gel eliminates the influence of the structure and charge, and proteins get separated only on the basis of length of polypeptide chain. SDS is a detergent which denatures proteins after binding to its backbone. The binding of SDS (reducing agent) cleaves disulfide bonds, help in unfolding of proteins into linear chains.

Procedure

Key points of the gel electrophoresis include:

- TAE (Tris acetate EDTA) buffer is used as a source of ions for setting up the electric field during electrophoresis.
- The percentage of gel used depends upon the size of molecules. A high percentage agarose gel (>1%) is prepared for separation of smaller size molecules (<500bp). High percentage of agarose gel creates small pores that increase the separation of small DNA segments.
- The agarose TAE buffer solution is poured into the casting tray. The gel solution gets solidified after cooling. The wells are created at the top of the gel using a comb.
- The solid gel is placed into a chamber filled with TAE buffer. The gel is positioned in such a way that wells are placed close to the negative electrode of the chamber. The wells are loaded with the DNA samples. A DNA ladder or marker with multiple molecules of known and varying molecular weights is also loaded as reference for size. The power supply is switched on to generate electric field.
- The negatively charged DNA samples migrate through the gel and move away from the negative electrode towards the positive one under the influence of electric field. The rate of migration is proportional to size. Smaller fragments move faster and reach bottom of the gel.
- Once the dye in the DNA samples migrates far enough through the gel, the power supply is turned off and the gel is removed. It is then placed into an ethidium bromide solution. Ethidium bromide intercalates between DNA. Illumination with ultraviolet light causes the intercalated dye to fluoresce.
- The stained gel is then exposed to UV light and a picture is taken. DNA bands are visualized from each lane corresponding to a chamber well. The DNA ladder gives us an estimation of the length of the DNA band.

Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gel electrophoresis (PAGE) is an analytical method for separation of components of a protein mixture based on their size. A polyacrylamide gel is formed as a thin slab between the two glass plates. After the gel gets polymerized, the slab is suspended between two compartments containing buffer and two electrodes. The protein sample is prepared in a solution containing sucrose or glycerol. Voltage is applied between the buffer compartments. The alkaline buffers help in the separation as they make the proteins negatively charged and cause them to migrate towards the positively charged anode (see Fig. 3.12). The current flows across the slab causing the proteins to move towards the oppositely charged electrode. The movement of proteins depends on the charge density (charge per unit mass). The greater the charge density, forcefully the protein molecule moves through the gel, and high is rate of migration. The larger the protein molecule, more it becomes entangled and slower it migrates. Compact globular proteins move more rapidly in comparison to fibrous proteins. The lower is the concentration of

acrylamide, less the cross linking in the gel and rapid is the protein migration. A gel containing 5% acrylamide is generally used to separate proteins of 60 to 250kDa, whereas the gel of 15% is used to separate molecules of size 10 to 50 kDa.

A tracking dye is used to assess the progress of electrophoresis. The dye Coomassie Brilliant Blue is commonly used to study proteins. The proteins can also be radiolabelled. The position of proteins can be determined by pressing the gel against the piece of X ray film. This results in production of an autoradiograph. The gel is sliced into fractions and individual proteins are isolated or gel is transferred by a second electrophoretic procedure to the nitrocellulose membrane to form a blot. The proteins get absorbed on the surface of the membrane.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis is the most commonly used gel electrophoresis. The technique facilitates better separation and analysis of proteins. Sodium dodecyl sulphate (SDS) a negatively charged detergent binds to large number of protein molecules. The polyacrylamide gel forms an inert matrix through which proteins migrate in SDS-PAGE. The SDS solubilized proteins when passed through the gel migrate towards the positive terminal electrode. SDS binds to the hydrophobic regions of the protein molecules and causes them to unfold into extended polypeptide chains. Mercaptoethanol, a reducing agent is also used which helps in breaking of S-S (disulphide) linkages so that protein subunits can be separated. Both SDS and mercaptoethanol help in unfolding the native structure of protein.

The unfolding of proteins into a rod shape eliminates the differences in shape of proteins. This helps in proteins of same size to behave similarly and proteins of different sizes form series of discrete bands. The number of SDS molecules that can bind to protein is roughly proportional to the molecular mass of the protein. The proteins get separated on the basis of molecular mass i.e. polypeptides are separated due to size. The gel can be stained with dye Coomassie Brilliant blue to visualize major bands while the minor bands can be visualized using silver stain (Fig. 3.14).

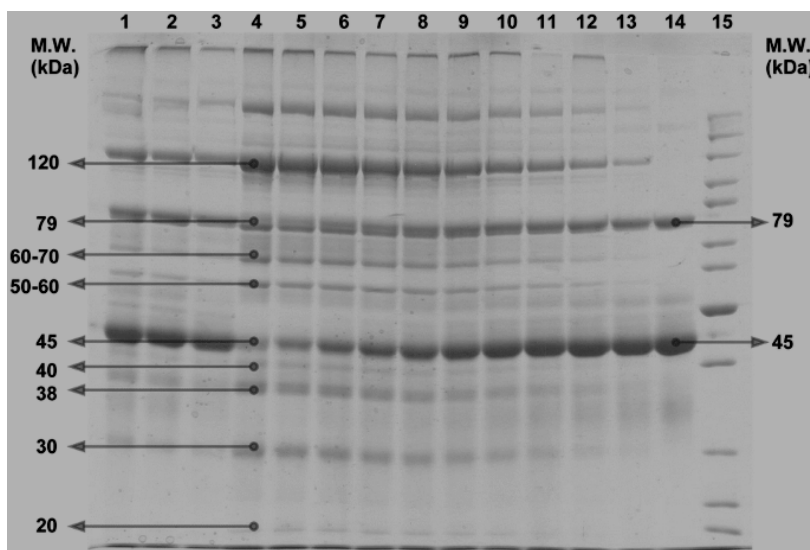


Fig. 3.14: Diagram Gel showing bands separated by SDS-PAGE.

Two-dimensional gel electrophoresis

In this type of electrophoresis, the mixture of proteins can be separated using two different properties of the molecules. Proteins are first separated in a tubular gel according to their isoelectric point via isoelectric focusing technique. The gel is removed and placed on the top of a slab of SDS saturated polyacrylamide and subjected to SDS- PAGE. The proteins move into the slab gel and get separated on the basis of molecular mass. The proteins are removed from the gel and digested into peptide fragments that can be analyzed by mass spectrometry.

This type of gel electrophoresis is used to detect changes in the proteins present in a cell under different conditions. The technique is suitable for distinguishing proteins that have high molecular mass, are highly hydrophobic or present in very low copy numbers per cell.

Applications

Electrophoresis technique is used for the analysis of biological or other polymeric samples. The electrophoresis techniques are used to separate various types of biomolecules, analyze their characteristics and study their interaction with a molecule of interest. Biomolecules such as DNA, RNA and proteins are separated using this technique.

- i) The gel electrophoresis technique helps to visualize, identify and distinguish molecules such as nucleic acids.
- ii) Small RNA or DNA molecules of few hundred nucleotides are separated by PAGE.
- iii) SDS-PAGE is a technique commonly used for identification and separation of proteins.
- iv) Pulse field electrophoresis is used for separation of DNA molecules greater than 25kb.
- v) Agarose Gel Electrophoresis is most commonly used for separation and identification of DNA molecules.
- vi) Denaturing PAGE (polyacrylamide gel electrophoresis) is commonly used for separation of RNA.

SAQ 3

- a) Answer in one word :
 - i) An instrument that measures the amount of light absorbed by a sample when a beam of light is passed through it.
 - ii) In spectrophotometer the concentration of coloured and colourless compounds can be determined by measuring this.
 - iii) In this type of centrifugation the components of the sample get distributed into different layers according to their density i.e. separation occurs under the influence of gravitational force.
 - iv) This type of centrifugation results in separation of cellular organelles.

- v) A high concentration of sucrose or cesium chloride is used for the separation of molecules.
 - vi) In this technique molecules such as proteins, amino acids, nucleotides are separated on the basis of differences in their net charge when placed in an electric field.
 - vii) The solution obtained after centrifugation.
- b) Fill in the blanks.
- i) solution is used in the density gradient centrifugation.
 - ii) A high concentration of is used in ultracentrifugation.
 - iii) During electrophoresis the gel can be stained using dye
 - iv) In two dimensional gel electrophoresis, proteins are first separated according to their
 - v) Gel electrophoresis is also used to separate nucleic acids of different
- c) State whether the statements are 'True' or 'False'.
- i) In spectrophotometry, the amount of light absorbed by a colored solution is directly proportional to the concentration of the solution.
 - ii) In ultracentrifugation, the components get separated on the basis of their speed.
 - iii) An incident beam of monochromatic X-rays interacts with a target material in X-ray diffraction analysis.
 - iv) The movement of proteins in a polyacrylamide gel electrophoresis depends on the size of the molecules.
 - v) SDS binds to the hydrophobic regions of the protein molecules.
 - vi) Gel electrophoresis is also used to separate nucleic acids of different charges.

3.6 SUMMARY

- A spectrophotometer is an instrument used to measure absorbance of the compound at different wavelengths. It is commonly used for the determination of the concentration of coloured and colourless compounds by measuring their optical density or absorbance.
- In centrifugation technique the contents of the sample get separated into various layers according to their density. The technique is used to isolate cell organelles. It is based on the principle that particles of different size and shape travel toward bottom of the centrifuge tube with respect to the surrounding medium when placed in a centrifugal field.

- X-Ray Diffraction is a method used to analyze the structure of crystalline materials. The protein crystals are bombarded with a fine beam of X-rays. The radiation gets scattered (diffracted) when the electrons strike the proteins atoms. The diffraction produced determines the structure within the protein and other crystalline materials.
- Electrophoresis is a technique by which the molecules such as proteins, amino acids, nucleotides are separated on the basis of differences in their net charge when placed in an electric field. The separation of the molecules depends on the ability of the charged molecules to migrate when placed in an electric field.
- SDS-PAGE is the electrophoresis technique that facilitates better separation and analysis of proteins. The gel is prepared by polymerization of monomers. The pore size in the gel regulates the passage of proteins. The proteins are held in by the solution having negatively charged detergent called sodium dodecyl sulphate (SDS). SDS binds to the hydrophobic regions of the protein molecules and causes them to unfold into extended polypeptide chains. When the SDS treated proteins are passed through the gel, proteins migrate towards the positive terminal electrode.

3.7 TERMINAL QUESTIONS

1. Describe the applications of:
 - a) Spectrophotometer
 - b) X-ray diffraction analysis
 - c) Ultracentrifugation
2. Enlist the differences between density gradient and differential centrifugation.
3. What governs the movement of proteins in Polyacrylamide gel electrophoresis (PAGE)?
4. Write short notes on:
 - a) Two dimensional gel electrophoresis
 - b) Principle of centrifugation

3.8 ANSWERS

Self-Assessment Questions

1.
 - a) The working principle of the spectrophotometer is based on Beer-Lambert's law. There is a linear relationship between absorbance and concentration of an absorber of electromagnetic radiation. It states that the amount of light absorbed by a coloured solution is directly proportional to the concentration of the solution and the length of a light path through the solution.
 - b) Refer to Fig. 3.2.

2.
 - a) Beer-Lambert's law
 - b) optical density or absorbance
 - c) Relative centrifugal force (RCF)
 - d) centrifugal force
 - e) microfuge
3.
 - a)
 - i) spectrophotometer
 - ii) optical density or absorbance
 - iii) density gradient centrifugation
 - iv) differential centrifugation
 - v) ultracentrifugation
 - vi) electrophoresis
 - vii) homogenate
 - b)
 - i) sucrose
 - ii) sucrose or cesium chloride
 - iii) Coomassie brilliant blue
 - iv) isoelectric point
 - v) molecular mass
 - c) i) True; ii) False; iii) True; iv) False; v) True; vi) False

Terminal Questions

1.
 - a) The spectrophotometer is used for determining the concentration of compounds by measuring their optical density or absorbance. The spectrophotometer works on the principle of Beer-Lambert's law. This law states that the amount of light absorbed by a coloured solution is directly proportional to the concentration of the solution and the length of a light path through the solution.
 - b) The protein crystals are bombarded with a fine beam of X rays. The radiation (X-rays) get scattered (diffracted) when the electrons of the protein atoms strike an electron sensitive detector placed behind the crystal. An incident beam of monochromatic X-rays interacts with a target material.
 - c) The molecules in this case can be separated on the basis of their buoyant density. The band of highest buoyant density is at the bottom and lowest at the top.
2. The main difference between differential and density gradient centrifugation is that fractionation is carried out based on the size in differential centrifugation whereas fractionation is carried out based on the density in density gradient centrifugation.

3. It is a technique by which the molecules such as proteins, amino acids, nucleotides are separated based on differences in their net charge when placed in an electric field. The separation of the molecules depends on the ability of the charged molecules to migrate when placed in an electric field. The technique is used in purification of enzymes.
4. a) Refer to Section 3.5.
b) Refer to Section 3.3.

Acknowledgement for figures

Fig. 3.1 : <https://www.google.co.in/imgres?imgurl=https%3A%2F%2Fwww.unicosci.com%2Fmedia%2Fcatalog%2Fproduct%2Fcache%2Fimage>

Fig. 3.3 : <https://www.google.co.in/imgres?imgurl=https%3A%2F%2Fus.vwr.com%2Fstibo%2Fbigweb%2Fstd.lang.all%2F77%2F39%2F4677739.jpg>

https://www.google.co.in/imgres?imgurl=https%3A%2F%2Fimg.medicaexpo.com%2Fimages_me%2Fphoto-g%2F131159-16310271.jpg

Fig. 3.6 : <https://www.google.co.in/imgres?imgurl=https%3A%2F%2Fwww.himac-science.com%2Fproducts%2Fhimac%2Fultra%2Fcpnx%2Fcpnx.jpg>

Fig. 3.13 : <https://www.google.co.in/imgres?imgurl=https%3A%2F%2Fwww.syngene.com%2Fwp-content%2Fuploads%2Fingenius-gel-doc-system-1-1.jpg>

Fig. 3.14 : <https://www.google.co.in/imgres?imgurl=https%3A%2F%2Fwww.researchgate.net%2Fprofile%2FJuwartina-Ida-Royani%2Fpublication%2F328710018%2Ffigure%2Ffig1%2FAS%3A688711058591744%401541212920226%2FSDS-PAGE-sodium>

You tube videos

<https://www.youtube.com/watch?v=xgxFBQZYXIE>

<https://www.youtube.com/watch?v=MhJT9yjnI88>

<https://www.youtube.com/watch?v=bYwq5oNZmq4>

<https://www.youtube.com/watch?v=4OJAzQsZnbo>