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1. Introduction

Bioanalytical techniques, are the analytical tools used to study the biological molecules; non-biological molecules involved with life, such as drugs and biological processes. These tools are routinely used to identify, estimate, purify, and characterize the biomolecules. Quantification of molecules in biological samples is at the heart of bioanalysis and is routinely used to diagnose various diseases and metabolic disorders. For example, estimation of thyroxine and triiodothyronine concentrations in blood provides information about the activity of thyroid gland. Home pregnancy test kits look for the human chorionic gonadotropin (hCG) hormone in the urine, presence of which above a threshold concentration is an indicator of pregnancy. Initially, nonspecific assays were used to quantify the drugs in biological samples. Evolution of the existing assays, advancement in instrumentation, and introduction of newer techniques have made it possible to distinguish the drug molecules and their closely related metabolites in complex biological specimens.

Estimation of the analytes

Identification and quantification of analytes is perhaps the most common application of bioanalytical methods. Various diseases and disorders including cancers are diagnosed by estimating the levels of the characteristic biomarkers in a particular tissue or organ. Semenogelase, for example, is a biomarker for prostate cancer, one of the most frequently diagnosed cancers in human males.

a. Qualitative versus quantitative analyses

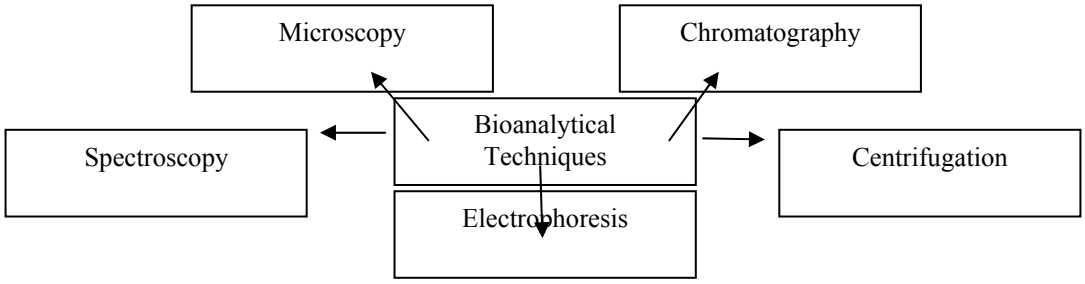
A qualitative analysis simply tells about the presence or absence of an analyte in a sample. An absence of analyte, however, may result due to concentrations below the detection level of the bioanalytical technique used. Qualitative analyses are used wherein detection of an analyte is sufficient to take further course of action. For example, doping test done for atheletes. In certain cases, however, it is important to estimate the concentration of the analyte. A quantitative analysis would result in the determination of actual amount of the substance present in the sample. During optimization of an enzyme purification, parameter yielding maximum levels of enzyme has to be known, hence quantitative determination is done.

b. Accurate and precise determination of analytes

Accuracy is the measure of how closely the measured values match the true values. Precision tells about the reproducibility of the measurement ***i.e.*** how closely the measured values are if repeated measurements are made on the sample. Bionalytical techniques, resulted in development of spectrophotometeric methods that allow accurate as well as reproducible result.

Identification and characterization of molecules

Researchers involved in the discovery of novel bioactive natural products often have to identify the bioactive component present in the crude sample; for example, isolation of novel antibiotics and antimicrobial peptides from various organisms. Individual components in the crude sample are isolated based on the differences in their physical and chemical properties. The bioactive component is identified by testing the activities of these isolated compounds. The bioactive compound is then characterized using various spectroscopic methods to arrive at its structure and function(s). Bioanalytical techniques can typically be classified as shown below



Spectroscopic tools such as infrared spectroscopy, circular dichroism spectroscopy, and nuclear magnetic resonance spectroscopy can provide structural information about the molecules which in turn provides insights into their functional aspects.

Studying biological processes

Life is an outcome of the complex interplay of biological molecules. These involve interactions between macromolecules (*e.g.* protein-protein interactions and DNA-protein interactions, RNA-protein interactions); interactions of biomolecules with small molecules (glucose channels, water channels, ligand-binding) and ions (K^+ channel, Na^+ and K^+ pump, Ca^{2+} channels); and interaction of molecules with light (chlorophyll, photoreceptors). Interactions of the molecules with their receptors/ligands, both *in vitro* and *in vivo*, are usually studied using various spectroscopic and microscopic tools. Fluorescence spectroscopy and microscopy are among the most commonly employed tools to study the biological processes. Discovery of the green fluorescent protein (GFP) and subsequent development of its analogs with different spectral properties have revolutionized the area of cellular research. Before discussing in detail the various tools that have gained importance in bioanalytical research, it is worthwhile to take a pause for very quickly reviewing the important structural aspects of major classes of biomolecules.

Bioanlytical techniques and their advancements have paved the way for studying the fields of Biochemistry and Molecular biology, which include:

- Study of the structure and function of the total protein component of the cell (proteomics) and of all the small molecules in the cell (metabolomics);
- the mechanisms involved in the control of gene expression;
- the identification of genes associated with a wide range of human diseases;
- the development of gene therapy strategies for the treatment of human diseases;

- the characterization of the large number of 'orphan' receptors, whose physiological role and natural agonist are currently unknown, present in the human genome and their exploitation for the development of new therapeutic agents;
- the identification of novel disease-specific markers for the improvement of clinical diagnosis;
- the engineering of cells, especially stem cells, to treat human diseases;
- the understanding of the functioning of the immune system in order to develop strategies for the protection against invading pathogens;
- the development of our knowledge of the molecular biology of plants in order to engineer crop improvements, pathogen resistance and stress tolerance;
- the application of molecular biology techniques to the nature and treatment of bacterial, fungal and viral diseases.

2. MICROSCOPY

Introduction: Microscopy is the technical field of using microscopes to view objects and areas of objects that cannot be seen with the naked eye (objects that are not within the resolution range of the normal eye). There are three well-known branches of microscopy: optical, electron, and scanning probe microscopy. Optical and electron microscopy involve the diffraction, reflection, or refraction of electromagnetic radiation/electron beams interacting with the specimen, and the collection of the scattered radiation or another signal in order to create an image. This process may be carried out by wide-field irradiation of the sample (for example standard light microscopy and transmission electron microscopy) or by scanning of a fine beam over the sample (for example confocal laser scanning microscopy and scanning electron microscopy). Scanning probe microscopy involves the interaction of a scanning probe with the surface of the object of interest. The development of microscopy revolutionized biology, gave rise to the field of histology and so remains an essential technique in the life and physical sciences.

In 1673, with the aid of a crude microscope consisting of a biconcave lens enclosed in two metal plates, Leeuwenhoek introduced the world to the existence of microbial forms of life, and this had led to the discovery of first microscope. Over the years, microscopes have evolved from the simple, single-lens instrument of Leeuwenhoek, with a magnification of 300, to the present-day electron microscopes capable of magnifications greater than 250,000.
Microscopes are designated as either light microscopes or electron microscopes. The former use visible light or ultraviolet rays to illuminate specimens. They include brightfield, darkfield, phase-contrast, and fluorescent instruments. Fluorescent microscopes use ultraviolet radiations whose wavelengths are shorter than those of visible light and are not directly perceptible to the human eye. Electron microscopes use electron beams instead of light rays, and magnets instead of lenses to observe submicroscopic particles.

There are basically two fundamentally different types of microscopes: The light microscope and the electron microscope (Figure 1.1). Light microscope uses beam of light while electron microscope uses beam of electron. There is huge difference in the sample preparation to image development process or its visualization which are listed in the table.

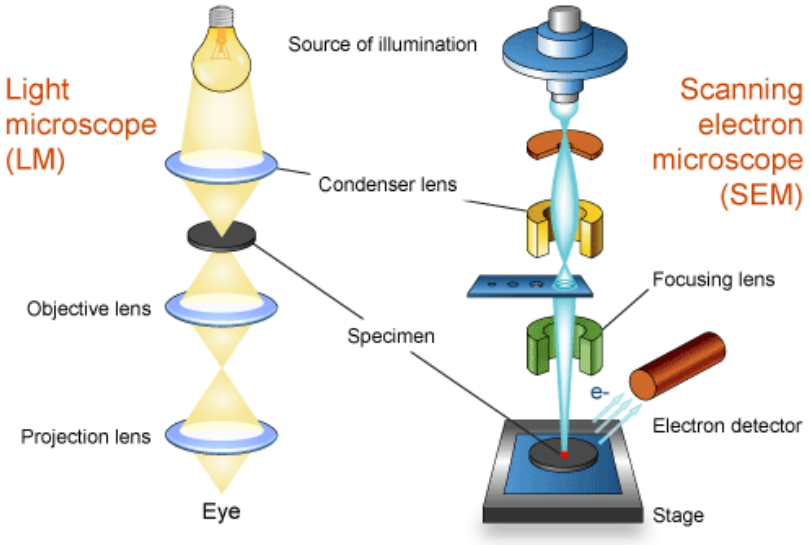


Figure 1.1: Schematic representation of Light microscope versus Electron Microscope

THE LIGHT MICROSCOPE v THE ELECTRON MICROSCOPE

FEATURE	LIGHT MICROSCOPE	ELECTRON MICROSCOPE
Electromagnetic spectrum used	Visible light 760nm (red) – 390nm Colours visible	Electrons app. 4nm Monochrome
Maximum resolving power	app. 200nm	0.2nm Fine detail
Maximum magnification	x1000 – x1500	↓ x500 000
Radiation source	Tungsten or quartz halogen lamp	High voltage (50kV) tungsten lamp
Lenses	Glass	Magnets
Interior	Air-filled	Vacuum
Focussing screen	Human eye (retina), photographic film	fluorescent (TV) screen, photographic film

The field of microscopy has undergone a renaissance over the past 20 years, with the 20years, with the addition of various technological advancements to the instruments. Most imaging produced by microscopes are now recorded electronically using digital imaging techniques- digital cameras, digital image acquisition software, digital printing and digital display methods. These advancements

have allowed many more applications of the microscope in biotechnology, ranging from routine observation of cells nd cell extracts to specialized techniques for directly measuring events in cells.

Essential Features of Various Microscopes

Bright field Microscope

This instrument contains two lens systems for magnifying specimens: the ocular lens in the eyepiece and the objective lens located in the nose-piece. The specimen is illuminated by a beam of tungsten light focused on it by a sub-stage lens called a condenser, and the result is that the specimen appears dark against a bright background. A major limitation of this system is the absence of contrast between the specimen and the surrounding medium, which makes it difficult to observe living cells. Therefore, most bright field observations are performed on nonviable, stained preparations.

Dark field Microscope

This is similar to the ordinary light microscope; however, the condenser system is modified so that the specimen is not illuminated directly. The con-denser directs the light obliquely so that the light is deflected or scattered from the specimen, which then appears bright against a dark background. Living specimens may be observed more readily with dark field than with bright field microscopy.

Phase-Contrast Microscope

Observation of microorganisms in an unstained state is possible with this microscope. Its optics include special objectives and a condenser that make visible cellular components that differ only slightly in their refractive indexes. As light is transmitted through a specimen with a refractive index different from that of the surrounding medium, a portion of the light is refracted (bent) due to slight variations in density and thickness of the cellular components. The special optics convert the difference between transmitted light and refracted rays, resulting in a significant variation in the intensity of light and thereby producing a discernible image of the structure under study. The image appears dark against a light background.

Fluorescent Microscope

This microscope is used most frequently to visualize specimens that are chemically tagged with a fluorescent dye. The source of illumination is an ultraviolet (UV) light obtained from a high-pressure mercury lamp or hydrogen quartz lamp. The ocular lens is fitted with a filter that permits the longer ultraviolet wavelengths to pass, while the shorter wavelengths are blocked or eliminated. Ultraviolet radiations are absorbed by the fluorescent label and the energy is re-emitted in the form of a different wavelength in the visible light range. The fluorescent dyes absorb at wavelengths between 230 and 350 nanometers (nm) and emit orange, yellow, or greenish light. This microscope is used primarily for the detection of antigen-antibody reactions. Antibodies are conjugated with a fluorescent dye that becomes excited in the presence of ultraviolet light, and the fluorescent portion of the dye becomes visible against a black background.

Electron Microscope

This instrument provides a revolutionary method of microscopy, with magnifications up to one million. This permits visualization of submicroscopic cellular particles as well as viral agents. In the electron microscope, the specimen is illuminated by a beam of electrons rather than light, and the focusing is carried out by electromagnets instead of a set of optics. These components are sealed in a tube in which a complete vacuum is established. Transmission electron microscopes require specimens that are thinly prepared, fixed, and dehydrated for the electron beam to pass freely through them. As the electrons pass through the specimen, images are formed by directing the electrons onto photographic film, thus making internal cellular structures visible. Scanning electron microscopes are used for visualizing surface characteristics rather than intracellular structures A narrow beam of electrons scans back and forth, producing a three-dimensional image as the electrons are reflected off the specimen's surface.

Theoretical Principles of Microscopy

The basic principles of microscopy include: magnification, resolution, numerical aperture, illumination, and focusing.

Magnification

Enlargement or magnification of a specimen is the function of a two-lens system; the ocular lens is found in the eyepiece, and the objective lens is situated in a revolving nose-piece. These lenses are separated by the body tube. The objective lens is nearer the specimen and magnifies it, producing the real image that is projected up into the focal plane and then magnified by the ocular lens to produce the final image.

The most commonly used microscopes are equipped with a revolving nose piece containing four objective lenses possessing different degrees of magnification. When these are combined with the magnification of the ocular lens, the total or overall linear magnification of the specimen is obtained. Thus, final magnification of the microscope is dependent on the magnifying power of the objective times the magnifying power of the ocular. Objective magnification powers range from 4X to 100X. Lower magnification is impractical on a compound microscope stand because of spatial constraints with image correction and illumination. Higher magnification is impractical because of limitations in light gathering ability and shortness of working distances required for very strong lenses. Ocular magnification ranges are typically 8X-12X though 10X oculars are most common. As a result, a standard microscope will provide a final magnification range of ~40X up to ~1000X.

Resolving Power or Resolution

Although magnification is important, unlimited enlargement is not possible by merely increasing the magnifying power of the lenses or by using additional lenses, because lenses are limited by a property called resolving power. By definition, resolving power is the ability of a lens to show two adjacent objects as discrete entities. When a lens cannot discriminate, that is, when the two objects appear as one, it has lost resolution. Increased magnification will not rectify the loss, and will, in

fact, blur the object. The resolving power of a lens is dependent on the wavelength of light used and the numerical aperture, which is a characteristic of each lens and imprinted on each objective. The numerical aperture is defined as a function of the diameter of the objective lens in relation to its focal length. It is doubled by use of the sub stage condenser; which illuminates the object with rays of light that pass through the specimen obliquely as well as directly. Resolution is determined by certain physical parameters that include the wavelength of light, and the light-gathering power of the objective and condenser lenses and the distance between objects. A simple mathematical equation defines the smallest distance (d_{min}) separating the two very small objects:
Resolving power is inversely related to wave length
d_{min} = 1.22 x wavelength / N.A. $_{objective}$ + N.A. $_{condenser}$

This is the theoretical resolving power of a light microscope. In practice, specimen quality usually limits d_{min} to something greater than its theoretical lower limit.
However; as with magnification, resolving power also has limits. It is clear that merely decreasing the wavelength will automatically increase the resolving power of a lens. Such is not the case, because the visible portion of the electromagnetic spectrum is very narrow and borders on the very short wavelengths found in the ultraviolet portion of the spectrum.
The relationship between wavelength and numerical aperture is valid only for increased resolving power when light rays are parallel. Therefore, the resolving power is dependent on another factor, the refractive index. This is the bending power of light passing through air from the glass slide to the objective lens. The refractive index of air is lower than that of glass, and as light rays pass from the glass slide into the air, they are bent or refracted so that they do not pass into the objective lens. This would cause a loss of light, which would reduce the numerical aperture and diminish the resolving power of the objective lens. Loss of refracted light can be compensated for by interposing mineral oil, which has the same refractive index as glass, between the slide and the objective lens. In this way, decreased light refraction occurs and more light rays enter directly into the objective lens, producing a vivid image with high resolution.

Numerical Aperture:

Based on the above statement on resolution, the shorter the wavelength, the greater the resolving power of the lens. Thus, short wavelengths of the electromagnetic spectrum are better suited than longer wavelengths in terms of the numerical aperture.
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Illumination

An essential factor in producing a good image with the light microscope is obtaining adequate levels of light in the specimen, or object plane. It is not only necessary to obtain bright light around the object, but for optimal imaging, the light should be uniform across the field of view. The best way to illuminate the specimen involves the use of yet another lens system, known as a condenser. The front element of the condenser is usually a large, flattened lens that sits directly beneath the specimen. Its placement on a movable rack provides the means to focus the light beam coming past the object and maximize the intensity and control the uniformity of illumination. Two apertures in the illumination system allows to regulate the diameter of the illumination beam by closing or opening iris diaphragms. One of these diaphragms, housed within the condenser and known as the condenser diaphragm, allows to increase contrast, but at the cost of worsening resolution. The second of these diaphragms, known as the field aperture diaphragm, does not affect resolution as dramatically and is regularly adjusted for optimal illumination.

Focusing of microscope:

This refers to the changing of the distance between objective to specimen to obtain a crisp picture. In most cases the stage is raised or lowered with the coarse and the fine focus knob.

During Fine Focus This focus knob moves the stage up and down in small steps. It is used to focus at different layers of the specimens while in coarse **a**lso referred to as rough focus, this knob raises and lowers the stage quickly. It should only be used in connection with the low magnification lenses.

Light Microscope:

The simplest form of light microscope consists of a single glass lens mounted in a metal frame – a magnifying glass (figure 1.2). Here the specimen requires very little preparation, and is usually held close to the eye in the hand. Focusing of the region of interest is achieved by moving the lens and specimen relative to one another. The source of light is usually the sun or ambient indoor light. The detector is the human eye. The recording device is a hand drawing or an anecdote.

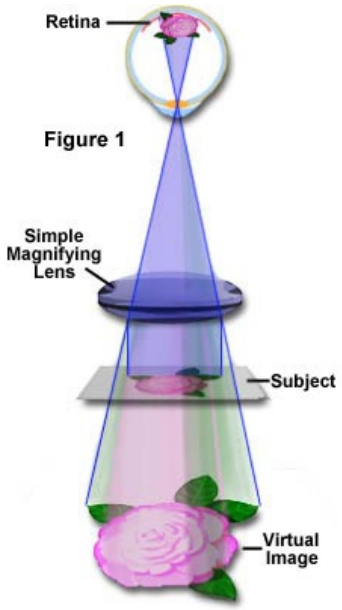


Figure 1.2: Magnification in a simple microscope

Compound light microscope:

A *compound light microscope* is an optical instrument that uses visible light to produce a magnified image of an object (or specimen) that is projected onto the retina of the eye or onto an imaging device. The word *compound* refers to the fact that two lenses, the objective lens and the eyepiece (or ocular), work together to produce the final magnification *M* of the image such that

Mfinal = Mobj - Moc

Two microscope components are of critical importance in forming the image: (1) the *objective lens,* which collects light diffracted by the specimen and forms a magnified real image at the real intermediate image plane near the eyepieces or oculars, and (2) the *condenser lens,* which focuses light from the illuminator onto a small area of the specimen.

The arrangement of these and other components is shown in Figure 1.3. Both the objective and condenser contain multiple lens elements that perform close to their theoretical limits and are therefore expensive. As these optics are handled frequently, they require careful attention. Other components less critical to image formation are no less deserving of care, including the tube and eyepieces, the lamp collector and lamp socket and its cord, filters, polarizers, retarders, and the microscope stage and stand with coarse and fine focus dials. At this point take time to examine Figure 1.4, which shows how an image becomes magnified and is perceived by the eye. The figure also points out the locations of important focal planes in relation to the objective lens, the ocular, and the eye. The specimen on the microscope stage is examined by the objective lens, which produces a magnified real image of the object in the image plane of the ocular. When looking in the

microscope, the ocular acting together with the eye's cornea and lens projects a second real image onto the retina, where it is perceived and interpreted by the brain as a magnified virtual image about 25 cm in front of the eye. For photography, the intermediate image is recorded directly or projected as a real image onto a camera.

Microscopes come in both inverted and upright designs (figure 1.5). In both designs the location of the real intermediate image plane at the eyepiece is fixed and the focus dial of the microscope is used to position the image at precisely this location. In most conventional upright microscopes, the objectives are attached to a nosepiece turret on the microscope body, and the focus control moves the specimen stage up and down to bring the image to its proper location in the eyepiece. In inverted designs, the stage itself is fixed to the microscope body, and the focus dials move the objective turret up and down to position the image in the eyepieces.

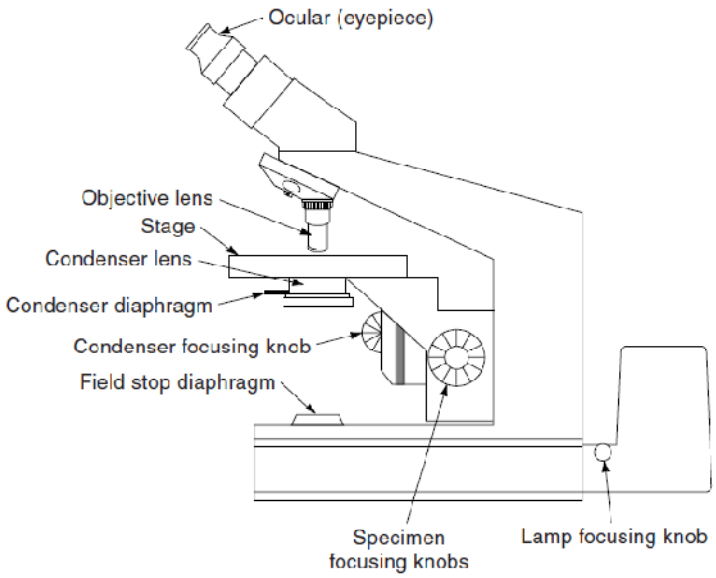


Figure 1.3: Components of a Compound light microscope

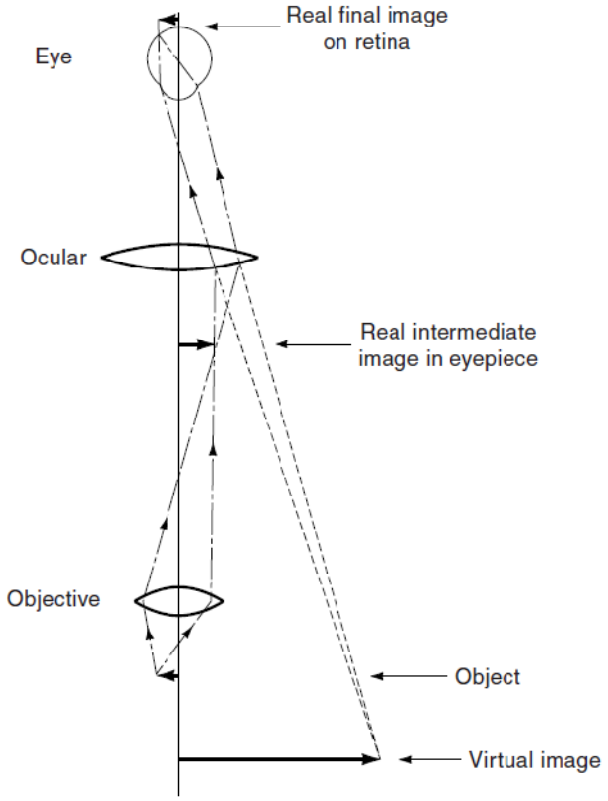


Figure 1.4 perception of a magnified virtual image of a specimen in the microscope

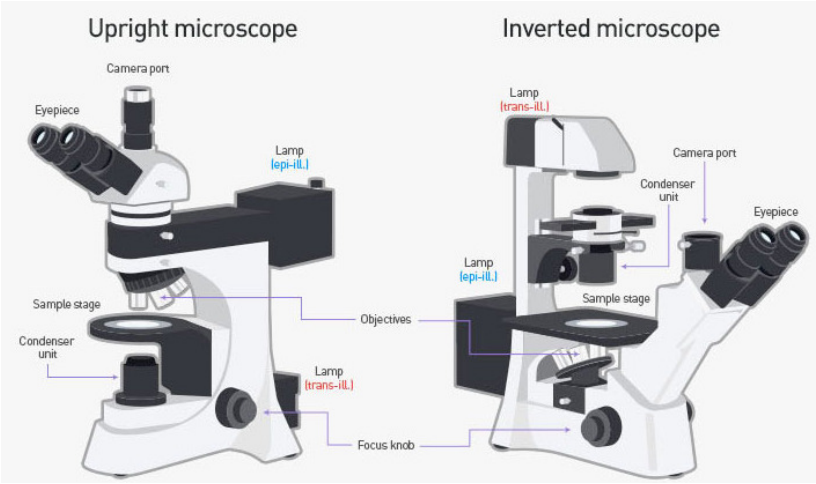


Figure 1.5: The two basic types of compound microscopes

Figure 1.6: Objective lenses used in compound microscope

Figure 1.7: Eye piece used in compound microscope

Parts of a Compound light microscope:

The following are the parts of a compound light microscope (figure 1.3);

1. Objective lenses:

The objective lenses are the most important components of microscopes and thus will be discussed in greater detail here. Their basic function is to gather the light passing through the specimen and then to project the image up into the body of the microscope. Then, the eyepiece lens system further magnifies the image for eye to see. Most quality microscopes use glass for the objectives and even for beginner microscopes, stay away from plastic objectives lenses as the quality level is quite inferior. The objectives are the lens system closest to the specimen.

There is one objective for each eyepiece in a compound microscope. For stereo microscopes, there are objective pairs (one objective lens for each eyepiece lens) which give the 3-D effect. On

compound microscope objectives, there is printed the following information on each one – power, DIN tube length, N.A., cover slip thickness, universal color ring. Tube length of the objectives usually have a DIN (interchangeable) of 185mm or 195mm. Objectives vary in power from 1x to 160x in compound microscopes but the most common power range is from 4x to 100x. Most compound microscopes have three or four (occasionally five) objectives usually of 4x, 10x, 40x, and 100x (oil immersion) which revolve on a nose piece (turret) to give different magnifying powers. The 4x, 10x, and 40x are called "dry" objectives which means they operate with air between the objective and the specimen. The 100x is called a "wet" objective which means it operates with immersion oil between the lens and the specimen. For stereo microscopes, they usually have one or two objectives lenses which normally are 1x, 2x, 3x, or 4x. In addition, there are zoom models which operate from about 0.5x up to 5x (figure1.6).

Eye Piece:

The eyepiece consists of a series of lenses mounted in a tube (barrel) at the upper end of the microscope (figure 1.7). Its basic function is to look at the focused, magnified image projected by the objective lens and magnify that image a second time before your eye looks at the image of the specimen. As with objective lenses, stay away from eyepieces where the optics are made of plastic as they will ensure very poor quality. The eyepieces are usually 10x but also come in 5x, 12.5x, 15x, and 20x. The "x" refers to the amount of magnification (power) that this lens adds as a multiplier to the magnification of the objective. They are inscribed with the magnification and its field number (which is the diameter in millimeters of the diaphragm opening (aperture) of the eyepiece. The aperture limits the field of view to the useful coverage of the eyepiece.

Condenser Lens (Sub-stage Condenser)

A glass lens or lens system located within or below the stage on compound microscopes. Its basic function is to gather the light coming in from the light source and to concentrate that light into a light cone onto the specimen (figure1.8). High power objective lenses have very tiny diameters and require concentrated light to work properly. A basic condenser is fixed in place while a moveable and more precise and more expensive condenser is the Abbe condenser. It usually can be moved up and down vertically, regulating the amount of light from the illuminator. It mounts under the stage and usually has an adjustable iris type aperture to control the diameter of the beam of light entering the lens system. By changing the size of the iris and moving the lens up or down from the stage, the diameter and focal point of the cone of light that goes through the specimen can be controlled. It is most useful at 400x and higher powers.

Diaphragm:

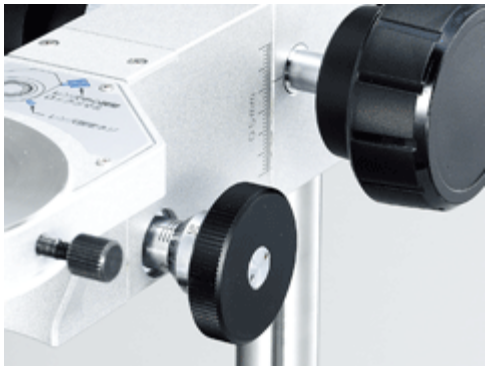
The diaphragm is also called the sub-stage diaphragm or aperture diaphragm (figure 1.8). The diaphragm is normally located under the stage of a microscope and it adjusts the amount of light passing into the slide or specimen. It is most useful at high powers.

Most compound microscopes have one of two types of diaphragm:

1. Disc Diaphragm – is the simplest and least expensive of the two types. It is located between the light source and the slide or specimen. It contains a rotating disk (usually fixed) with five to ten openings of differing diameters which limit the amount of light passing through to the specimen.
2. Iris Diaphragm – is the better and more expensive of the two types. It has a continuously variable diameter (like the iris of an eye or a camera shutter) which has a function to limit the size of the opening through which light passes from the light source to optimize resolution, contrast, and sharpness. It is usually controlled by a lever.

Figure 1.8: Image of a basic condenser and its respective diaphragm

A)



B)

Figure 1.9: A) Fine Knob and Coarse Knob B) Coaxial Coarse/Fine focusing Knobs

Figure 1.10 Microscopes with Monocular, binocular and trinocular heads

Illumination Systems (Light Source)

Since specimens rarely generate their own light, illumination is necessary. Illumination is the application of light onto an object or specimen in a microscope. The illuminator is the source of light which illuminates the object or specimen to be observed. Illumination of the object or specimen should be bright, free of glare, and evenly dispersed in the field of view. The simplest means of illumination can be provided by overhead lights, desk or table lamps, or indirect sunlight. Many compound microscopes are provided with adjustable plano/concave mirrors which reflect an external light source into the microscope. The flat slide (plano) of the mirror usually provides the sharper image but if stronger and brighter illumination is needed then use the concave side. These methods are the least expensive illumination methods but it can be difficult to direct the light source for proper illumination. The more expensive and common illumination is by using built-in or attached light sources using bulbs or lamps that provide direct and intense illumination. These light sources can be from above the specimen or object which is used mainly with low power stereo microscopes and is called incident (reflected) light or from below a specimen (typically a slide specimen) which is light passing up through the specimen from inside the base and called transillumination (transmitted light). Lighting from both top and bottom at the same time can provide enough light for the most thick and irregular specimens. These illuminators may be of a fixed intensity, or of a variable intensity, which use a control knob (rheostat) to control the intensity of the light produced.

Illumination lamps or bulbs come in various types:

Tungsten – is an incandescent bulb filament which is the most common and least expensive. They give off a yellowish hue and give off moderate heat. They are typically 15-watt or 20-watt.

Halogen – is a lamp which generally is the hottest light source for a microscope. The light is very bright, very white, and concentrated. The halogen type is more expensive than the tungsten. They are typically 15-watt or 20-watt.

Fluorescent – is a lamp that is cool in temperature. The light is bright and white and very sharp while being comfortable to the eye. The fluorescent is great for observing live specimens. They are typically 5-watt to 10-watt and generate the same brightness as the tungsten or halogens do. They can be built in the base of a microscope or they can be attached (called a ring light) to observe from above.

LED – these are light emitting diodes which provide a bright light source with virtually no heat. The white beam is brighter and cooler than the other illumination systems. They are typically battery operated and thus are cordless and great for outdoor use also.

There are various forms of illumination produced by varying the amount of light or the quality of the light allowed to impinge on the specimen:
Bright Field – this is the most fundamental and common form of lighting for microscopes. It is a highly directional and intense light source. Light aimed from beneath the stage through a condenser lens, through the specimen, through an objective lens, and through the eyepiece to the eye.
Diffuse – this is where you place a ground glass, some translucent plastic, some opalescent material, or other similar material in front of the condenser (between the illuminator source and the condenser lens) and will cause the light of a bright field source to be scattered. Often this broadens the field illumination and brings subtle changes in the image.
Phase Contrast – this form is used mainly because a large spectrum of living biological specimens (blood, tissue, and cultured cells), are virtually transparent or have poor contrast when observed with a bright field microscope. By utilizing a phase annulous (ring) mounted in the condensers front focal plane partially modulates the light ray bundles that pass through and around the specimen, where they are slowed ¼ wave, then are retarded another ¼ wave when they pass through the phase plate in the rear focal plane of the objective. This system also diminishes background light about 85% providing a darkened background to contrast with the illuminated structure of the phase object. While the affect diminishes the resolution of the image, it makes detail visible that one could not see without it.
Dark Field – this form is a method to examine transparent or semi-transparent specimens which cannot be distinguished from the background. It shuts out background light and allows only scattered light to reach the specimen in order to heighten textural detail.
Koehler – this form is a technique to optimize light quality and sharpness by aligning and adjusting each component of the optical system starting with a focusing illuminator. The light quality will be even and bright. The Koehler is the best form of illumination possible with a microscope and is offered on only the most expensive microscopes.

Focus Systems

A focus control allows to adjust the focus of the microscope. Every microscope includes a focusing control (knob) forquick (coarse) focusing of the image. More expensive compound microscope models include a coarse (quick) and fine focusing control. The fine focus is particularly advantageous in high power applications and required for 400x and higher but is not available on stereo microscopes since they are only low power.
Coaxial controls (focus) are where both the course and fine focus knobs are on one large control (on top of each other). The larger knob is typically for the coarse focusing and the smaller knob for the fine focusing. The smaller knob is usually centered on the inside of the larger one.

Head (Body)

The head is the upper part of the microscope that connects the eyepiece to the nosepiece or turret. Some heads are fixed in place and allow you to tilt them from angles of 0° up to 60°. More expensive microscopes usually have heads that can be rotated 360° allowing two or more users to see the specimen without the microscope itself having to be picked up and rotated.

There are several types of heads (figure 1.10):

Monocular – this is a microscope with a single eyepiece. These types are the more economical models and are very satisfactory for their usage. A monocular head with a second vertical viewing port is called a teaching head (dual view head) which can be used by a second person (or teacher) to observe the same image as the first person. Or, the second port can be used with various cameras.

Binocular – this is a microscope with two eyepieces, one for each eye. They are generally used on high power compound microscopes and all low power stereo microscopes and are generally more comfortable to use than a monocular type.

Trinocular – this is a microscope with a binocular head for viewing and an additional port that can be used for a third eyepiece for a second person (or teacher) to use or it would be used for various photo applications.

Nosepiece (Turret or Revolving Nosepiece)

The nosepiece is a rotating turret located above the stage on compound microscopes that can hold multiple objective lenses of various magnifications. By rotating the objectives into the light path and over the specimen you can observe various magnifications of the specimen during your examination.

Arm

The arm (also called the stand or limb) is the component of a microscope which contains the focus mechanism and supports the stage, as well as the body or head which contains the eyepieces. It provides the rigidity of a microscope as it rises from the base.

Base

The base is the bottom support part of the microscope. It provides balance and rigidity. It houses electrical components for illumination.

Eyepiece Tubes

The eyepiece tubes are also called observation tubes or drawtubes. They are attached to the arm above the nosepiece. They are usually set at angles of 45° or 30° for comfortable viewing. The bottom of the eyepiece tubes hold a special lens called the eyepiece (tube) lens.

Tube Lens

At the bottom of the eyepiece tubes is a tube lens. Its function is to gather the parallel rays of light projected by the objective lens and bring those rays to focus at the plane of the fixed diaphragm of the eyepiece. On some microscopes, the tube lens is built into the body of the microscope itself.

Stage

The platform beneath the objectives on which the slide or object to be observed is placed is called a stage. It has a smooth, flat surface and can be rectangular or circular. On most compound microscopes, the stage moves up and down and the nosepiece is stationary but on some microscopes just the reverse takes place. The stage has an opening for passing light.

The simple type of stage is called a plain stage and the more sophisticated stage is called a mechanical stage.

Applications:

Compound microscopy is used extensively in microelectronics, nanophysics, biotechnology, pharmaceutic research, mineralogy and microbiology. It is used for medical diagnosis, the field being termed histopathology when dealing with tissues, or in smear tests on free cells or tissue fragments.

In industrial use, binocular microscopes are common. Aside from applications needing true depth perception, the use of dual eyepieces reduces eye strain associated with long workdays at a microscopy station. In certain applications, long-working-distance or long-focus microscopes are beneficial.

FLUORESCENCE MICROSCOPY

Fluorescence microscopy has become an essential tool in biology as well as in materials science as it has attributes that are not readily available in other optical microscopy techniques. The use of an array of fluorochromes has made it possible to identify cells and submicroscopic cellular components and entities with a high degree of specificity amid nonfluorescing material. The fluorescence microscope can reveal the presence of a single fluorescing molecule. In a sample, through the use of multiple staining, different probes can simultaneously identify several target molecules. Although the fluorescence microscope cannot provide spatial resolution below the diffraction limit of the respective objects, the detection of fluorescing molecules below such limits is readily achieved. There are specimens that autofluoresce when they are irradiated and this phenomenon is exploited in the field of botany, petrology, and in the semiconductor industry. In the study of animal tissues or pathogens, autofluorescence is often either extremely faint or nonspecific.

Principle:

Fluorescence microscopy requires that the objects of interest fluoresce. Fluorescence is the emission of light that occurs within nanoseconds after the absorption of light that is typically of shorter wavelength. The difference between the exciting and emitted wavelengths, known as the Stokes shift, is the critical property that makes fluorescence so powerful. By completely filtering out the exciting light without blocking the emitted fluorescence, it is possible to see only the objects that are fluorescent. This approach to contrast is superior to absorption techniques in which objects are

stained with agents that absorb light. With absorbance dyes, the amount of light absorbed becomes only infinitesimally different from the background for small objects. In fluorescence, however, even single fluorescent molecules are visible if the background has no autofluorescence. Fluorophores molecules that are used by virtue of their fluorescent properties are called fluorophores. The outermost electron orbitals in the fluorophore molecule determine both its efficiency as a fluorescent compound and the wavelengths of absorption and emission. When fluorescent compounds in their so-called 'ground state' absorb light energy (photons), alterations in the electronic, vibrational and rotational states of the molecule can occur. The absorbed energy sometimes moves an electron into a different orbital that is on average farther away from the nucleus. This transition to an 'excited state' occurs very rapidly (in femtoseconds). Usually the excitation process also sets in motion molecular vibrations in which the internuclear distances vary over time. All this absorbed energy is eventually shed. Vibrational relaxation and fluorescence emission are the chief ways a fluorophore returns to its low-energy ground state. Whereas many organic substances have intrinsic fluorescence (autofluorescence), and a few are useful for specific labeling of components in biological systems, the typical approach to fluorescence microscopy is to take advantage of synthesized compounds that have some degree of conjugated double bonds. Such compounds often have ring structures (aromatic molecules) with pi bonds that easily distribute outer orbital electrons over a wide area. These compounds are optimal for fluorescence microscopy because the energy differences between excited state and ground state orbitals are small enough that relatively low-energy photons in the visible part of the electromagnetic spectrum can be used to excite electrons into excited states.

Lit of few Fluorophores is mentioned in the table below

Dye	Excitation maximum (nm)	Emission maximum (nm)
Commonly used fluorophores		
Fluorescein (FITC)	496	518
Bodipy	503	511
CY3	554	568
Tetramethylrhodamine	554	576
Lissamine rhodamine	572	590
Texas Red	592	610
CY5	652	672
Nuclear dyes		
Hoechst 33342	346	460
DAPI	359	461
Acridine Orange	502	526
Propidium iodide	536	617
TOTO3	642	661
Ethidium bromide	510	595
Ethidium homodimer	528	617
Feulgen	570	625
Calcium indicators		
Fluo-3	506	526
Calcium Green	506	533
Reporter molecules		
Green fluorescent protein (GFP)	395/489	509
DsRed	558	583
Mitochondria		
JC-1	514	529
Rhodamine 123	507	529

DAPI, 4′,6′-diamidino-2-phenylindole.

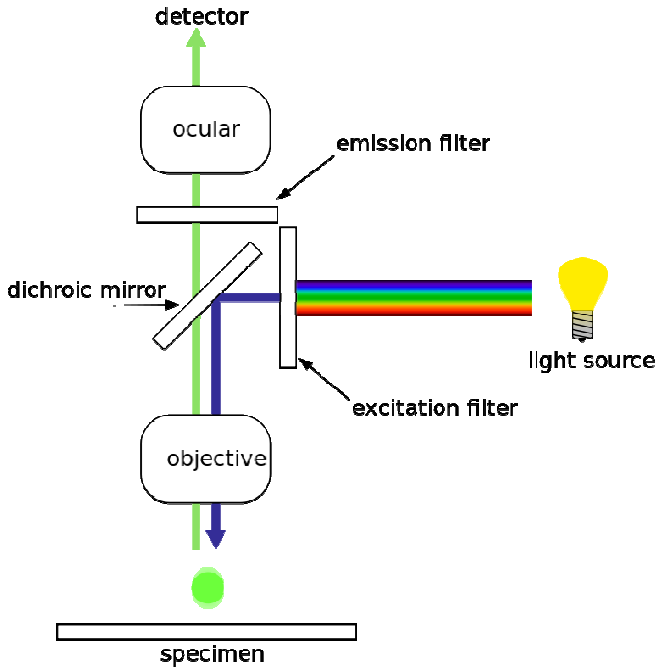


Figure 1.11: Schematic representation of a Fluorescent Microscope

Light source:

Fluorescence microscopy requires intense, near-monochromatic, illumination which some widespread light sources, like halogen lamps cannot provide. Four main types of light source are used, including xenon arc lamps or mercury-vapor lamps with an excitation filter, lasers, supercontinuum sources, and high-power LEDs. Lasers are most widely used for more complex fluorescence microscopy techniques like confocal microscopy and total internal reflection fluorescence microscopy while xenon lamps, and mercury lamps, and LEDs with a dichroic excitation filter are commonly used for wide field epifluorescence microscopes.

Sample Preparation:

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

Filters :

Basically, there are three categories of filters: exciter filters, barrier filters, and dichromatic beam splitters (dichroic mirrors). Fluorescence filters were formerly almost exclusively made of colored

glass or colored gelatin sandwiched between glass plates. Now, interference filters are used for exciter filters to pass or reject wavelengths of light with great selectivity and high transmission. Dichromatic beam splitters are specialized interference filters. Barrier filters may be either made of colored glass or interference filters.

Limitations of Fluorescent microscopy:

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

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

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

CONFOCAL MICROSCOPE

The technique **confocal imaging** which was first proposed by Nipkow and pioneered by a postdoc at Harvard named Minsky who made the first stage scanning confocal microscope in 1957. His microscope was commercially unfeasable because the technology needed to produce useful images was not available at the time. In 1986-87, a confocal microscope with the capabilities of producing very useful images could be built by combining the technologies of the laser, the computer, and microelectronics.

Principle:

"**Confocal**" is defined as "having the same focus." What this means in the microscope is that the final image has the same focus as or the focus corresponds to the point of focus in the object. The object and its image are "confocal." The microscope is able to filter out the out-of-focus light from above and below the point of focus in the object. Normally when an object is imaged in the fluorescence microscope, the signal produced is from the full thickness of the specimen which does not allow most of it to be in focus to the observer. The confocal microscope eliminates this out-of-focus information by means of a confocal "pinhole" situated in front of the image plane which acts as a spatial filter and allows only the in-focus portion of the light to be imaged. Light from above

and below the plane of focus of the object is eliminated from the final image. A diagram of the confocal principle is shown below (figure 1.12).

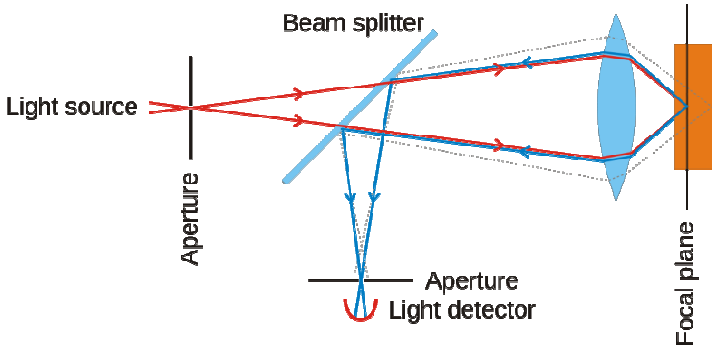


Figure 1.12: Principle of Confocal microscope

While the image that is seen with confocal filtering is all in-focus information, this creates another problem. Compared to a normal fluorescence microscope, the amount of light that is seen in the final image is greatly reduced by the pinhole, sometimes up to 90-95%. To compensate for this loss of light somewhat, two components have been incorporated into modern confocal microscopes. First, lasers are used as light sources instead of the conventional mercury arc lamps because they produce extremely bright light at very specific wavelengths for fluorochrome excitation. Second, highly sensitive photomultiplier-detectors (PMTs) were employed as imaging devices to pick up the reduced signal. The signal for detection in the original design of modern confocal microscopes is created by scanning a focused laser beam across a square or rectangular field. A system of motorized scanner mirrors sequentially scans a horizontal beam across the specimen. A third technology that is incorporated into the confocal microscope is the modern microcomputer. The computer is used to control the microscope's scanner mirrors and motorized focussing mechanism as well as collect, store, and analyze the data. Data is stored in the form of digital images which may be observed on a computer video monitor or sent to a hardcopy output device such as a film graphics recorder or a video or digital color printer. The computer allows the system to scan sequential planes in the Z-direction, store them, and create overlays of all the in-focus Z sections. This information can also be used to create three dimensional images, or movie rotations of well stained specimens.

Another useful feature of the confocal microscope is the ability to show colocalizations of signals from different fluorochromes. In specimens double-labeled for different molecules or structures, the different fluorochromes can be collected in different channels and combined to make color images which along with the three dimensional information obtained by confocal sectioning can more precisely show colocalizations of the signals than with the normal fluorescence microscope.

Confocal microscopy provides the capacity for direct, noninvasive, serial optical sectioning of intact, thick, living specimens with a minimum of sample preparation as well as a

marginal improvement in lateral resolution. Biological samples are often treated with fluorescent dyesto make selected objects visible. However, the actual dye concentration can be low to minimize the disturbance of biological systems: some instruments can track single fluorescent molecules. Also, transgenic techniques can create organisms that produce their own fluorescent chimeric molecules (such as a fusion of GFP, green fluorescent protein with the protein of interest).

Types of Confocal microscopes:

Four types of confocal microscopes are commercially available: Confocal laser scanning microscopes use multiple mirrors (typically 2 or 3 scanning linearly along the x and the y axis) to scan the laser across the sample and "descan" the image across a fixed pinhole and detector.

Spinning-disk (Nipkow disk) confocal microscopes use a series of moving pinholes on a disc to scan spots of light. Since a series of pinholes scans an area in parallel each pinhole is allowed to hover over a specific area for a longer amount of time thereby reducing the excitation energy needed to illuminate a sample when compared to laser scanning microscopes. Decreased excitation energy reducesphoto-toxicity and photo-bleaching of a sample often making it the preferred system for imaging live cells or organisms.

Microlens enhanced or dual spinning disk confocal microscopes work under the same principles as spinning-disk confocal microscopes except a second spinning disk containing micro-lenses is placed before the spinning disk containing the pinholes. Every pinhole has an associated micro-lens. The micro-lenses act to capture a broad band of light and focus it into each pinhole significantly increasing the amount of light directed into each pinhole and reducing the amount of light blocked by the spinning disk.

Programmable array microscopes (PAM) use an electronically controlled spatial light modulator (SLM) that produces a set of moving pinholes. The SLM is a device containing an array of pixels with some property (opacity, reflectivity or optical rotation) of the individual pixels that can be adjusted electronically. The SLM contains microelectromechanical mirrors or liquid crystal components. The image is usually acquired by a charge coupled device (CCD) camera.

Each of these classes of confocal microscope have particular advantages and disadvantages. Most systems are either optimized for recording speed (i.e. video capture) or high spatial resolution. Imaging frame rates are typically slower for single point laser scanning systems than spinning-disk or PAM systems. Commercial spinning-disk confocal microscopes achieve frame rates of over 50 per second– a desirable feature for dynamic observations such as live cell imaging. In practice, Nipkow and PAM allow multiple pinholes scanning the same area in parallel as long as the pinholes are sufficiently far apart. Cutting-edge development of confocal laser scanning microscopy now allows better than standard video rate (60 frames per second) imaging by using multiple microelectromechanical scanning mirrors.

Confocal X-ray fluorescence imaging is a newer technique that allows control over depth, in addition to horizontal and vertical aiming, for example, when analyzing buried layers in a painting.

Applications:

Confocal microscopy finds its applications in numerous biological sciences disciplines, from cell biology (figure 1.13), genetics, microbiology to developmental biology. It is also used in quantum optics and non-crystal imaging and spectroscopy.

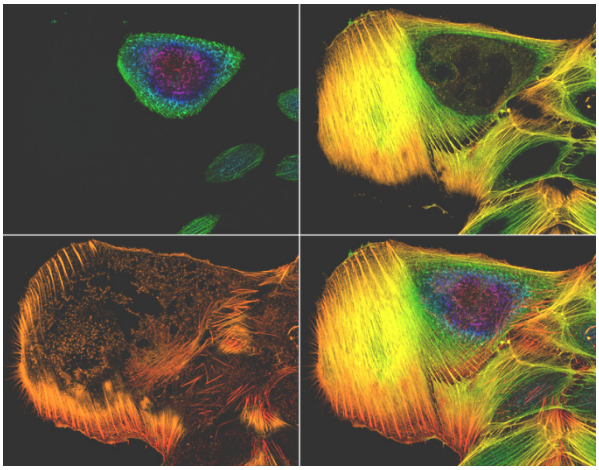


Figure 1.13: Stack of confocal images show actin filaments through out a cell

ELECTRON MICROSCCOPE

An electron microscope is a microscope that uses a beam of accelerated electrons as a source of illumination. As the wavelength of an electron can be up to 100,000 times shorter than that of visible light photons, the electron microscope has a higher resolving power than alight microscope and can reveal the structure of smaller objects. A transmission electron microscope can achieve better than 50 pm resolution and magnifications of up to about 10,000,000x whereas most light microscopes are limited by diffraction to about 200 nm resolution and useful magnifications below 2000x.

The transmission electron microscope uses electrostatic and electromagnetic lenses to control the electron beam and focus it to form an image. These electron optical lenses are analogous to the glass lenses of an optical light microscope.

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

There are two different types of electron microscopy: The Transmission electron microscope (TEM) and the Scanning electron microscope (SEM). In TEM, electrons that pass through the specimen are imaged. In the SEM, electrons that are reflected back from the specimen (secondary electrons) are collected, and the surfaces of specimen are imaged.

The equivalent of the light source in an electron microscope is the electron gun. When high voltage between 40000 and 100000V is passed between the cathode and the anode, a tungsten

filaments emits electrons (figure 1.14). The negatively charged electrons pass through a hole in the anode forming an electron beam. The beam of electrons passes through a stack of electron magnetic lenses. Focusing of the electron beam is achieved by changing the voltage across the electromagnetic lenses. When the electron beam passes through the specimen some of them are scattered while others are focused by the projector lens onto phosphorescent screens or recorded using photographic film or a digital camera. The electrons have limited penetration power, which means that specimens must be thin (50-100nm).

Electron microscopes are high-vacuum systems. In the area of the electron source a vacuum of 10^{-7} to 10^{-10} mbar is necessary to prevent oxidation/burning of the heated filament. The column and the area of the specimen are evacuated steadily to a vacuum of 10^{-5} to 10^{-7} mbar. The mean free path, referred to as the distance a molecule can fly before hitting another particle, accounts to about 50 meter at a vacuum of 10^{-6} mbar.

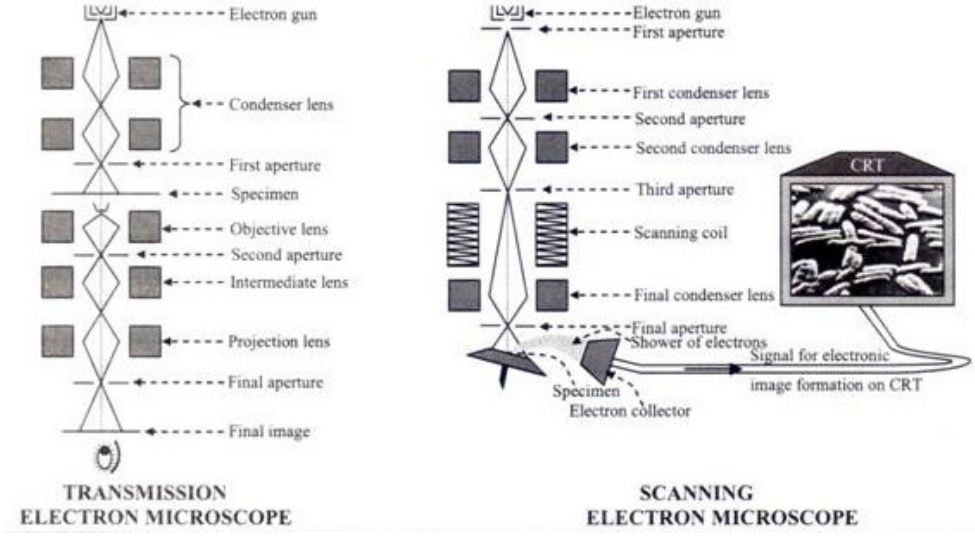


Figure 1.14: Principle of TEM and SEM

The electron source

Electrons can either be produced by thermionic emission or in a process called cold field emission. During thermionic emission, a very fine tip of a tungsten filament, LaB6 crystal or a ZrO/W Schottky emitter is heated by an electrical current flowing through the electron source enabling the escape of electrons. The electrons leaving the filament have a low energy and, therefore, need to be accelerated to the desired speed before entering the electron column. A high voltage between the electron source (cathode) and an anode plate is applied leading to an electrostatic field through which the electrons are guided and accelerated. During cold field emission, the electrons can escape from an extremely fine tungsten tip without heating (room temperature). The advantage of cold field emission sources is the very high yield of electrons and the very low chromatic aberration of

the electrons allowing imaging at atomic resolution. These instruments are very costly and require particularly high vacuum.

Electromagnetic lenses

Electromagnetic lenses consist of a huge bundle of windings of insulated copper wire, a soft iron cast and pole piece (figure 1.15). A magnetic field is induced by the current in the winding and reaches its main strength at the pole piece of the lens. The accelerated electrons entering the magnetic field are deviated following the law of a charge passing a magnetic field. The resultant force is always perpendicular to the plane defined by the direction of the magnetic field and the direction of the electrons. In conclusion, the electrons take a circular path through the lens system.

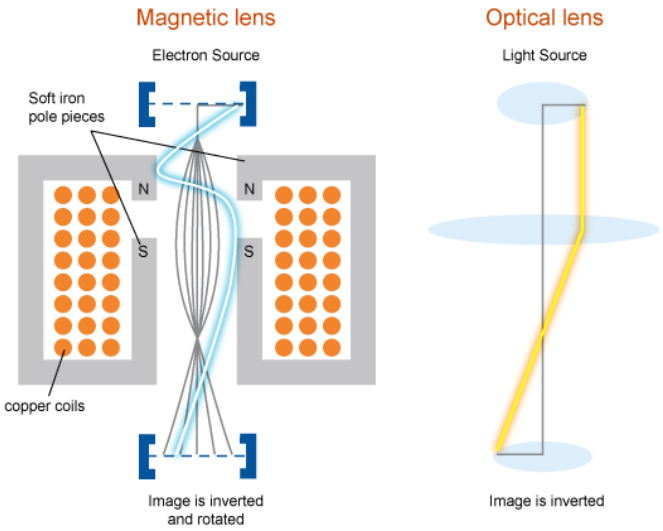


Figure 1.15: Comparison of Optical lens with electromagnetic lens

Specimen holders and stages

In TEM, the electron column does not offer a lot of space for the specimen. Additionally, the specimen must be very thin so that the electrons can penetrate the specimen and form an image. The average thickness of a biological specimen should be around 70 nm for a TEM with an acceleration voltage for the electrons of ~100 kV (the higher the voltage, the thicker specimens can be examined). Thin sections of the specimen are mounted on copper grids of 3 mm diameter, which are available in a wide variety of materials and mesh sizes. The grids with the sections on top are attached in a holder and introduced into the goniometer of the TEM through a vacuum lock, since the system always stays under high vacuum. The goniometer is the mechanical setup which enables highly precise and stable control of the specimen holder during imaging. Any drift or instability results in an unsharp image, in particular at high magnifications (figure 1.16).

The specimen chamber of SEMs are large and offer much more space for the specimen. In routine SEM the specimen can be at least 10 cm in diameter and 2 cm thick, since it does not have to be

permeable to electrons (bulk specimens). The specimen usually attached on a small aluminium stub (1 cm diameter) is mounted on a holder able to carry 8 stubs for example. The stub holder then is mounted on a 5 axis stage of the microscope, allowing controlled movement in x, y, z, tilting and rotation. The stub holder can be inserted in the microscope by hand requiring venting of the whole chamber or by using a vacuum lock (figure 1.17).

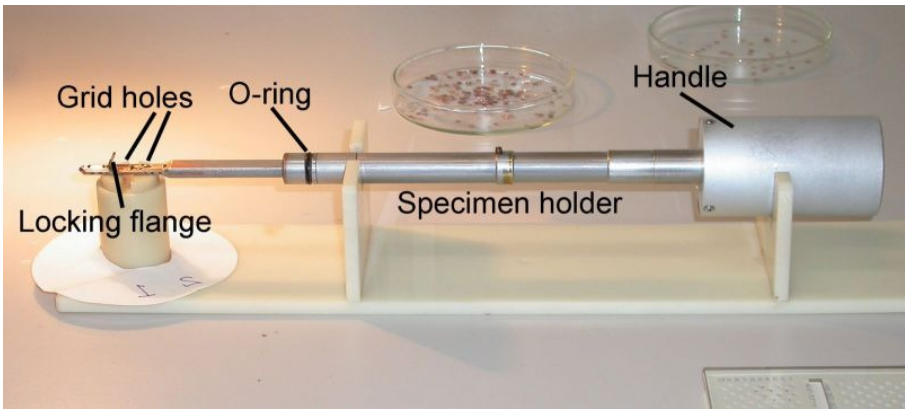


Figure 1.16: Specimen holder in TEM

Figure 1.17 : Specimen holder in SEM

Sample preparation:

Materials to be viewed under an electron microscope may require processing to produce a suitable sample. The techniques required varies depending on the specimen and the analysis required:

- **Chemical** fixation **–** This step for biological specimens aims to stabilize the specimen's mobile macromolecular structure by chemical crosslinking of proteins with aldehydes such as formaldehyde and glutaraldehyde, and lipids with osmium tetroxide.
- Negative stain **–** suspensions containing nanoparticles or fine biological material (such as viruses and bacteria) are briefly mixed with a dilute solution of an electron-opaque solution such as ammonium molybdate, uranyl acetate (or formate), or phosphotungstic acid. This mixture is applied to a suitably coated EM grid, blotted, then allowed to dry. Viewing of this preparation in the TEM

should be carried out without delay for best results. The method is important in microbiology for fast but crude morphological identification, but can also be used as the basis for high resolution 3D reconstruction using EM tomography methodology when carbon films are used for support. Negative staining is also used for observation of nanoparticles.

- Cryofixation – freezing a specimen so rapidly, in liquid ethane, and maintained at liquid nitrogen or even liquid helium temperatures, so that the water forms vitreous (non-crystalline) ice. This preserves the specimen in a snapshot of its solution state. An entire field called cryo-electron microscopy has branched from this technique. With the development of cryo-electron microscopy of vitreous sections (CEMOVIS), it is now possible to observe samples from virtually any biological specimen close to its native state
- **Dehydration –** or replacement of water with organic solvents such as ethanol or acetone, followed by critical point drying or infiltration with embedding resins. Also freeze drying.
- **Embedding, biological specimens –** after dehydration, tissue for observation in the transmission electron microscope is embedded so it can be sectioned ready for viewing. To do this the tissue is passed through a 'transition solvent' such as propylene oxide (epoxypropane) or acetone and then infiltrated with an epoxy resin such as Araldite, Epon, or Durcupan; tissues may also be embedded directly in water-miscible acrylic resin. After the resin has been polymerized (hardened) the sample is thin sectioned (ultrathin sections) and stained – it is then ready for viewing.
- **Embedding, materials –** after embedding in resin, the specimen is usually ground and polished to a mirror-like finish using ultra-fine abrasives. The polishing process must be performed carefully to minimize scratches and other polishing artifacts that reduce image quality.
- **Metal shadowing –** Metal (e.g. platinum) is evaporated from an overhead electrode and applied to the surface of a biological sample at an angle. The surface topography results in variations in the thickness of the metal that is seen as variations in brightness and contrast in the electron microscope image.
- **Replication –** A surface shadowed with metal (e.g. platinum, or a mixture of carbon and platinum) at an angle is coated with pure carbon evaporated from carbon electrodes at right angles to the surface. This is followed by removal of the specimen material (e.g. in an acid bath, using enzymes or by mechanical separation) to produce a surface replica that records the surface ultrastructure and can be examined using transmission electron microscopy.
- **Sectioning –** produces thin slices of specimen, semitransparent to electrons. These can be cut on an ultramicrotome with a diamond knife to produce ultra-thin sections about 60–90 nm thick. Disposable glass knives are also used because they can be made in the lab and are much cheaper.
- Staining – uses heavy metals such as lead, uranium or tungsten to scatter imaging electrons and thus give contrast between different structures, since many (especially biological) materials are nearly "transparent" to electrons (weak phase objects). In biology, specimens can be stained "en bloc" before embedding and also later after sectioning. Typically thin sections are stained for several minutes with an aqueous or alcoholic solution of uranyl acetate followed by aqueous lead citrate.
- **Freeze-fracture or freeze-etch –** a preparation method particularly useful for examining lipid membranes and their incorporated proteins in "face on" view. The fresh tissue or cell suspension is frozen rapidly (cryofixation), then fractured by breaking or by using a microtome while maintained

at liquid nitrogen temperature. The cold fractured surface (sometimes "etched" by increasing the temperature to about −100 °C for several minutes to let some ice sublime) is then shadowed with evaporated platinum or gold at an average angle of 45° in a high vacuum evaporator. A second coat of carbon, evaporated perpendicular to the average surface plane is often performed to improve stability of the replica coating. The specimen is returned to room temperature and pressure, then the extremely fragile "pre-shadowed" metal replica of the fracture surface is released from the underlying biological material by careful chemical digestion with acids, hypochlorite solution or SDS detergent. The still-floating replica is thoroughly washed free from residual chemicals, carefully fished up on fine grids, dried then viewed in the TEM.

- **Ion beam milling –** thins samples until they are transparent to electrons by firing ions (typically argon) at the surface from an angle and sputtering material from the surface. A subclass of this is focused ion beam milling, where gallium ions are used to produce an electron transparent membrane in a specific region of the sample, for example through a device within a microprocessor. Ion beam milling may also be used for cross-section polishing prior to SEM analysis of materials that are difficult to prepare using mechanical polishing.
- **Conductive coating –** an ultrathin coating of electrically conducting material, deposited either by high vacuum evaporation or by low vacuum sputter coating of the sample. This is done to prevent the accumulation of static electric fields at the specimen due to the electron irradiation required during imaging. The coating materials include gold, gold/palladium, platinum, tungsten, graphite, etc.
- Earthing **–** to avoid electrical charge accumulation on a conductive coated sample, it is usually electrically connected to the metal sample holder. Often an electrically conductive adhesive is used for this purpose.

Electron specimen interactions

The electron beam (TEM and SEM) hits the sample and either passes the sample unaffected or interacts with it.

Three different interactions of electrons with an atom can be generally discerned :

i) An electron of the primary beam is scattered by the electrostatic interaction with the positively charged nucleus of an atom at an angle of more than 90°, yielding **backscattered electrons**. This type of electrons has practically the same energy as the ones of the primary beam.

ii) An electron of the primary beam is scattered by the electrostatic interaction with the positively charged nucleus of an atom at an angle of less than 90°, yielding **elastically scatterd electrons**. Also these electrons do not loose energy and therefore are referred to as elastically scattered electrons.

iii) Electrons can also loose energy while interacting with the “electron cloud” of the atom. These are called inelastically scattered electrons.

In biological TEM analysis, the elastically scattered electrons are mainly used for imaging. The inelastically scattered electrons will be recorded as well with the imaging device (e.g. CCD camera). They have a slightly lower energy (a longer wavelength) than the primary electrons. In

conclusion, they are deviated less strongly in the electron lenses and do not merge at the same spot as the elastically scattered electrons (chromatic aberration). They contribute to unsharpness of the image. In high-end instruments, the inelastically scattered electrons can be filtered out with an energy filter (figure 1.18).

In SEM, mainly secondary electrons are used for imaging of biological specimens. These electrons have a very low energy (around 50 eV) compared to the energy of the primary electrons (up to 30 keV). Due to the low energy, these electrons can escape only from the surface area of the specimen and therefore provide information about the surface topography. Also backscattered electrons are frequently used for imaging with an according backscatter detector (figure 1.18). The number of electrons which are backscattered from a certain spot of the specimen depends on the local elemental density of the specimen. Hence, backscattered electrons provide a "density image" and information about the elemental composition, respectively. Backscattered electrons are also more resistant to charging due to their high energy.

X-rays are always produced when matter interacts with an electron beam. The energy of a part of the emitted x-rays is specific for different atoms and can be used for elemental analysis in TEM and SEM using special detectors.

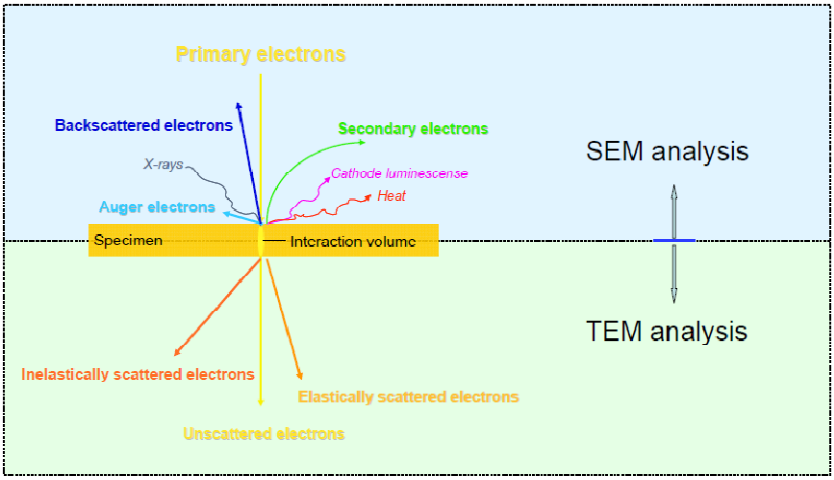


Figure1.18: Interaction of electrons with specimen in SEM and TEM

Contrast formation and imaging in TEM

The image formation in TEM is basically the same as for regular light microscopy, since the properties of electronsare very similar to the ones of light.

The primary electron beam passes through the thin specimen and generates non-diffracted and diffracted electromagnetic waves. The objective lens collates the diffracted and non-diffracted waves in the image plane and forms the primary image. Biological objects usually consist mainly of very light elements like C, O, H, N, S, P and lead to poor interaction with the electrons and, hence, provide almost no contrast. The specimen (thin section) must be treated to provide enough contrast for imaging. This is done by impregnation of the thin section with heavy metals like uranium and lead. The positively charged uranium and lead ions bind specifically to different constituents of

biological matter and to a different extent. The high atomic number and density of the heavy metal ions leads to a much higher interaction and diffraction of the electrons. The main contrast formation is achieved by trapping the numerous diffracted electrons using the objective aperture located in the back-focal plane of the objective lens. Spots of the sample containing more heavy metals will lead to a higher number of diffracted/scattered electrons, which will hit the objective aperture. These electrons do not reach the imaging device (CCD, screen) and will cause a "dark" or "electron dense" area in the image. Therefore, the image formation of biological specimens is based on **amplitude contrast** (figure 1.19). For many decades, images were taken by exposing electron sensitive films to the electron beam and the negatives were developed by conventional film development in a dark room. Nowadays, specialized CCD cameras are used for image acquisition. CCD cameras are available which reach the resolution of a conventional chemical film.

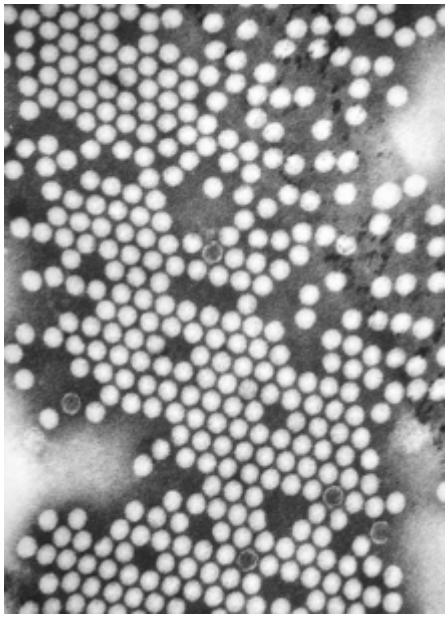


Figure 1.19: image of polio virus obtained by TEM

Contrast formation and imaging in SEM

In SEM, an electron beam with a tiny spot is guided pixel by pixel over the surface of a specimen, e.g. 1024 x 768 pixels. At every pixel, the beam stays for a defined time and generates a signal (e.g. secondary electrons) which are detected, amplified and displayed on a computer screen. The scanning of the beam is synchronized with the display of the computer screen. The signal deriving from every single pixel on the sample is simultaneously displayed on the corresponding pixel of the computer screen and finally forms the image.

The image consists of displayed grey levels, e.g. 256 gray levels in an 8 bit image. The magnification is changed by scanning a smaller area with the same number of pixels. The pixel size therefore gets smaller and the resolution is increased. The beam of electrons which hits a spot of the surface of the specimen interacts with a whole volume of the specimen. The interaction volume

looks like a pear. Secondary electrons are produced in the whole volume but can escape only from a small volume close to the surface of the specimen due to their low energy (around 50 eV). Backscattered electrons manage to escape from a larger depth since their energy is the same as the primary electrons. On their way through the sample, backscattered electrons can again interact with atoms and produce secondary electrons (called SE 2 electrons). X-rays can escape from the whole interaction volume. The size of the interaction volume is dependent on the acceleration voltage of the primary electrons and the atomic number of the atoms close to the surface. Heavy atoms decelerate the beam more than light elements and therefore reduce the interaction volume.

Imaging using secondary electrons

Secondary electrons (SE) are mainly used in SEM for imaging the surface topography of biological specimens. They are detected with an Everhart Thornley detector. The principle of detection is shown in figure 1.20. The SE are collected by a collector grid. A voltage of + 200 to 500 V is applied to the collector grid which attracts the low energy electrons. The SE then hit a scintillator which converts the electrons to photons. The photons are guided by a light conducting tube on the photomultiplier tube, where the photons again are converted to electrons that are amplified finally leading to an electrical signal. The current is translated into a gray value and displayed on the screen of the monitor.

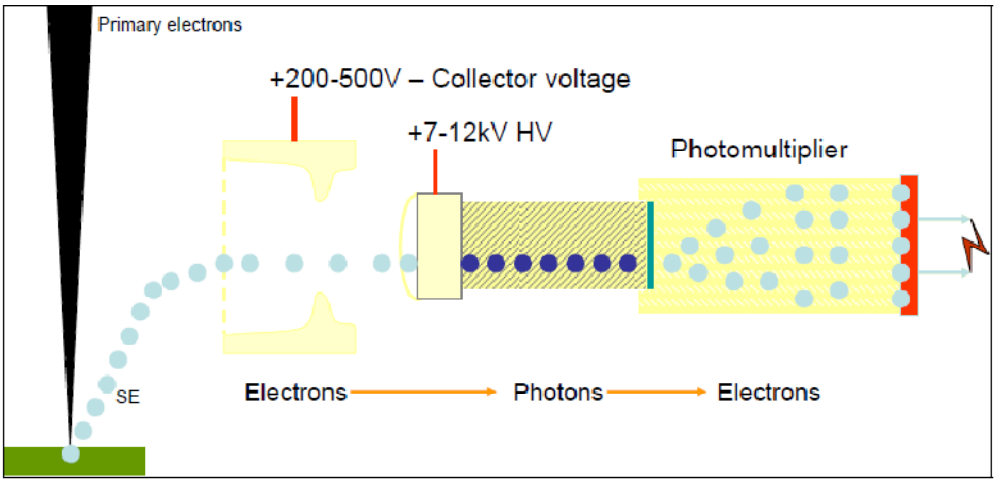


Figure 1.20: Principle of SE detector

Applications of Electron microscopy:

TEM:

A Transmission Electron Microscope is ideal for a number of different fields such as life sciences, nanotechnology, medical, biological and material research, forensic analysis, gemology and metallurgy as well as industry and education.

-TEMs provide topographical, morphological, compositional and crystalline information.

-The images allow researchers to view samples on a molecular level, making it possible to analyze structure and texture.

- This information is useful in the study of crystals and metals, but also has industrial applications.

- TEMs can be used in semiconductor analysis and production and the manufacturing of computer and silicon chips.

-Technology companies use TEMs to identify flaws, fractures and damages to micro-sized objects; this data can help fix problems and/or help to make a more durable, efficient product.

SEM

SEMs have a variety of applications in a number of scientific and industry-related fields, especially where characterizations of solid materials is beneficial.

-From topographical, morphological and compositional information, a Scanning Electron Microscope can detect and analyze surface fractures, provide information in microstructures, examine surface contaminations, reveal spatial variations in chemical compositions, provide qualitative chemical analyses and identify crystalline structures.

- SEMs can be as essential research tool in fields such as life science, biology, gemology, medical and forensic science, metallurgy.

- In addition, SEMs have practical industrial and technological applications such as semiconductor inspection, production line of miniscule products and assembly of microchips for computers.

PRACTICE QUESTIONS

1. The 100x objective lens is also called _____ as it can operate in oil immersion
   (A) Oil objective (B) Wet objective
   (C) Dry objective (D) Refractive objective

2. _____ is a technique to optimize light quality and sharpens by aligning and adjusting each component of the optical system starting with a focusing illuminator.
   (A) Dark field (B) Phase contrast
   (C) Koehler (D) Diffuse

3. Secondary electrons are used for imaging in which technique _____
   (A) Photo-ionization
   (B) SEM
   (C) TEM
   (D) Confocal microscopy

4. Out of focus information is eliminated in _____ microscopy.
   (A) Co axial focusing (B) Confocal (C) Trinocular focusing (D) TEM

5. The three categories of filters used in microscopy are,
   (A) Exciter filter, diaphragm filters, barrier filters
   (B) Exciter filters, barrier filters, UV filters
   (C) UV filters, barrier filters, prism filters
   (D) Exciter filters, barrier filters, and dichromatic beam splitters

6. The two types of diaphragms used in compound microscopes are:
   (A) Disc diaphragm & Iris diaphragm
   (B) Light diaphragm & Dark diaphragm
   (C) Iris diaphragm & Source diaphragm
   (D) Iris diaphragm & Filter diaphragm

7. Fluorescence microscope, involves treatment of samples with fluorphores
   _____ and _____ are the chief ways by which a flurophore returns to ground state
   (A) Photon emission & electron emission
   (B) Vibrational relaxation & electron emission
   (C) Vibrational relaxation & Fluorescence emission
   (D) Photons emission & Fluorescence emission

8. Fluorescence microscope, involves treatment of samples with fluorphores
Which among the following are fluorophores?
(A) Fluorescein
(B) Texas Red
(C) Ethidium homodimer
(D) Calcium green
(E) GFP
(G) DAP1
(A) All of the above
(B) A, C, E
(C) A, C, E, F
(D) A, E

9. What are electro-magnetic lenses?
(A) Lenses that are magnets
(B) Lenses with magnetic properties
(C) They have huge bundles of windings of insulated copper wire, a soft iron cost and a pin hole.
(D) They have huge bundles of soft iron cast and a pin holes.

10. Which method of sample preparation in electron microscopy involves, freezing the sample, breaking it in liquid nitrogen and then shadowing with gold & platinum.
(A) Gold plating
(B) Freeze-Etch method
(C) Cryopreservation
(D) (A) & (B)

ANSWER KEYS

1	B	**2**	C	**3**	B	**4**	B	**5**	D	**6**	A	**7**	C
8	A	**9**	C	**10**	B								

EXPLANATIONS

1. 100 x objective lens is also called as wet objective.

2. Koehler is a technique to optimize light quality. It is the best form of illumination possible with a microscope and is offered in expensive microscopes

3. Secondary electrons are mainly used in SEM for imaging the surface topography of biological specimens.

4. Confocal means having the same focus. This microscope is able to filter out the out-of-focus light from above and below the point of focus in the object.

5. The three basic categories of filters are, Exciter filters, barrier filters, and dichromatic beam splitters.

6. Most compound microscopes use Disc diaphragm and Iris diaphragm. They are used to adjust the amount of light passing through the specimen.

7. Vibrational relaxation and fluorescence emission are the chief ways a fluorophore returns to its ground state.

8. All the above are Fluorophores.

9. Electromagnetic lenses consist of huge bundles of windings of insulated copper wire, a soft iron cast and pole piece.

10. Freeze-etch or freeze fracture involves the above procedure.

3. Centrifugation

Introduction:- Centrifugation is one of the important separating techniques used in the analysis of subcellular components and bio molecules. This is developed by Svedberg during late 1920s and its refinement has led to Preparative centrifugation technique by Albert Claude et al during 1940's. Now it is an indispensable technique applied in various fields that are devoted for separation of cells, intracellular structures, membrane vesicles, subcellular molecules, macromolecules etc. To simply illustrate its significance an example of Lectin - Aggulation method can be given. To purify plasma lemma vesicles from skeletal muscles, this technique is used, in which lectins are added that lead to agglutination of vesicles by binding to their protein. This complete can be separated from other cellular components, which are present in the cytosol, by simple centrifugation process.

Basic Principles of sedimentation:-

The basic components of centrifugal are:-

i) A metal rotor with holes in it to accommodate a vessel that has the pimple.

ii) A motor that can spin the rotor at a selected speed.

A centrifuge has several other accessories that enable it to maintain environment for better centrifugation.

And a moving object that is moving in a circular path at a steady angular velocity will experience a force, F, directed outwards. This is the basis of centrifugation. Angular velocity in radians is ω, and the radius of rotation r in centimeters, collectively determine the magnitude of force F.

$$F = \omega^2 r \qquad \ldots (1)$$

F can be expressed in terms of earth's gravitational force, when divided by 980. The resultant is referred to as the relative centrifugal force, RCT, and is referred to as" number times g".

$$\therefore RCF = \frac{\omega^2 r}{980} \qquad \ldots (2)$$

The relationship can be further better expressed in terms of "Revolutions per minute", rpm. Generally the operating speed of centrifuge is expressed in terms of rpm, and the relationship between angular velocity and rpm is:

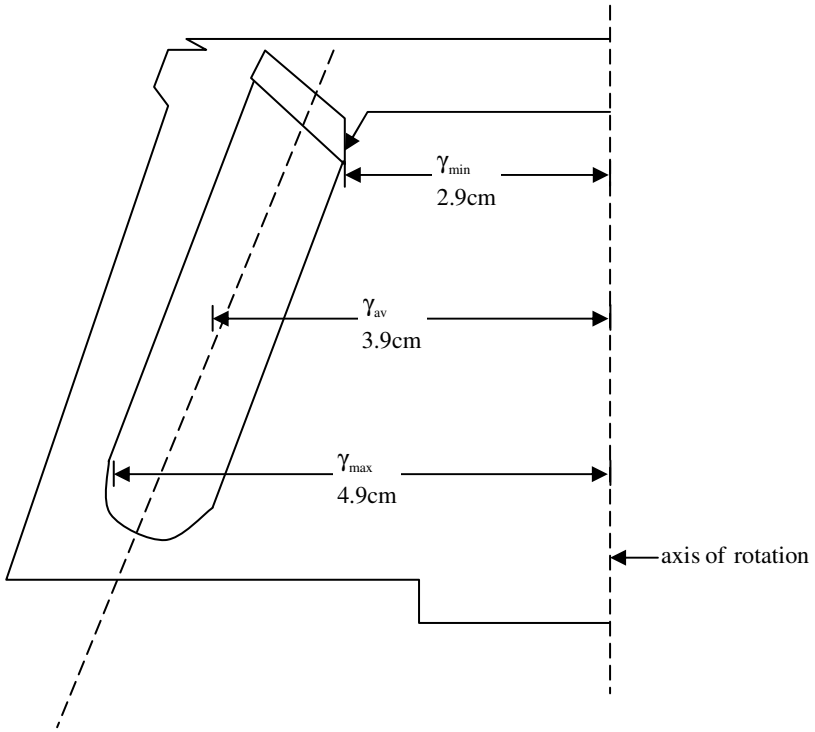
$$\omega = \frac{\pi(rpm)}{30} \qquad \ldots(3)$$

Substituting Eq. 3 in Eq 2.

$$RCF = \frac{\pi^2 (rpm)^2}{\frac{(30)^2}{980}} r = (1.119 \times 10^{-5})(rpm)^2 r$$

For a given centrifuge radius 'r' is constant and hence rpm determines RCF value.

But for few centrifuges, the sample holder shape varies, i.e., the distance of particles in the vessel progressively varies as it goes down the tube holder, i.e., it increases (figure 2.1). In such cases average of radius is taken to determine RCF value.



Calculating RCF for all the radii by fixing rpm as 12,000.

(i) $RCF_1 = (1.119 \times 10^{-5})(12,000)^2 2.9 = 4672.94 \times g$

(ii) $RCF_2 = (1.119 \times 10^{-5})(12,000)^2 3.9 = 6284.30 x g$

(iii) $RCF_3 = (1.119 \times 10^{-5})(12,000^2)4.9 = 7895.66 x\ g$

Problems on Relative centrifugal Field:-

1. Consider two centrifuges operating at an rpm of 20,000, having different radii of 4.8 and 8.0 am. Calculate their RCF values.

(i) $RCF_1 = (1.119 \times 10^{-5})(20000)^2 4.8 = 21484.8 \times g$

(ii) $RCF_2 = (1.119 \times 10^{-5})(20000)^2 8.0 = 38080 \times g$

Problems for students:-

A fixed angular rotor exhibits a minimum radius, r_{min} at the top of the centrifuge of 3.5 cm, and a maximum radius, r_{max} at the bottom of the tube of 7.0 cm. If the centrifuge rotates at a speed of 8,000 rpm, calculate RCF generated at both the points.

Sol.

$RCF_1 = Relative$ centrifugal force at top of tube

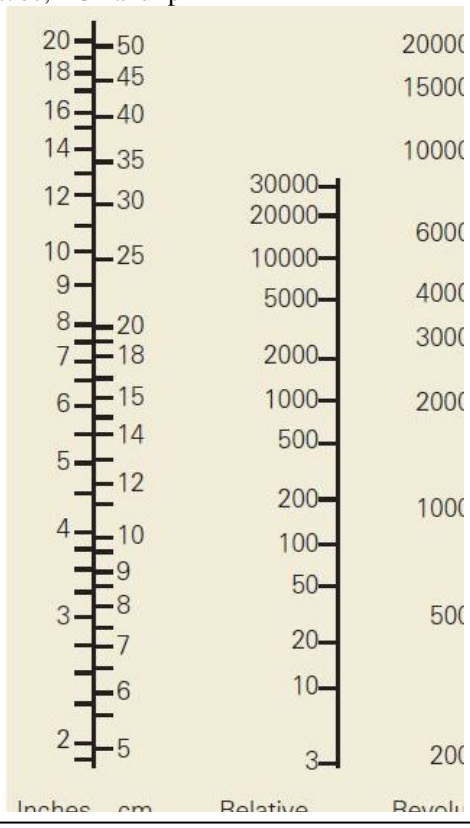
$RCF_2 =$ Relative centrifugal force at bottom of tube.

$RCF_1 = (1.119 \times 10^{-5}) \times (8000)^2 \times 3.5 = 2506.56 \times g$

$RCF_2 = (1.119 \times 10^{-5}) \times (8000)^2 \times 7 = 5013.12 \times g$

The following figure (figure 2.2) reveals the relationship between radius of tube, RCF and rpm.

Figure 2.2: Relationship between radius of tube, RCF and rpm



A part form rpm and radius, RCF is also influenced by other characteristics of particles, like density & viscosity of solution.

Consider that centrifugal force is being applied to a particle. As the particle sediments, it will have to displace some amount of the medium of suspension. This displacement will result in an upthrust felt by the particle. The upthrust will be equal to the weight of the liquid

displaced. For a spherical particle then, the net outward force (F), if the sedimentation is being done at ω radius per second, will be

$$F = \frac{4}{3}\pi r_p^3 \left(\rho_p - \rho_m\right)\omega^2 r \qquad \ldots(4)$$

Where ρ is due density of particle,

ρm is due density of medium

r is the distance of the particle from the centre of rotation.

and $\left(\frac{4}{3}\right)\pi r_p^3$ is the volume of a spherical particle of radius r_p.

A moving body always generates friction and a equation describing the force acting on the particle, this phenomenon has to be factored in. If we assume now that the spherical particle is also rigid and moving at known velocity, then the frictional force (F_0) is given as

$$f_0 = vf \qquad \ldots (5)$$

Where, v is the sedimentation velocity and *f* is the frictional coefficient of the particle in that particular medium Fractional coefficient depends upon such particle characteristics as its radius, shape, and hydration, as well as upon the viscosity of the medium

$$f = 6\pi\eta r_p \qquad \ldots(6)$$

Where $\eta$is the viscosity coefficient of the medium.

Thus, an unhydrated spherical particle accelerates under a centrifugal force till the net force of sedimentation of becomes equal to the frictional force acting in the opposite direction i.e., till F becomes equal to F_0, or

$$\frac{4}{3}\pi r_p^3 \left(\rho_p - \rho_m\right)\omega^2 r = 6\pi\eta r_p v \qquad \ldots(7)$$

When these two forces balance out, the particle sediments at constant velocity. The rate of sedimentation (V) is then gives as,

$$v = \frac{dr}{dt} = \frac{2}{9}\frac{r_p^2\left(\rho_p - \rho_m\right)\omega^2 r}{\eta} \qquad \ldots (8)$$

From the above expression, it is clear that sedimentation rate of the particle is determined by the size of the particle. If all conditions are equal, then from the equation the following conclusions can be drawn,

1. The large the particle, the faster, it will move
2. The more dense a particle, the faster it will move .
3. The more dense a solution is, the slower will be the movement of the particle.
4. The higher the viscosity of the medium, the slower will be the particle movement.

The time of sedimentation is another variable which is of importance, and can be obtained from the following equation,

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

Where, t = sedimentation time in seconds

r_t = radial distance from the centre of rotation to the liquid meniscus, and

r_b = radial distance from the centre rotation to the bottom of the tube.

From the above equation, it is clear that heterogeneous particles can be separated by centrifugation on the basis of their densities and/or size. This is achieved by differential centrifugation or by letting the particles sediment for a fixed time at a fixed sedimentation velocity.

Instrumentation

Centrifuges are categorized into 3 different types which are as follows:

1. Desktop centrifuges
2. High speed centrifuges
3. The ultracentrifuge.

1. Desk top Centrifuges:-

These are very simple and inexpensive. They are used to rapidly sediment and collect blood cells, yeast cells, bulk precipitates of chemical/Biochemical reactions. They are also known as clinical centrifuges and operate at a speed of 3000 rpm. They do not have any temperature regulation. They have the maximum sample carrying capacities varying from 10, 50 to $100cm^3$ tubes. The example tubes are placed evenly with equal volumes in opposite slots of the rotor spaces, to balance weight on the fast rotating shaft.

2. High speed centrifuges:- These operate at speeds of 25,000 rpm providing a centrifugal force of $\sim 90,000g$. These are equipped with the refrigeration to remove heat which is generated due to fiction between air and the spinning rotor. This instrument is used to collect microorganisms, cell debris, cells, large cellular organelleles, precipitates of chemical reactions and Immune precipitates. The subcellular components like nuclei and mitochondria can be separated, while these are of less used to get organelleles of smaller 's' value like lysosomes, microsomes etc.

3. The ultra centrifuges:- These can operate at high speeds of around 75,000 rpm generating a centrifugal force of $\sim 5,00,000$. Also the speed of rotations are high, more heat will be generated due to fiction, and to avoid this the rotor chamber is sealed and evacuated using pumps to create vacuum. A part from this, these centrifuges are also equipped with cooling system, which can maintain the temperature of the rotor at around 0° to $4^\circ C$.

The driving shaft on which the rotor is mounted is around 1/16 inches in diameter. This allows the shaft to be flexible during high speeds, and accumulate a certain degree of imbalance without damaging the spindle. The rotor is also equipped with a speed controlling device, and excessive speeds at time, may lead to explosive expulsion of rotor. To contain such explosions, the rotor chamber is always enclosed in heavy armor plates.

The resolution capacities of these centrifuges are very high. They can separate DNA molecules which differ in their nitrogen isotope content $\left(^{14}N \& ^{15}N\right)$. These are also used for isolation of viruses and purification protocols.

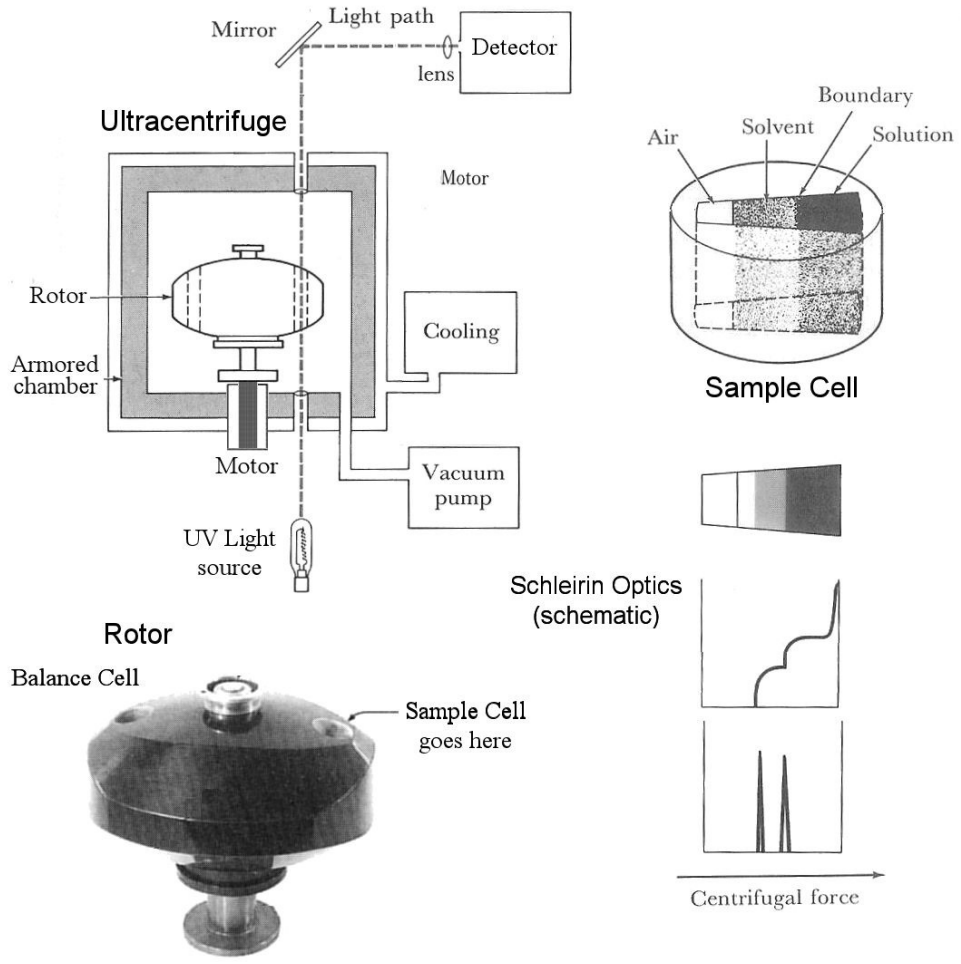
Ultra centrifuges are of two types based, upon their usage. Some are required for isolation purification purposes and these are called Preparative centrifuges, while the other one is for characterization of sample, and these are called Analytical centrifuges. Hence the two types of ultra centrifuges :-

i) Analytical ultra centrifuge and

ii) Preparative ultra centrifuge.

i) Analytical ultracentrifuge (figure 2.3)

Figure 2.3: Analytical ultracentrifuge

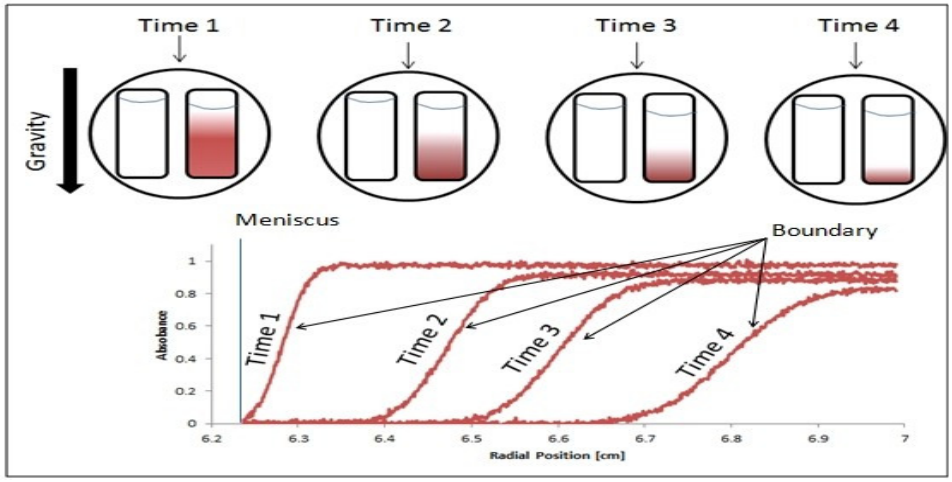


Both analytical and preparative centrifuges have a common description of components as mentioned above. However, the analytical ultra centrifuges differ with respect to monitoring the progress of centrifugation.

The rotor is elliptical in shape and has 2 holes for holding centrifuge cells. One cell is known as the analytical cell while the other one is counter poise cell. These two cells are vertical in position in the rotor and are placed at same distance in opposite direction to balance each

other. The analytical cell holds the sample and its sample volume capacity is $\sim 1 cm^3$. The upper and lower planes of the analytical cell are transparent having quartz or synthetic sapphire windows that allow light to pass through to monitor the progress of centrifugation.

Figure 2.4: Moving boundary of protein during centrifugation



The optical system used in called as the Schlieren optics Rayleigh interference option. The Rayleigh interference operates on the basis that, the region of the solution in analytical cell, harboring macromolecules will have a refractive index higher than rest of the solution. At the beginning of sedimentation, the peak of refraction will be at meniscus. With the progress of sedimentation, however, as the macromolecules move down the cell, the peak shifts giving information of the sedimentation characteristics of the macromolecules (figure 2.4). the Analytical ultracentrifuges can be used to measure sedimentation coefficients of macromolecules and even determination of molecular weights.

ROTORS

Centrifuges used 4 types of rotors. These includes:-

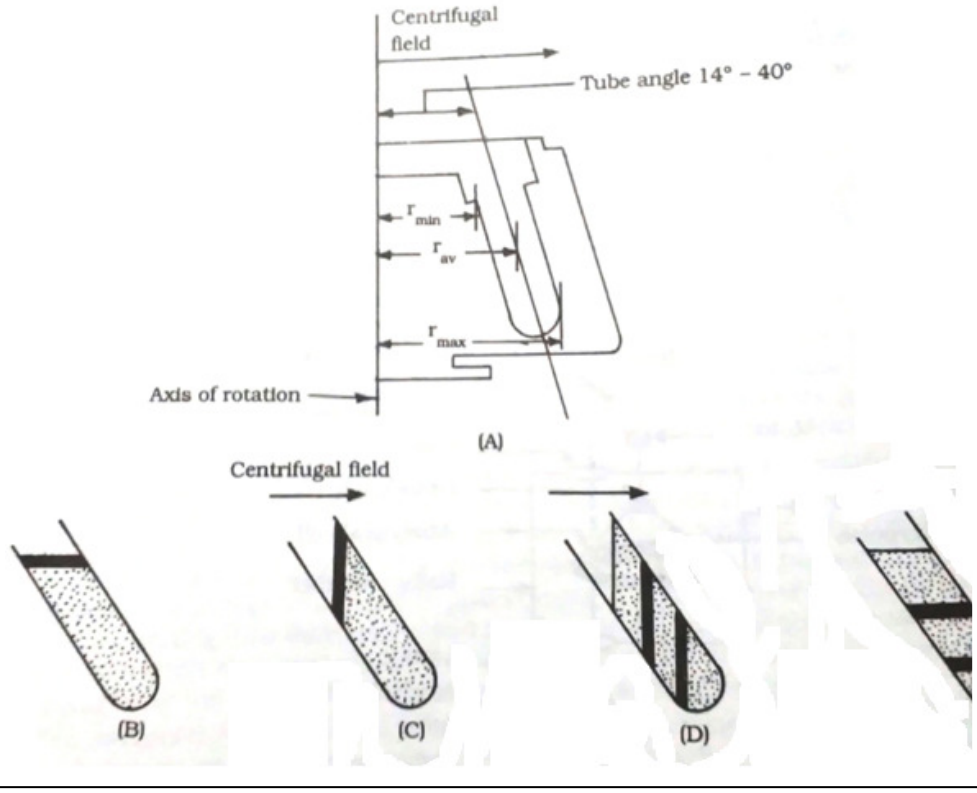
1. Fixed angle rotors
2. Vertical tube rotors
3. Swinging bucket rotors and
4. Zonal rotors.

1. **Fixed angle rotors:-**

These rotors have holes within them at a fixed angle, and the centrifuge tubes are just slided down within them. The sample holes are at angles between 14^o to 40^o to the centre shaft and therefore the solution within the tubes also takes same angle (figure 2.5). When centrifugal force is applied on the particles contained in the tube, they move radially outwards, travel a

short distance as they strike wall of the tube and them slide down along the wall to form pellet at the outer most point in the tube. The sliding down along the wall makes sedimentation process quick and this is termed Wall-effect.

Figure 2.5: Fixed Angle rotor: A) Cross section of Fixed angle rotor, B) Initial stage of centrifugation with sample layered on tube, C) Reorientation of sample and gradient during at the start of centrifugation, D)Separation of sample components, E)Gradient reorientation as centrifugation stops



However, the disadvantage with this rotor is that particles at different places (radii) within the tube experience different RCF values. Hence, can be used to separate molecules with huge difference in their sedimentation characteristics.

2. Vertical tube Rotors

These rotors have holes that lie parallel to the shaft. As the rotor accelerates and centrifugal field is applied, the solution within the tube reorients through 90^{o}. This makes particles perpendicular to the axis of rotation and particles get separated. As the rotor speed decelerates, the solution orients back to its original position.

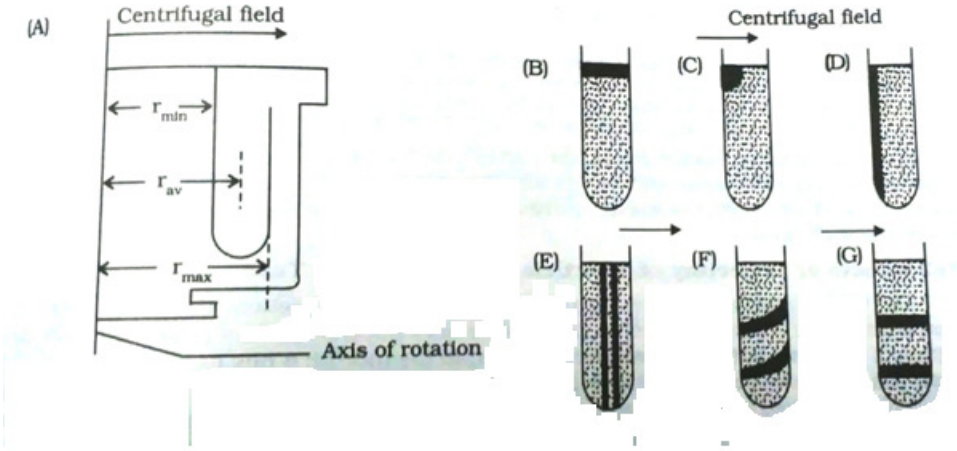


Figure 2.6: A) Cross section of Vertical Tube rotor , B) Tube filled with sample, C) Orientation of sample at the start of centrifugation, D) Final Orientation due to centrifugal force, E) Separation of samples F) Reorientation as centrifugal force ceases, G) At the end of separation

In these rotors, sedimentation occurs across the diameter of the tube, hence particles have to transverse short distance before they sediment. As these have holes at their edges, radius is maximum (r_{max}) importing high RCF in minimum time, thereby speeding up the process. At times pellets even deposits on the outer wall and they fall back in solution leading to no net separation and if it falls back partially it will finally result in loss of sample.

3. Swinging bucket rotors (figure 2.7)

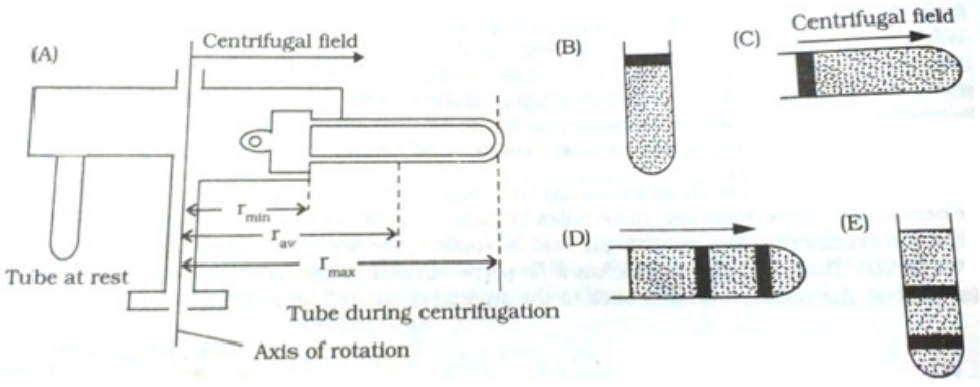


Figure 2.7: A) Cross section of Swinging Bucket rotor, B) Sample loaded on medium C) As centrifugal field is applied, tube swings out, D) Sample separation, E) Reorientation of sample at the end of centrifugation

These rotors have buckets that swing out to a horizontal position when the rotor accelerates. The solution in the tube reorients itself to lie perpendicular to the axis of rotation and parallel to the applied centrifugal field. As the rotor decelerates, the tubes fall back to their original position and the solution too, regains its original orientation. These have application for preparative purposes.

4. Zonal Rotors (figure 2.8)

These rotors are flattened spheres, interiorly divided into four equal quadrants called as the Sectors. These are used for density gradient centrifugation and operate at 3000 to 4000 rpm. Each quadrant has a special hole to load the gradient solution and sample port. The gradient holes are continuous with the holes onto the perimeter of the rotors interior and specially movable seal and bearing assembly.

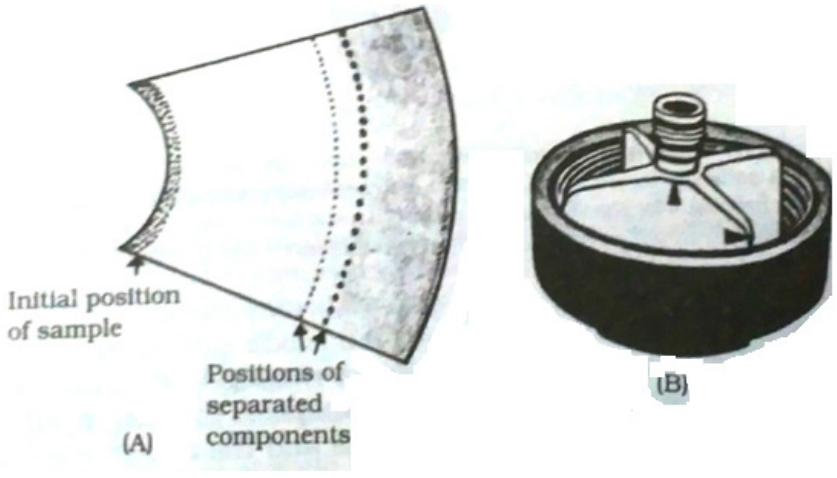
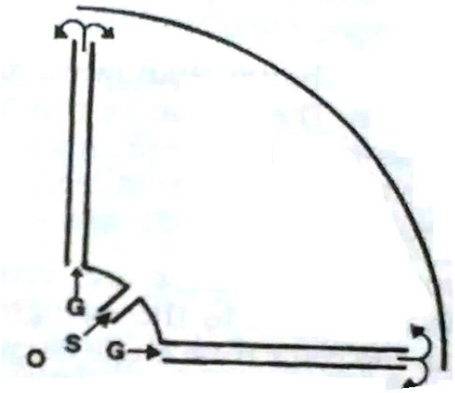


Figure 2.8: A) Sector shaped cell of a zonal rotor
B) Design of Zonal rotor

The gradient is pumped into the sectors as the rotor is moving. First, the material with lowest gradient is pumped, and subsequently high dense gradient materials are pumped. Usually a high dense solution pushes the low dense material towards the centre of rotation, there by generating a density gradient. The sample is loaded on the top of gradient towards the centre and the rotor is centrifuged. Based upon their buoyant densities, sample components get separated in the sector of the rotor. When the centrifugation process is over, separated components are collected by pumping in highly dense solution from periphery, which allows sample collection from the sample port.

This is best used for separating or fractionation of samples based on their buoyant densities. Wall effects are avoided and organalleles fractionation or protein purification/separation can be done effectively.

Figure 2.9: Cross section of a sector with holes for Gradient (G) and sample (S)



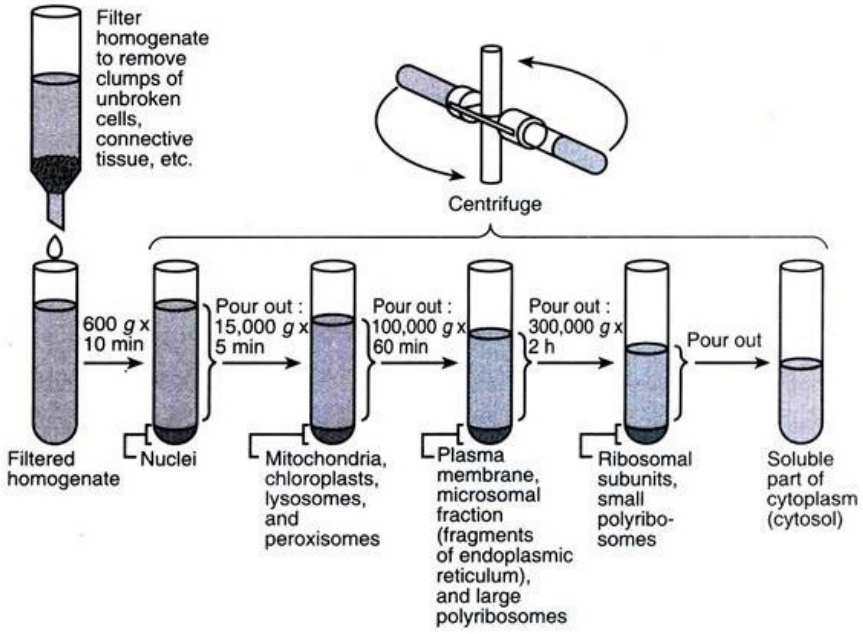


Figure 2.10: Outline of typical Fractionation using Differential Centrifugation

Detailed study of characteristics, variations and applications of Preparative and analytical centrifugations.

A. Reparative centrifugation, B. Analytical centrifugation.

A. Preparative Centrifugation:- It is concerned with isolation of biological material for subsequent biochemical investigations. Depending upon the medium of suspension, used for the separation, this is of two types. 1. Differential centrifugation, in which suspension

medium is homogeneous and 2. Density gradient centrifugation, in which the suspension medium is having density gradient.

1. **Differential Centrifugation:-** A mixture of homogeneous particles can be separated based an densities and/or size. This is achieved easily if the time required for sedimentation at a fixed centrifugal field is known. According to the equation below, if the shape, density of particles, medium characteristics are known, at a specified rpm, the time required for sedimenting intracellular particles if fixed.

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

A fair the estimate of sedimentation required for a cell suspension can be had and used for cell fractionation. Example- A field of 1000 g sediments cell debris in 10 minutes and 15 minutes would lead to sedimentation of nuclei. If the homogenate is subjected to centrifugation for 15 minutes at 10,000g, mitochondria and lysosomes would pellet out. This strategy can be adopted (figure 2.10) the cell homogenate can be separated into several fractions. Advantage with this procedure, is its relative ease, convenience and time economy. But has a disadvantage that, the resultant yield is not 100% pure.

2. **Density gradient configuration**

This has 2 variations

a) Rate zonal centrifugation and

b) Isopycnic centrifugation

a) Rate zonal centrifugation (figure 2.11)

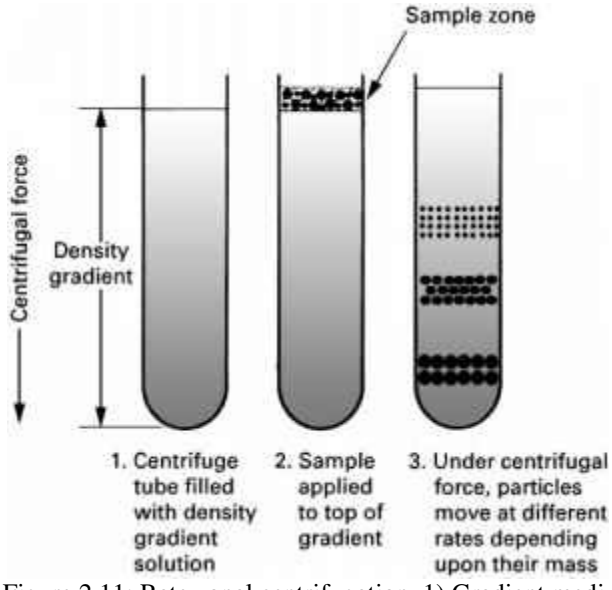


Figure 2.11: Rate zonal centrifugation, 1) Gradient medium, 2) Sample application, 3) Separation of sample based on size

In this technique, the density of the maximum gradient of the gradient medium is less than that of the density of least dense particle. The gradient generated is also shallow. The prepared sample is layered over the liquid gradient and process is operated at low speed for less time. Because of the RCF, particles move down the gradient and get separated. This can be used to separate molecules which differ in size, but not in their densities.

Rate zonal centrifugation cannot used for fractionation of cell homogenates, but can be used to separate two protein molecules, having same molecules weight, but vary in their densities.

b) Isopycnic centrifugation:-

Recall the equation

$$V = \frac{dr}{dt} = \frac{2}{9} r_p^2 \left(\frac{\rho_p - \rho_m}{\eta} \right) \omega_r^2$$

From this equation, if the density of medium and the density of particles become same, then the rate of sedimentation becomes zero. The same principle is applied in this technique. A density gradient is generated in the centrifuge tube (figure 2.12).

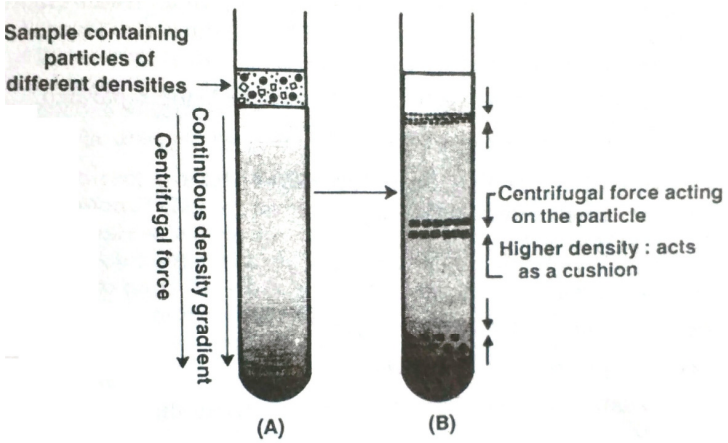


Figure 2.12: Isopycnic centrifugation, A) Sample layered on continuous gradient medium B) Sample separated based on Buoyant density

Different particles have different buoyant densities, and as they travel down the medium, they become stationary at regions, where their density becomes equal to the density of medium, and they start floating on layer of the medium, whose density is greater than their own buoyant density. This is called is isopycnic centrifugation or sedimentation equilibrium centrifugation.

This method differs from rate zonal centrifugation in the following ways (Table 1)

Table 1:- Differences between Rate-zonal and density gradient centrifugation.

	Rate-Zonal	Isopycnic
Synonym	S-zonal. Sedimentation velocity	Density equilibrium. Sedimentation Equilibrium
Gradient	Shallow, Maximum density of the gradient medium is less than the density of least dense particle species, gradient is continuous.	Steep, Maximum density of the gradient medium is greater than the most dense species. Gradient either is continuous or discontinuous
Centrifugation	Incomplete sedimentation low speed & short time	Complete sedimentation till equilibrium is achieve, high speed & longer duration
Separations	RNA-DNA hybrids, Ribosomal subunits etc	DNA, plasma, Lipo proteins, Lysosomes, Mitrochondria, Peroxisomes etc.

The following table reveals, the density of sucrose solution and approximate density of sub cellular particle that can be purified accordingly.

Table 2: Approximate densities of particles in sucrose solution.

Particle	Density (gm/cm^3)
Golgi apparatus	1.06 – 1.10
Plasma Membrane	1.16
Smooth endoplasmic Reticulum	1.16
Intact oncogenic virus	1.16 – 1.18
Mitochondria	1.19
Lysosomes	1.21
Peroxysomes	1.23
Plant viruses	1.30 – 1.45
Soluble Proteins	1.30
Rhino- and entero viruses	1.30–1.45
Nucleic acid & Ribosomes	1.60 – 1.75
Glycogen	1.70

Gradient Materials:-

Sucrose remains the most common material used. For Rate zonal centrifugation, sucrosse is used to generate density gradient, while for isopycnic centrifugation caesium chloride is used. Substance to be used as gradient material should have the following properties.

i) It should not affect the biological activity of the sample being separated.

ii) It should not interfere with the assay techniques.

iii) It should be easily removable from the purified product.

iv) It should not absorb any wavelength in UV range

v) It should not be corrosive to the rotor.

vi) It should be easily sterilizable.

vii) It should be cheap and readily available, and

viii) It should preferably be recoverable for reuse

Some common materials used to generated density gradient are as follows:

Sucrose (66%)

Polyvinyl chloride

Silica solutions

Diodon

Glycerol

RbBr

CsCl

Rbcl

Cs acutate

Potassium formate

Cs formate

Na formate

Ficol

Me trizamide

Sorbitol

Renografin

Urograffin

Among these, sucrose interacts with particles and can affect the results obtained. The interaction varies with the particles being separated.

Non-membrane bound solid particles are not affected as much as membrane bound particles which have high water of hydration. And many commercial preparation using sucrose, have it as an impurity. Hence selection of approximate material, as well as development of procedures to completely get rid of gradient material from end product is of importance.

Preparation of Density Gradients:-

Two types of gradients are generally prepared, which include step or discontinuous density gradient and continuous density gradient.

Discontinuous density gradient is that in which the density increases abruptly from one layer to another. This is prepared by layering the density gradient solutions, starting with the densest, which is placed at the bottom, to the less dense that is layered at the top of the centrifuge tube. The sample is layered and centrifugation is done at appropriate conditions. This is used for separation of whole cells, sub cellular organalleles from tissue homogenate etc.

In continuous gradient method, the density increases gradually as one moves down the tube. This can be generated by adopting several methods.

The simplest being allowing the solution of gradient material to stand still for some time as the gradient material which is viscous starts setting down a continuous and gradual increase in density is seen. But this is not an appropriate method. Instead special devices are developed to generate density gradient of continuous nature.

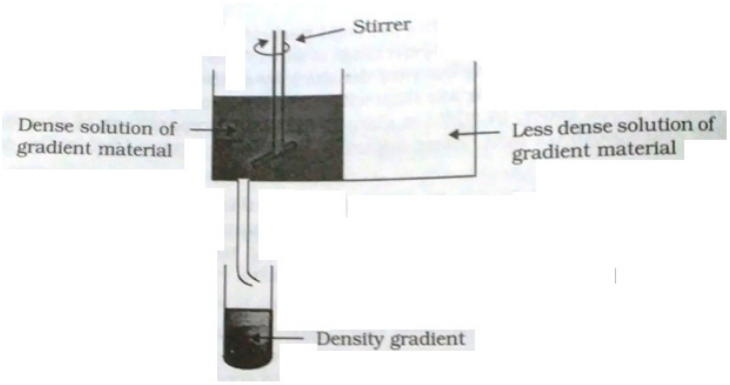


Figure 2.13: Making of density gradient

The device has two chamber, one having a solution of lowest density and the other one having solution with highest density. The outlet of first chamber having high dense material is placed in the tube. Both the chambers are connected by a pipe through which flow is regulated. The first chamber is also equipped with a stirring device to attain homogeneity. The flow from first chamber is initiated, while parallely allowing solutions to mix, and during this process the collecting tube receives densest material first, followed by less dense substance. As the process continuous a gradient is generated within the tube (figure 2.13). This can be used for separation of many proteins, enzymes, ribosomes and even viruses.

Sample Application to the gradient:-

The Sample is usually layered above the separating medium and is same in any of the gradient centrifugation method. Most of the time the solution itself, has the sample for separation. The volume of sample varies with the centrifuge tube's cross sectional area and it

varies between 0.5 to $1.0 cm^3$. Large volumes generally do not serve the purposes. Even the concentration of sample is to be limited, and it is generally 10 times less than the concentration of the starting gradient.

Application is done using a syringe. It is held slightly above the solution at an inclined angle of 45^o and is layered along the wall of the tube. At times broad mouthed pipettes are used while working with fragile solutions.

Recovery of Sample :-

Two method are in use, Puncturing method & Displacement method (figure 2.14). In the first method the bottom of the centrifuge tube is punctured using a needle and the dripping solution is collected as fractions.

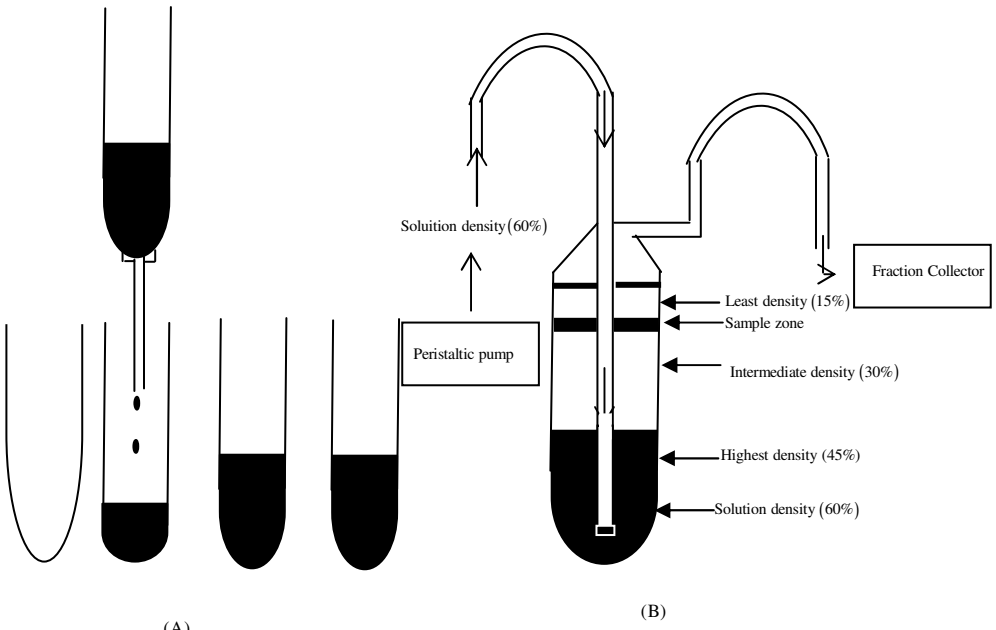


Figure 2.14: Sample recovery: A) Puncture method
B) Displacement technique

In the displacement method, the centrifuge tube itself has a special lid that has an inlet for pipe of a peristaltic pump and also an outlet for collecting fractions. Through the centre hold which has tubing that reduces the bottom of tube, dense material is pumped. Its density is much greater than the highest density of the medium. This displaces the density gradient material up the tube. As the process continuous samples are collected from another outlet.

The fractions collected can be analyzed for separated components. If the centrifugation has been carried out to obtain sub cellular organelles, their presence can be known by analyzing for appropriate markers or enzymes.

Choices of Rotor:- If rate zonal centrifugation is being done, swinging bucket rotors can be preferred as they offer maximum path length. The particles get separated effectively and no

sample mixing is seen with these rotors. For better separation, longer durations of centrifugation can be adopted and if time consumption is to be reduced, half filled tubes can be used.

While for Isopycnic centrifugation, fixed angle rotors or vertical tube rotor can be used. This is quick method to separate samples based on density. As the particles become stationary as their buoyant densities and will not move any further, even if time of centrifugation exceeds little. Even samples do not get disturbed during deceleration of rotor.

Zonal rotors are best ones while working with density gradient centrifugation. But the volume of gradient material is more or less equal to swinging bucket rotors (100 ml). The advantage is that this can be operated for several days to get the desired separation.

B. Analytical ultra centrifugation

1. Basic Principles of Sedimentation:-

According to equation (8) represented previously, the rate of sedimentation of particles depend on angular velocity, radius (distance from the axis of rotation), density of the medium & particle and viscosity of the medium. This can be improved by considering other important parameters about particles and friction.

The particles strokes radius or effective radius is to be considered as, Assymetric particles sediment at lower rate due to layer friction that they experience. Hence the mathematical expression is modified and represented as equation 10.

$$V = \frac{dr}{dt} = \frac{2}{9}\frac{r_p^2\left(\rho_p - \rho_m\right)\omega^2 r}{\eta\left(f / f_0\right)} \qquad \ldots (10)$$

Where, f is the frictional coefficient for asymmetrical for hydrated particles.

f_0 – frictional coefficient for unhydrated particles which are spherical.

f / f_0 – Frictional ratio of a given molecule.

For globular proteins, the value is between 1 to 1.4. For fibrous or rod like long particles the ratio is larger. This is of signification in understanding the difference in rates of sedimentation of particles having same molecules weight but of different shape.

Factors affecting sedimentation velocity:-

i) Effect of concentration:- If the concentration of molecules is high their rate of sedimentation is less. This is because of frequent collisions among themselves and with the solvent molecules. Increase in concentration also contributes to in increase in effective viscosity around particles, which substantially decreases rate of sedimentation. High concentration of samples also effect resolution of sample into its constituents. Hence ideally diluted samples are used.

ii) Effect of Speed: The rate of sedimentation is proportional to the centrifugal field. But if too high speeds are used trailing of fast moving molecules will be left among the next set of migrating molecules leading to impaired resolution of sample mixture.

Based on the composition of mixture, the rpm is to be decided. Huge variation in size will enable working at high speeds and even with isopycnic centrifugation. Else slow speeds for longer duration are to be preferred.

iii) Effect of charge:- As biomolecules are charged suitable strength of counter ionic solutions are to be used to prevent retardation in their rate of sedimentation. An ionic strength of 0.05 M is preferred for neutralizing the charge of molecules.

2. Sedimentation Coefficient:-

It is the velocity of a particle per unit centrifugal field. It is denoted by the symbol 's' and is expressed mathematically as,

$$s = \frac{v}{\omega^2 r} \qquad \ldots (11)$$

or

$$v = s\omega^2 r \qquad \ldots (12)$$

As v is a measure of $\omega^2 r$, s is a measure solely of the properties of particle like the size, shape and density (Equations 8 &10). The studies for determining sedimentation rate are to be performed under ideal conditions. The experimentally determined value of s is affected by temperature, solution viscosity and density.

By convention, the observed value of s is corrected for that value which would be obtained if water was used as the suspension medium at 20°C. This is done by substituting the observed value of s into the following formula.

$$\delta_{20,\omega} = S_{obs} \times \frac{\eta_T}{\eta_{20}} \times \frac{\eta_c}{\eta_o} \times \frac{(1-\bar{v}\rho_{20,\omega})}{(1-\bar{v}\rho_r)} \qquad \ldots (13)$$

Where,

S_{obs} – Experimentally obtained value of sedimentation coefficient

$S_{20,\omega}$ – Sedimentation coefficient that would be obtained with water as solvent 20°C

- It is the standard sedimentation coefficient.

η_T – Viscosity of water at temperature T

η_{20} – Viscosity of water at 20°C

η_c – Viscosity of solvent at a given temperature

η_0 – Viscosity of water at that temperature.

$\rho_{20,\omega}$ – Density of water at 20°C

ρ_T – …. Density of solvent at that temperature

$\bar{v}$ – Partial specific volume of the particle.

The 's' values for biological molecules vary between 10^{-13} to 10^{-11} seconds. For convenience, S value is reported as Svedberg(s) value, and 1 Svedberg is a value equal to 10^{-13} seconds. Prokaryotic ribosomes have 70S value, which means their sedimentation coefficient is 70×10^{-13} seconds.

Factors Affecting Sedimentation Coefficient:-

i) Molecules Shape:- Spherical molecules face less fiction than other large and long molecules. However, if the large, rigid molecules conformation is destroyed, their sedimentation coefficient will increase markedly.

Example:- A double stranded intact DNA has less sedimentation coefficient than a single stranded DNA. Melting of DNA can be monitored by determining 's' value.
Analytical centrifuges are used to determine the value of DNA (figure 2.18).

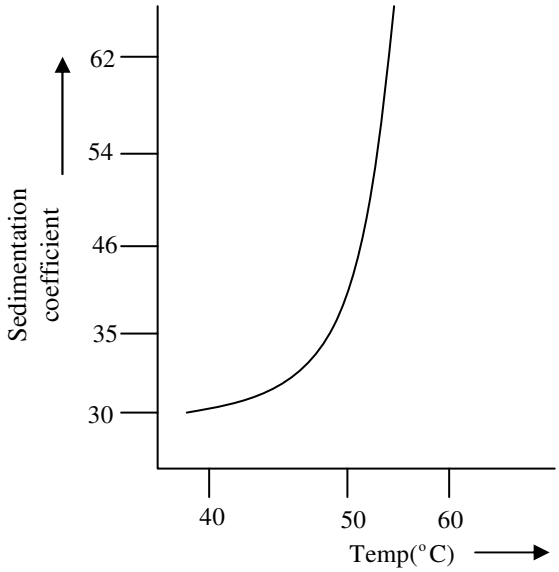


Figure 2.18 : Plot of sedimentation Coefficient of T_7DNA versus Temperatures

(ii) Molecules weight : Smaller molecules have lower 's' values. This is illustrated with the same example mentioned above, It temperature is further increased, the DNA molecules become single stranded and this leads to drop in molecular weight, which inturn is indicated in the sudden drop in 's' value (figure 2.19).

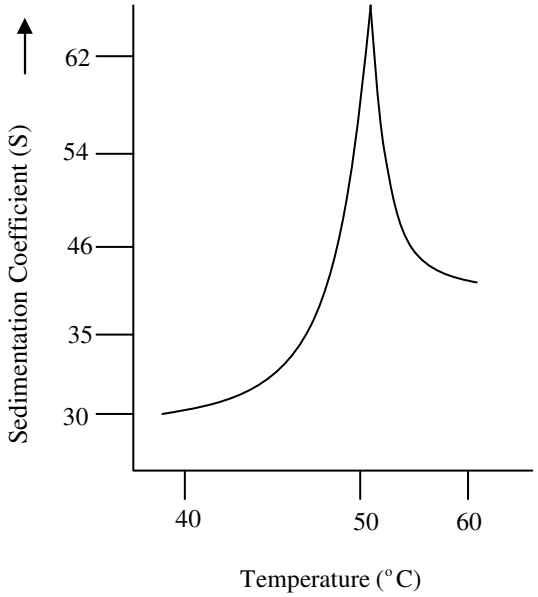


Fig.2.19. The drop in 's' value indicating separation of DNA stands

iii) Solute Binding – Presence of sufficient solute particles in the solution taken for centrifugation is essential to completely neutralize their charge or to give it sufficient coiling, there by contributing to faster rate of sedimentation.

iv) Concentration distribution:- Based upon the composition of sample and concentration of particles, discrete separation can be obtained which can be viewed through the optical system. This is based on measuring the absorption of particles or by getting refractive index.

Initially particles will be dispersed through the cell and hence no boundary indicates difference in refractive index is seen. As process continuous clear solvent remains in the upper part of tube while molecules will keep moving down. This leads to a difference in refractive index and as the sedimentation continuous particles reach bottom of tube and the boundary of refractive index is seen at the bottom (fig 2.20).

Figure 2.20 – Concentration distribution plot as viewed by schlieren optics.

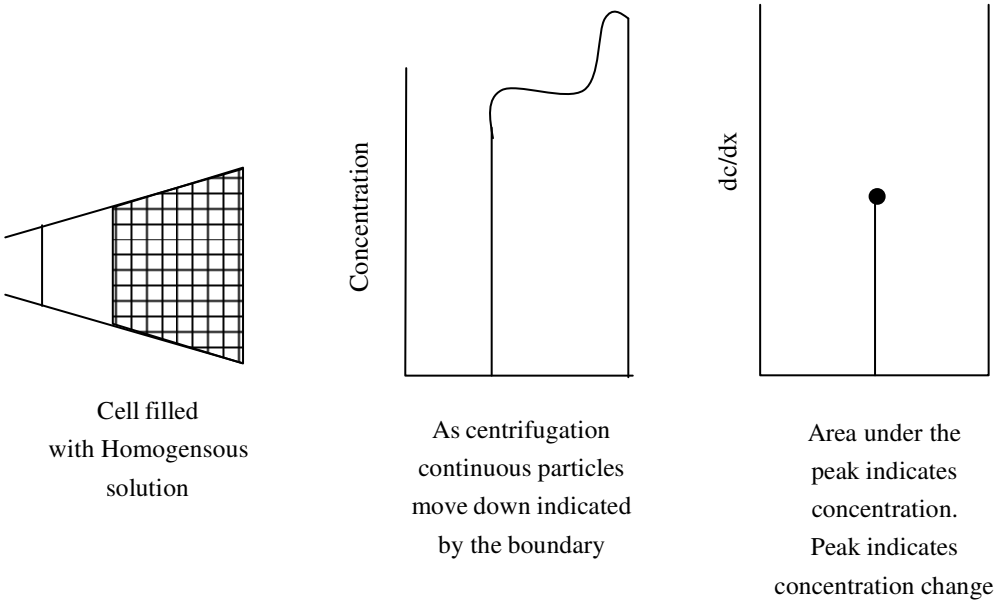


Figure 2.21 indicates that the sample has two components, while figure 2.22 reveals the schlieren plots for a homogeneous solution having several components differing very slightly in their 's' value.

Figure 2.21- Schleiren plot for a sample that has 2 components.

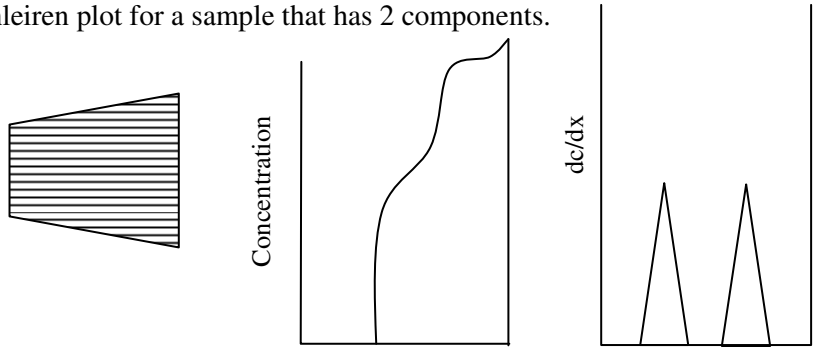
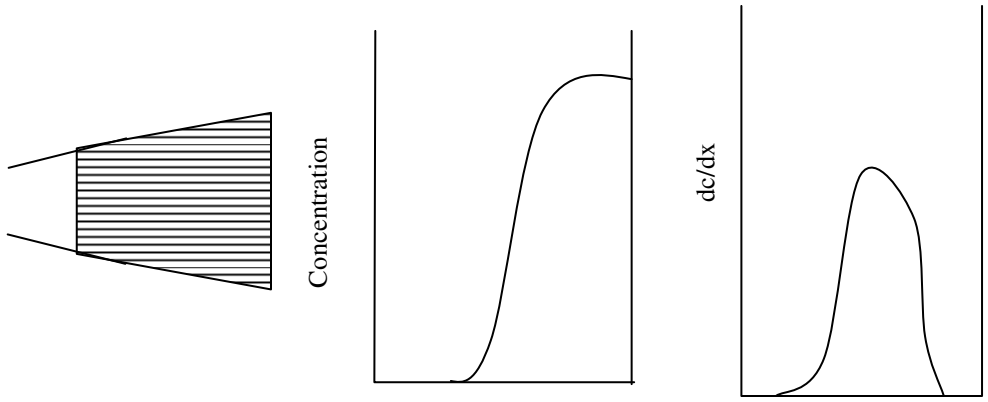


Figure 2.22 – Schleiren plot for a homogeneous mixture having several components



From these figures, the following points can be understood.

i) A homogeneous solution gives shape boundary

ii) Presence of 2 particles will give two sharp boundaries which appear like steps.

ii) Sharpness of peak is an indication of diffusion coefficient of the component.

Determination of Molecular weights:-

There are two approaches to determine molecular weight of samples by using centrifugation technique.

i) Sedimentation velocity ii) Sedimentation Equilibrium

i) Sedimentation velocity:- Used to measure the speed of moving boundary using Rayleigh interference optics or schlieren optics and determine the molecular weight of sample using the following equation.

$$M = \frac{RTS_{20}w}{D(1-\bar{v}e)}$$

Where,

M= Anhydrous Molecular weight of the molecule

R = Gas constant

T = Absolute Temperature

$S_{20}, \omega =$ Sedimentation coefficient of molecule

D = Diffusion coefficient of the molecule

$\bar{v} =$ Partial specific volume of macromolecule

ρ = Density of the medium

ii) Sedimentation Equilibrium:- Centrifugation of molecules leads to their separation as bands, which do exhibit a tendency to diffuse back if centrifugation is stopped. The

centrifugation speed at which a balance occurs between sedimentation and diffusion is called sedimentation Equilibrium. The values can be used to determine the molecular weight of sample using the following equation,

$$M = \frac{2RT\ln\left(C_2 / C_1\right)}{\omega^2\left(1-\overline{\nu}e\right)\left(r_1^2 - r_2^2\right)}$$

Where,

R = gas constant

T = Absolute temperature

ω= Angular velocity

ρ = Density of the solvent

$\overline{\nu}$ = Partial specific volume

C_1 & C_2 = Concentration of solute at distances r_1 and r_2.

PRACTICE QUESTIONS

1. Calculate RCF experienced by particles in a centrifuge at 4.8 cm radius operating at an rpm of 12000.

   (A) 16,113g (B) 7,734g (C) 3,867g (D) None

2. Which if the following rotors have tubes that reorient to lie perpendicular to axis of rotation and parallel to the applied centrifugal field?

   (A) Fixed –angle rotor

   (B) Vertical tube rotor

   (C) Swinging bucket rotor

   (D) Zonal rotor

3. Which among the following statements is false?

   (A) The force exerted on molecules during centrifugation is directly proportional to angular velocity

   (B) Desktop centrifuges are also known as clinical centrifuges

   (C) High speed centrifuges operate at speeds of 3000 to 1000rpm

   (D) Ultra centrifuges operate at above 500,000g

4. In which type of centrifugation, the gradient used has maximum density which is less than of the least desire particle.

   (A) Density gradient centrifugation (B) Rate- zonal centrifugation

   (C) Isopycnic centrifugation (D) Zonal centrifugation

5. The velocity of a particle per unit centrifugal field is referred to as _____.

   (A) Angular velocity

   (B) Sedimentation velocity

   (C) RCF

   (D) Sedimentation coefficient

6. Among the following statements, identify the ones that refer about zonal rotor.

   (A) Can be used for isolation of glyoxysomes

   (B) Used for preparation of antibodies

   (C) It is a disc shaped rotor with four sectors

   (D) It is a flattened disc with single sector

   (E) Can be used for density gradient separation

(F) The densest material is towards the outer end

(G) Speed of rotor is maintained at around 3000-4000 rpm

(A) A,B, E,F,G (B) All of the above (C) A, B, C, E, F, G (D) A, B, D, E, F, G

7. Ultra centrifuges operate at high speeds and are used for preparative as well as analytical purposes The shaft of the rotor has a diameter of _____ which allows its flexibility

(A) 10 cm (B) 1/16 inches (C) 16 inches (D) 1 cm

8. Ultra centrifuges operate at high speeds and are used for preparative as well as analytical purposes The components of analytical ultra centrifuge are

(A) Rotor with 32 holes

(B) Equipped with Schlieren optics

(C) Rotor with two holes, one for counterpoise and the other for test sample

(D) Counter poise accommodate air or vacuum

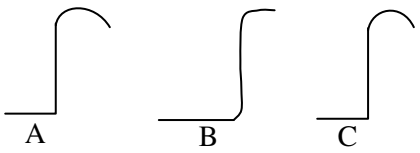
(E) Counter poise is for placing reference

(F) Separation can be seen with eye

(G) Used to determine sedimentation rate for an analyte

(A) B, C,E,G (B) A, B, D, F, G (C) All of the above (D) A, C, E, G

9. In an experiment stating the necessity for the presence of Mg^{2+} as cofactor for endonuclease activity, the following images are obtained.



A is the Schleiren optical image of sedimenting DNA.

Figure B indicates

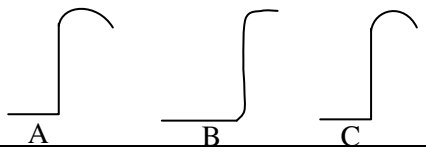
(A) Migration of DNA

(B) Migration of fragmented DNA by endonucleases

(C) Migration of DNA is not changed

(D) Endonuclease is inactive as per figure B

10. In an experiment stating the necessity for the presence of Mg^{2+} as cofactor for endonuclease activity, the following images are obtained.



A is the Schleiren optical image of sedimenting DNA.

The reasons for figure C being similar to A can be,

(A) Lack of enzyme

(B) R is simple DNA migration

(C) Chelation of Mg^{2+} can result in graph same as A

(D) All of the above

ANSWER KEYS

1	B	**2**	C	**3**	C	**4**	B	**5**	D	**6**	C	**7**	B
8	A	9	B	10	D								

EXPLANATIONS

1. $RCF=\left(1.119\times10^{-5}\right)\left(rpm\right)^2 r$
$=\left(1.119\times10^{-5}\right)\left(12{,}000\right)^2\ 4.06$
$=7734.528g$

2. Swinging backed rotors have backets that swing out to horizontal position upon acceleration and come back down parallel to the axis of rotation as it deceleration.

3. High speed centrifuges oparate at 25,000rpm and 90,000g.

4. The gradient used in rate zonal centrifugation has its maximum density below that of the least dense particles.

5. The velocity of particle per unit centrifugal field is referred to as its sedimentation coefficient which is expressed as, $S=\frac{v}{w^2 r}$

6. Features of Zonal rotor
   (A) Can be used for isolation of glyoxysomes
   (B) Used for preparation of antibodies
   (C) It is a disc shaped rotor with four sectors
   (E) Can be used for density gradient separation
   (F) The densest material is towards the outer end
   (G) Speed of rotor is maintained at around 3000-4000 rpm

7. The shaft is kept thin so as to allow is flexibility to accommodate any degree of imbalances and to prevent spindle breakage.

8. The components of analytical ultra centrifuge are
   (B) Equipped with Schlieren optics
   (C) Rotor with two holes, one for counterpoise and the other for test sample

(E) Counter poise is for placing reference

(G) Used to determine sedimentation rate for an analyte

9. Endonuclease with Mg^{2+} cleaves DNA and forms bands that are indicated in figure B.

10. The reasons for figure C being similar to A can be,

(A) Lack of enzyme

(B) R is simple DNA migration

(C) Chelation of Mg^{2+} can result in graph same as A

4. Spectroscopy

This chapter deals with exploitation of optical properties of biomolecules. The absorption and emission of optical signals by bio-molecules help to understand their structural details. Apart from determination of concentration of the molecules, methods have been developed to assertion sample purity and determine even chemical structure.

Basic principles:

Light has dual characteristics, i.e. corpuscular form and wave form. A beam of light is understood as an electromagnetic wave form or a photon of energy propagated at speed of light i.e. $3.0x10^8$m/sec. Light is made up of an electrical and a magnetic wave which are in phase and perpendicular to each other and to the direction of propagation (fig. 3.1). The magnitude of electrical vector is denoted by the symbol E and that of magnetic vector is denoted by the symbol H.

A beam of light from a bulb consists of many randomly oriented plane polarized components being propagated in the same direction. The distance along the direction of propagation for one complete cycle is known as wavelength (λ). It is measured in centimetres (cm), micrometers (μm), nanometers (nm) or Angstrom (A).

The relationship among these is,

$$1nm = 10^{-3}\mu m = 10^{-6}mm = 10^{-7}cm = 10^{-9}m \;\&\; 1A = 10^{-8}cm$$

Frequency (υ) is the number of waves passing through a fixed point on the time axis per second. The relationship between frequency and wavelength is, $\upsilon = c\,\lambda$

Where c= velocity of light $-3x10^{-8} m/sec$.

Wave number ($\bar{\upsilon}$), it is the number of complete cycles occurring per centimetre,

$$\bar{\upsilon} = 1/\lambda$$

Energy (E) of photon in relation to wavelength and frequency is represented as,

$E = h\upsilon = hc/\lambda$, where h= Planck's constant

Here wavelength and frequency are inversely related. Energy of photon decreases with increase in photon wavelength and vice versa, while frequency and energy have a direct relationship

$$E \propto \bar{\upsilon} \qquad \& \qquad E \propto 1/\lambda$$

Usually a beam of light from a bulb has several wavelengths (polychromatic). If a beam of light has single wavelength it is known as Monochromatic.

Electromagnetic radiations are produced due to events at molecular, atomic or nuclear levels. The events that involve emission of radiation include oscillation of nuclei and electrons in electrical or magnetic fields, molecular bonding & vibrations, excitation or ejection of electrons, rearrangements of electrons or breakup of nuclei. The radiation thus emitted has a range of wavelengths as represented in figure (3.2).

Figure 3.1: The electromagnetic wave

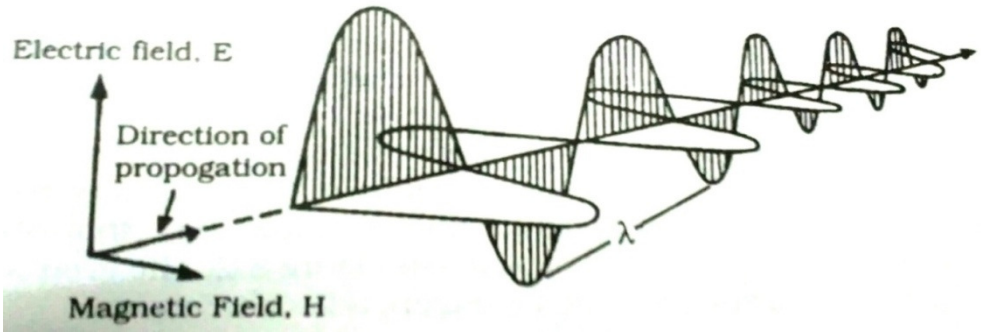
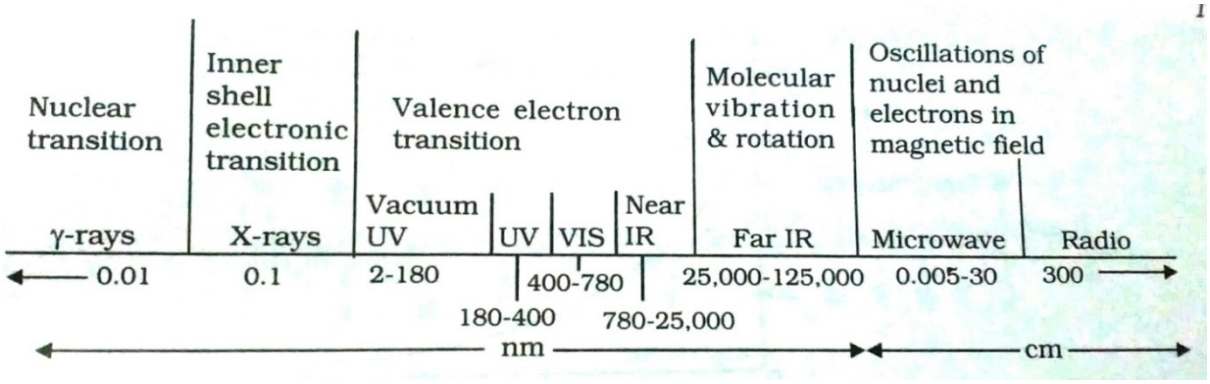


Figure 3.2: The Electromagentic spectrum, and the main regions of their wavelengths



The Laws of Absorption

The absorption of light by any absorbing material is governed by two laws:

Bouger-Lambert's law and

Beer's law

Bouger-Lamberts law states that, the amount of light absorbed is proportional to the thickness of the absorbing material and is independent of the intensity of the incident light. This is illustrated by the figure 3.3.

As the light is passing through the absorbing material of thickness 'b', it has the ability to absorb 50% of incident light. If a second vessel is placed next to it of the same thickness, it also absorbs 50% of the incident light that it receives and the transmitted light, out of second vessel is 25% and this continues. If incident light is assigned the value '1', the transmitted light will be 0.5, for first

vessel and it is so for others. The successive light intensities are of the sequence, $(0.5)^1$, $(0.5)^2$,$(0.5)^3$,etc. This is an exponential function and may be expressed as ,

$$\frac{I}{Io} = e^{-kb}$$

Where, I = The intensity of transmitted light

Io = The intensity of incident light

b = The absorbing thickness (path length)

k = The linear absorption coefficient of the absorbing material.

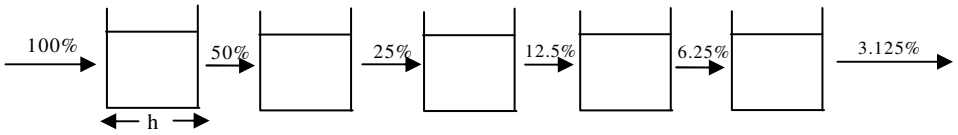
The above equation can be represented in conventional logarithmic forms,

$$\ln \frac{I}{Io} = -kb \quad \text{or} \quad \ln \frac{Io}{I} = kb$$

Changing to common logarithms we get,

$$2.303 \log_{10} \frac{Io}{I} = kb$$

Figure 3.3: Pattern of light absorption by successive equal path-lengths



Beer's law:- It states that, the amount of light absorbed by a material is proportional to the number of absorbing molecules, i.e. the concentration of absorbing solution. This is expressed mathematically as

$$2.303 \log_{10} \frac{Io}{I} = k'C$$

Where, k' = absorptivity constant and

C = The concentration of absorbing material

When the two equations of the two laws are combined, keeping a as a common constant for k & k' , the combined equation becomes,

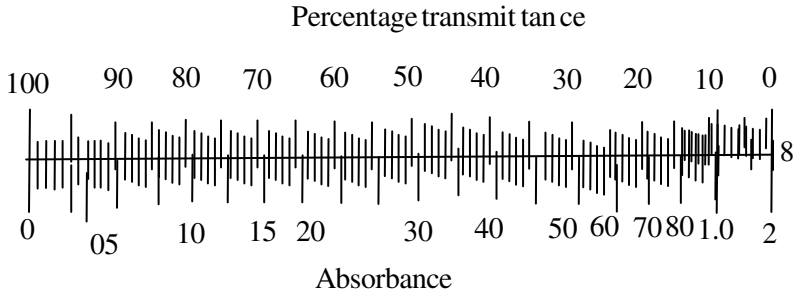
$$\log_{10} \frac{Io}{I} = abc$$

This equation is referred Beer-Lambert Law. The combined law states that, the amount of light absorbed is proportional to the concentration of the absorbing substance and to the thickness of the absorbing material.

The quantity $\frac{Io}{I}$ is known as the absorbance or the optical density (OD). The reverse of it, $\frac{I}{Io}$ is known as the transmittance (T), which indicates the amount of light that escapes absorption and is transmitted.

Absorption shares a linear relationship with sample concentration, while the relationship between transmitted and sample concentration is non-linear. Hence, absorbance by sample is generally measured. The relation between absorbance with transmittance (%) is better represented in the figure (3.4).

Figure 3.4: The relationship between percentage transmission and absorbance



Mathematical relationship between then is written as,

A (absorbance) = $\log Io - \log I$

Io is always set at 100%, hence $\log Io = 2$

$\therefore A = 2 - \log I$

Beer-Lambert's law can also be used to calculate path length, and also concentration of unknown sample.

$$= Concentrqation\ of\ the\ unknown = \frac{OD\ of\ the\ unknowan\ x\ conc.\ of\ standard}{OD\ of\ the\ standard}$$

At times OD of substance is interfered with absorbance of solvent. In order to prevent addition of absorbance by solvent to the value of substance, a blank is used. OD of blank is first nullified followed by taking absorption of sample which gives the value of sample.

The most accurate method of obtaining concentration of sample is by preparing a calibration curve using a series of standard solutions. The standard curve must be prepared in such a way that proportionality of concentration versus OD is maintained. Linearity of standard curve is important which is influenced by several factors of which the important one is wavelength of source.

Deviations from laws are seen under following condition:

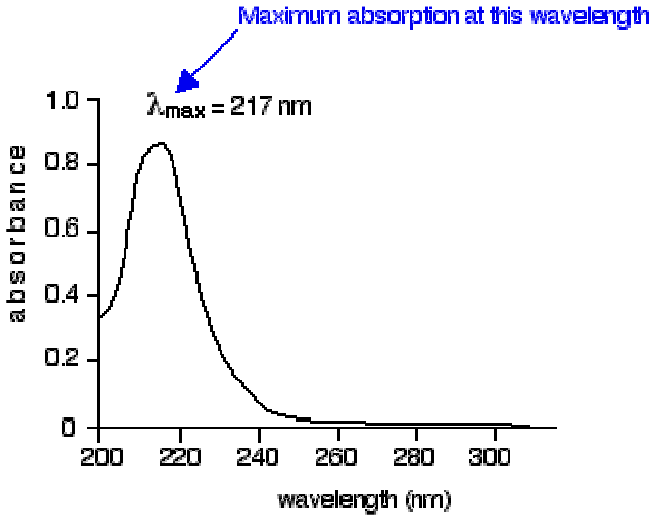
1. High sample concentration effects linearity of graph. This is also due to limitation with respect to range of the instrument. Temperature affects properties of solutes and molecules. It affects the degree of solubility, dissociation/association properties of solute, hydration and other factors.
2. Sample colour instability can occur as in the case of Fiske Subbarow method of estimating phosphate.
3. Some molecules fluoresce and the emitted fluorescent intensity interference with transmitted light.

4. Turbidity of solution if any interference with transmitted light decreasing OD.

Absorption Spectrum:-

The pattern of emergency by a substance when light of varying wavelength passes through it is uniquely characteristic of the substance and is known as ***Absorption spectrum.*** It is characteristic for a substance (Figure 3.6). The wave length at which the compound absorbs most of the wavelength of light is termed as its absorption maxima λ_{max}. Determination of λ_{max} is important as it is used in quantitative estimation of samples by photometric methods.

Figure 3.5: A simplified Absorption spectrum curve



The reason for λ_{max} is explained by the concept of quantized energy levels of atoms say an atom 'x' absorbs wave length between 210-220nm. The energy of this wavelength of radiation is sufficient to excite the electrons to higher energy levels, and 'x' does accept energy from radiation other than $\lambda_{max.}$

According to quantum theory, electrons of an atom exist in orbitals which involve different energies. To excite from their ground level say from n=1 to n=2, atoms absorb radiation of that wavelength which has exact quantized energy to push it from ground level to excited level. This absorption intensity of light which is transmitted and thereby it results in absorption spectrum.

Complex molecules have multiplicity of energy levels and thus broad bands of absorption are formed which are again specific for a molecule, i.e. Electrons of different atoms result in discrete bands.

Apart from these, vibrational and rotational energy levels are also associated with each energy level of electronic transition. These also contribute to broad bands.

The types of electronic transitions that can take place for a substance are mentioned in figure 3.6.

1) **Bonding σ-orbitals :-** These are extremely strong and constitute single bonds between atoms. The electrons are not delocalized and the distribution of electrons is cylindrically symmetrical about the bond.
2) **Bonding Π-orbitals** :- These constitute multiple bonds between atoms and are based on the combination of atomic p-orbitals. The electrons are strongly delocalized and interact with surrounding environment with relative ease.
3) **Π-orbitals :-** Certain molecules contain hetero atoms (eg- oxygen, nitrogen). The occupied orbitals with highest energy in such molecules are those of lone pairs which are non-bonding and thus retain their atomic character.

Figure 3.6: Schematic molecular orbital energy level diagram depicting relative energylevels and allowed transitions

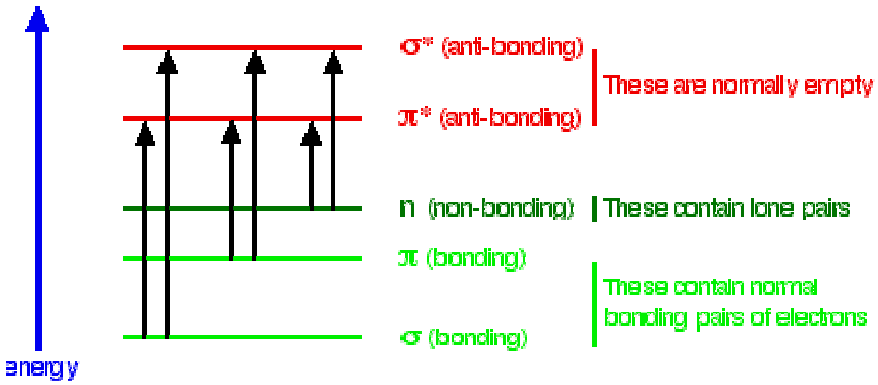
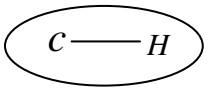
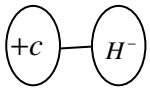
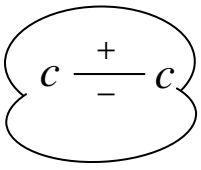
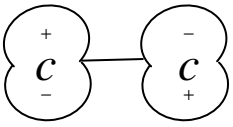
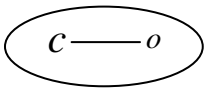
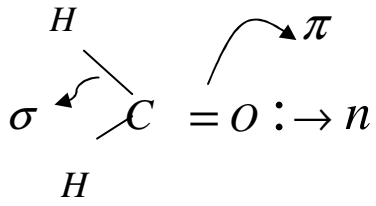


Table:- Few examples of some bond types, transitions and molecular orbitals.

Types of bond	Transition of Lowest energy	Shape of molecular orbitals	
		Ground State	Excited state
H_3C-N	$\sigma \rightarrow \sigma^*$		
$R_2C=CR_2$	$\pi \rightarrow \pi^*$		
$R_2C=0$	$\eta \rightarrow \pi^*$		Π electron system perpendicular to the plane of the page

All the above three types are illustrated in formaldehyde as below:



According to the molecular orbital theory, when a molecule is excited by the absorption of energy (UV or visible light), its electrons are promoted from a bonding to an anti-bonding orbital. The anti bonding orbital associated with σ-bond is called σ^* orbital and that associated with π bond is known as π^* orbital. Since non-bonding orbitals are not directly involved in bonding, there is no corresponding anti-bonding orbital for them.

The major electronic transitions within the ultraviolet and visible regions are:

$$\sigma \to \sigma^*, \eta \to \eta^*, \pi \to \pi^* and \ \eta \to \pi^*$$

The energies associated with these transitions are in increasing order. Therefore, $\sigma \to \sigma^*$ transition is of high order and requires more energy, while $\eta \to \pi^*$ transition is of low order and requires less energy. For most of the molecules, absorption of radiation occurs in UV or visible region and the transitions associated with these are $\pi \to \pi^*$ or $\eta \to \pi^*$ transitions.

Apart from these, there are factors that effect λ_{max} ,which include pH of solvent, polarity effects and orientation effects. pH effects the ionisation state of a compound and hence changes the λ_{max} of absorption spectrum. Polarity of solvent shifts $\eta \to \pi^*$ transitions to shorter wavelengths, $\pi \to \pi^*$ transitions.

The chromatophore concept :-

Chomatophore is defined as any isolated covalently bonded group that shows a characteristic absorption in the ultraviolet or the visible groups of ethylenic or acetylenic groups. Chromatophores are of two types:

1.Chromatophores containing π electrons involved in $\pi \to \pi^*$ transitions. Example- Acetylenes & Ethylenes

2. Chomatophores containing both π and ηelectrons and involved in $\pi \to \pi^*$ and $\eta \to \pi^*$ transitions. Example: Carbonyls, nitrites and azo compounds.

AUXOCHROMES:- These are groups which by themselves do not act as chromatophores, but whose presence brings about a shift of the absorption band. Important auxochromes include, -OH, -OR, $-H_2$, -NHR, $-NR_2$, -SH etc. Auxochromes exert its effects by virtue of its ability to extend the conjugation of a chromophore by sharing of non-bonding electrons. This results in a difference in λ_{max}. This shift in absorption is of four types (figure 3.7)

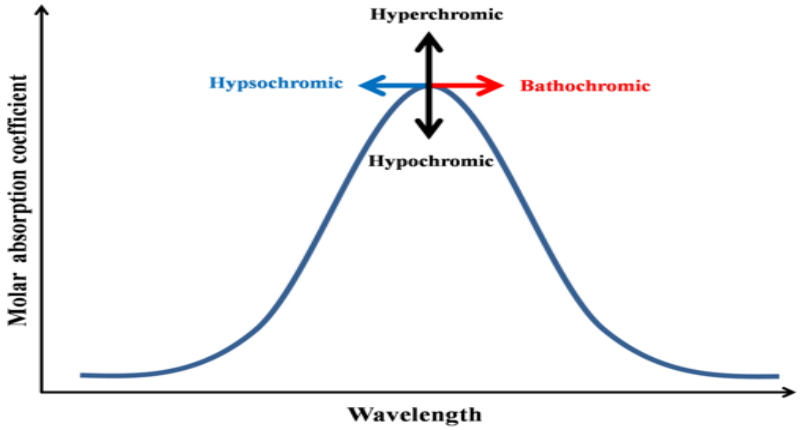
i) Bathochromic Shift :- This shift due to presence of auxochrome by virtue of which the absorption maximum shifts towards higher wavelengths. This is also called as Red shift. Red shift may also be due to decreasing polarity of the solvent.

Hypsochromic shift :- This is opposite of bathochromic shift. This shift is due to removal of conjugation and a change in the polarity of the solvent due to which the absorption maximum is shifted towards shorter wavelength.

Hyperchromic effect :- This signifies an increase in the intensity of the absorption maximum or a change in the extinction coefficient to a higher value at the same absorption maximum.

Hypochromic effect :- A decrease in the intensity of absorption of λ_{max} due to distortion of molecule, that is caused by introduction of group.

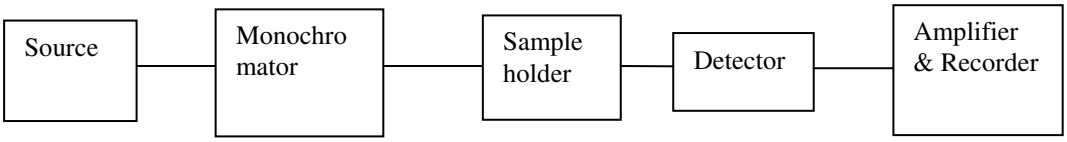
Figure 3.7: Different types of absorption and intensity shifts



INSTRUMENTATION OF ULTRAVIOLET, VISIBLE AND INFRARED SPECTROPHOTOMETRY

The spectrophotometers have the following components:

1.Source
2.Monochromator
3.Sample holder
4.Detector
5.Amplifier and Recorder



1)Radiant Energy Sources:-

Electrons of material can be excited to a high energy state by application of high voltage electricity. As these come down to ground state, energy is emitted in the form of radiation which is continuous and is of broad range. In practice, various sources are chosen to get wavelengths of different regions of spectrum.

a)Sources for UV radiation:- The commonly used sources are hydrogen lamp and deuterium lamp. The lamp source is a glass tube with quartz window that is filled with either hydrogen or deuterium gas at low pressure. Application of high voltage emits radiation in the range 180 to 350 nm. Xenon lamps also act as sources for UV radiations.

b)Sources of visible radiation :- Tungsten filament lamps are commonly used. These are inexpensive and emit radiations in the region between 350 to 2500 nm. Carbon arc provides more intense radiations and are less commonly used at commercial level.

c)Sources of infrared radiation:- Nernst Glower and Globar are the most common sources of IR radiation. The Globar consists of silicon carbide rod which when heated to around $1200°C$, emits radiations in the range 1-40 μm . Nernst Glower uses hollow rod of Zirconium and yttrium. It must be heated to $1500°C$, and it emits radiations in the range of 0.4-20 μ .

2)Monochromator (wave length selector):-

Monochromator allow working with monochromatic radiation, or select a narrow band width which is very close to λ_{max} for better absorption spectrum formation. These instruments are called wave length selectors which are of 2 types:

a) Filters
b) Monochromators
c)

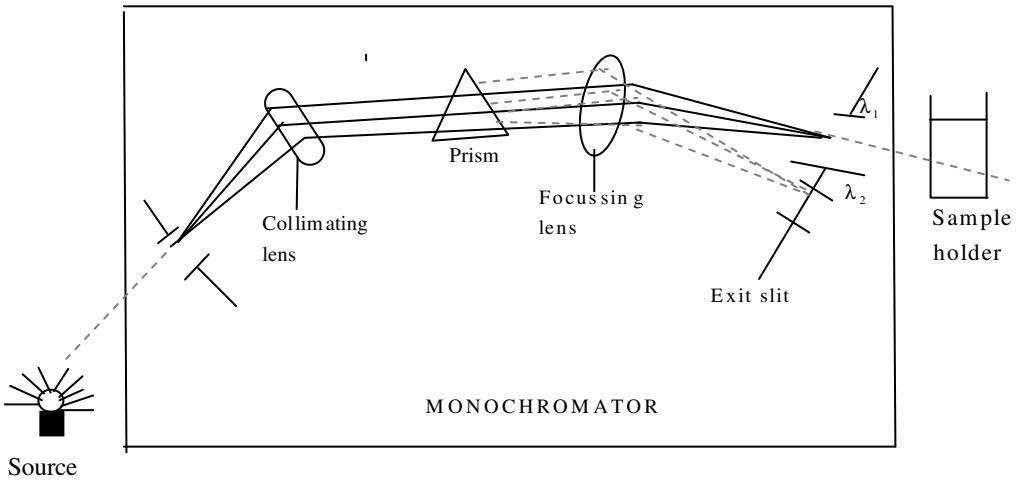
a)Filters:- They operate by absorbing light in all other regions except one. They have film of Gelatin dispersed with organic dyes sandwiched between glass plates. These filters narrow down the polychromatic light to a band width of ~40nm. They are commonly used in calorimeters. The disadvantage is that they have low transmittance (5-20% low).

b) Monachromator: (Figure 3.8) These are divces which resolve polychromatic radiation into its individual components and isolate these wavelength into narrow bands of light, that generate good absorption spectrum. The essential components of manochromator are

(i) An entrance slit, that allows polychromatic light from the source
(ii) A collimating device such as a lens or a mirror which collimates the polychromatic light on to a dispersing device.
(iii) A wave length resolving device like a prism or grating.
(iv) A focusing lens or a mirror
(v) An exit slit that allows monochromatic light to escape

From entrance slit to exit slit, as light passes, it will result in the narrow beam of light having wave length that is required for sample detection or estimation.

Figure 3.8: Monochromator

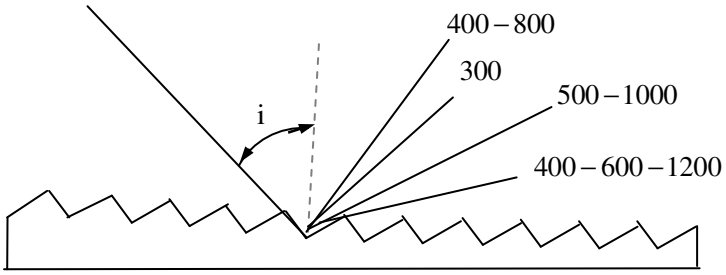


Dispensing devices used are prism or gratings. Prism are commonly used devices and are of 2 types- 60° croner quartz prism and 30° Littrow prism (commonly used). Glass prism are used when working with visible range of light while for UV regions silica, fused silica or quartz prisms. Fluorite is used in vaccum ultra violet range . Ionic crystalline materials are used in the infrared region. Some examples are NaCl, KBr, CsBr and this mixed crystallive material is referred to as KRS-5.

Gratings (figure 3.9) are used in spectrophotometers that operate in the ultraviolet, visible and infrared regions. These have highly aluminized surface which is etched thereby having large number of parallel groves (600 to 2000 lines/mm). When light falls on the etched surface, it is subjected to dispersion. A collimator followed by exit slit must allow selection of desired radiation.

Often, prism with grating is used in manochromator. The prism placed before grating is known as fore prism, and such arrangement helps in selection of radiation of single wave length.

Figure 3.9: Diffraction grating and the dispersion of polychromatic radiation



3. **Sample Containers :** Samples to be studies in UV and visible region are either gases or liquids and they are placed in cells called curettes. Spectrum of gases is taken in closed curettes which are preevaculated. The path length of cells varies from 0.1 to 100mm. if the sample is solid, it is formed into a pellet that is placed in a pellet holder for absorption measurements.

Material with which glass cuvettes are made varies with source of light. Quartz cuvettes are used while working with UV light, while simple glass cuvettes are used when working with visible light. The cuvettes have four sides and generally two surfaces are made up of rough ground glass, while two sides are maintained clean and clear.

The solvent must not exhibit any absorption and interfere with spectrum of solute. The solvents which can be used in UV and visible region are water, methyl-, ethyl-, isopropyl-alcohols, chloroform, hexane etc.

Infrared gas cells are made up of glass that has NaCl, KBr or CaF_2 windows that allow passage of IR radiation.

4. Detection Devices : The detectors must have following characteristics,

(i) High sensitivity
(ii) Short response time
(iii) Long term stability
(iv) Electronic signal to generate reaction amplified output.

The UV visible radiation on detectors are of three types - Photo cells, Photo tubes and Photomultiplier tubes (figure 3.10)

(i) **Photovoltaic or Barrier Layer Cells :** They employ semiconductor material which are crystalline, the bonding electrons between the crystals of some semiconductors can be knocked out by radiation. Although a number of materials are used in Photocells (Cadmium Sulphide, Silicon, Selenium), Selenium based photocells are common. They have thin coating of selenium over a thin transparent silver film on a steel base which allows easy passage of electrons from selenium to silver. A steel back plate acts as the other electrode and current blow is measured by a micrometer. Photocells have a long life and are inexpensive and reliable.

(ii) **Phototubes or Photoemmisive Tubes :** The components of phototube include

(a) Evacuated glass envelope with quartz window.
(b) A semi- cylindrical cathode whose inner is coated with alkali or alkaline with oxide
(c) A centrally located metal wire anode.

A potential difference 90 Volts is applied across electrodes. The quartz window allows the passage of radiation which strikes the photo emissive surface of the cathode. The energy of the photon is transferred to the loosely bound electrons of the electron surface. The

electrons become excited and finally leave the surface and travel towards the anode causing current to flow in the circuit. If the electron collection is 100% efficient, the phototube current should be proportional to the light intensity. Yet phototubes current are quite small and require amplification.

(iii) **Photo multipliers :** These detectors are designed to amplify the initial photoelectric effects and are suitable for use at very low light intensities . A photo multiplier consist of

(a) An evacuated glass tube into which are sealed the cathode and anode and

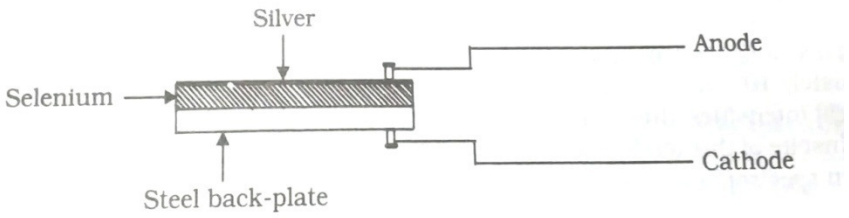
(b) Additional intervening electrodes known as dynodes.

The external circuitry is arranged so that a high voltage (1000Volts) exists between the anode and the anode. External voltage is arranged so that high voltage exists between anode and cathode. As radiations strike photocathode the applied potential difference accelerates electrons towards first dynode which liberates electrons. These are dragged to the next dynode where more electrons are released and this proceeds till the last dynode. By the time electrons reach the collecting anode, the amplification goes upto 10^6 times, giving ample amplification of week signals. Used in modern spectrophotometers. Linearity of instrument is lost when high intensities of radiation are used.

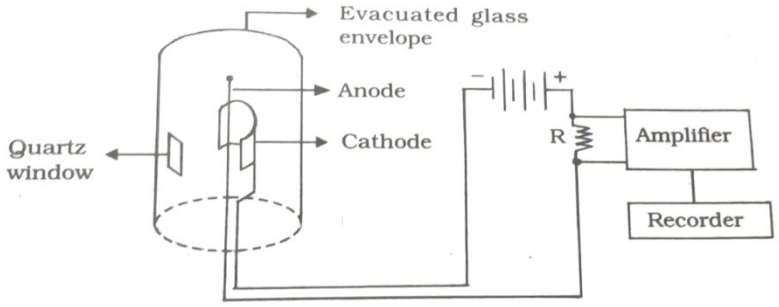
(iv) **Photodiodes :** these are semiconductors that change their charges voltage upon strike of light and this voltage change is converted into current and is measured. A photodiode array is a two dimensional matrix composed of hundreds of thin semiconductors spaced very close together. Light from the instrument is dispersed by either a grating or a prism onto the photodiode array. Each position of diode is calibrated to corresponding specific wavelength. Each diode is scanned and the resultant electronic change is calculated to be proportional to absorption. The entire spectrum is essential recorded within milliseconds.

Figure 3.10: a) Photovoltaic cell b) Photoemission tube c) Photoamplifier tube

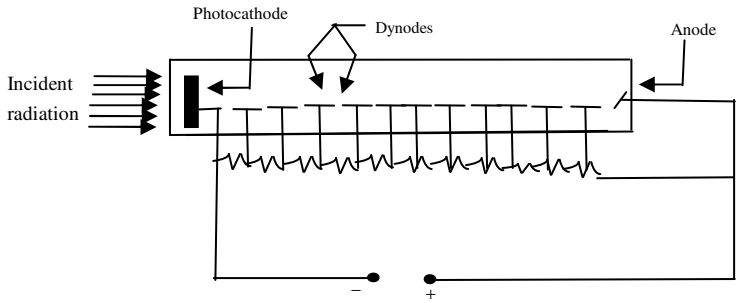
a)



b)



c)



Near Infrared Detection : These are usually photon conductive cells which detect infrared radiation in the range 0.8 - 3.0μ . The sensing element is a semiconductor (germanium, lead sulphide or lead telluride). Upon illumination by appropriate wavelength, the electrons of the semiconductor are raised to conduction bands, that in turn cause a drop in resistance. Therefore, if a small voltage is applied, a large increase of the system is such that the current may be amplified and finally indicated on a meter and is recorded.

Middle and far Infrared Detectors:

Middle and infrared photons when absorbed, cause rise in temperature to detect responses of this type, thermocouples resistance. Thermometers and gas thermometer are used in detectors. Thermocouples used in infrared receives typically consist of blackened gold lead tellurium metal pin junction which develops high voltage that is temperature dependent.

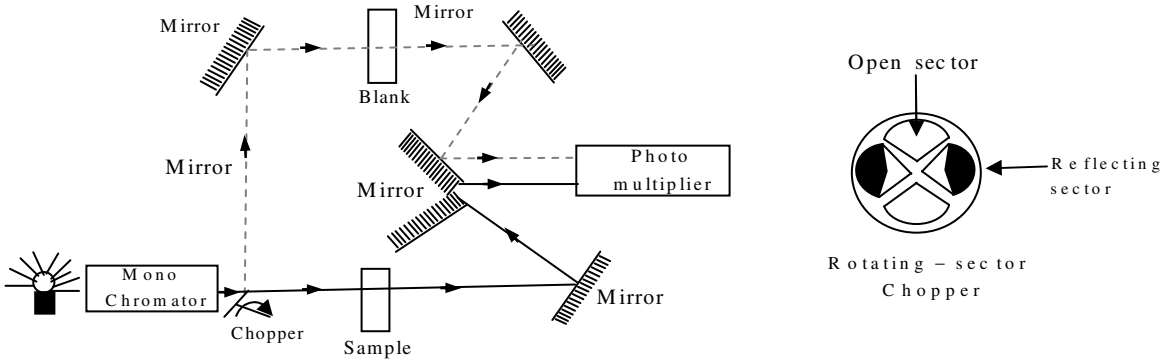
(v) Amplification and Readout:

Radiation detectors generates electronics signals which are proportional to the transmitted light these signals need to be translated into a form that is easy to interpret. This is accomplished by using amplifiers , ammeters, potentiometers and potentiometer recorders.

Variations in Spectro Photometers :

1) Double Beam Spectrophotometer: This is instrument employ beam splitter prior to sample container one split of beam passes through blank, while the other passes through blank. The two transmitted beams are then compared either continuously or alternately several times a second, which compensators any fluctuations in source intensity the final output will remain same without getting affected due to input variations (figure 3.11)

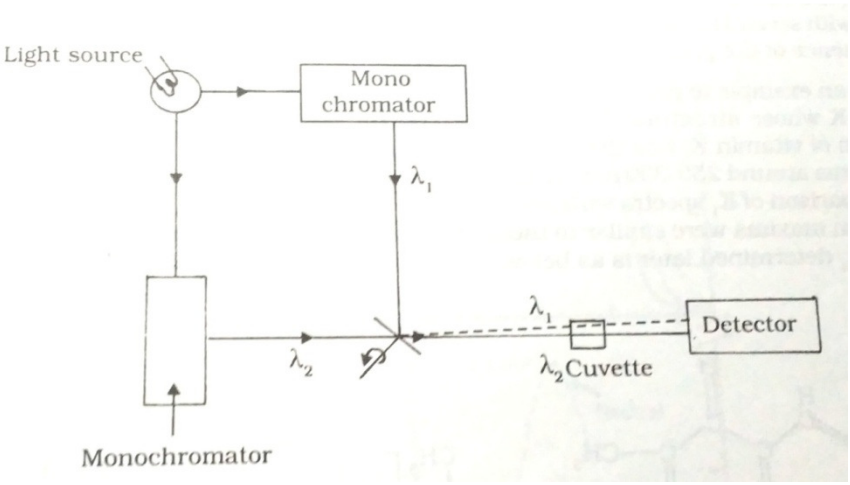
Figure 3.11: Optical arrangement of a double beam Instrument



2) Dual wave length spectrophotometer (figure 3 12)

At times few estimations need studies at two different wavelengths, eg: enzyme kinetics, metal chelation studies. These rate determining studies are done by taking OD at two wavelengths, but a gap of time persists which can effect results. In such situations two manochromators are used and the beams are made to pass through the sample alternately. This equipment has a single detector and the time difference between the two absorption studies is negligible.

Figure 3.12: Optical arrangement of a Dual wave length spectrophotometer



Applications of UV-visible Spectrophotometry

1. Quantitative and quantitative analysis: As absorption maximum (λ max) is specific for a biological molecules and as per Beer-Lambert's law the absorption of light intensity is proportional to concentration of a biomolecules. These equipment are used for determining their concentrations.
   Decrease in transmitted light indications presence of molecule. If absorbed light in terms of OD is taken and compared to a standard curve, concentration of molecules can be known.

2. Enzyme assay: Spectrophotometers enable enzymatic studies which involves development of a color or its disappearance. The other strategy involves formation of product that has different λmax as compared to substrate.

   Eg: Enzyme lactate dehydrogenase activity is determined by measuring NADH formation.

   $$Lactate + NAD^+ \rightleftharpoons pyruvate + NADH + H^+$$

   NAD^+ does not exhibit any absorption properties, while NADH exhibits maximum absorption at 340mm. Increase in OD indicates progress of forward reaction. From this rate of formation of pyruvate can be measured which reveals enzyme's rate of reaction.

3. Molecular weight determination:
   Although the extinction coefficient, $\propto$ of the absorption band remains constant in all the derivatives, the optical density, OD varies from compound to compound as they have different molecular weights. The molecular weight, M, of a compound can be calculated based on absorption data,
   $$M = awb / OD$$
   or
   $$M = 10 \propto / E^{1\%}_{1cm}$$
   Where, 'w' is weight of the compound is grams per litre and b is path length

4. Study of Cis-Trans Isomerism
   Geometrical isomers differ in the spatial arrangement of groups about a plane. Trans-isomers are more elongated than their Cis counterparts, and they have higher wave length of maximum absorption than cis-isomers. Thus, these can be used in the study of cis-trans isomerism.

5. In physico-chemical studies:
   Spectrophotometers can be used in the study of phenomena associated with physico – chemical studies of molecules like heat of formation of molecular addition compounds and

complexes in solution, determination of empirical formulas, formation constants of complexes in solution, hydration equilibrium of carbonyl compounds, association constants of complexes in solution, protein - dye associations, dissociation constants of acids and bases etc.

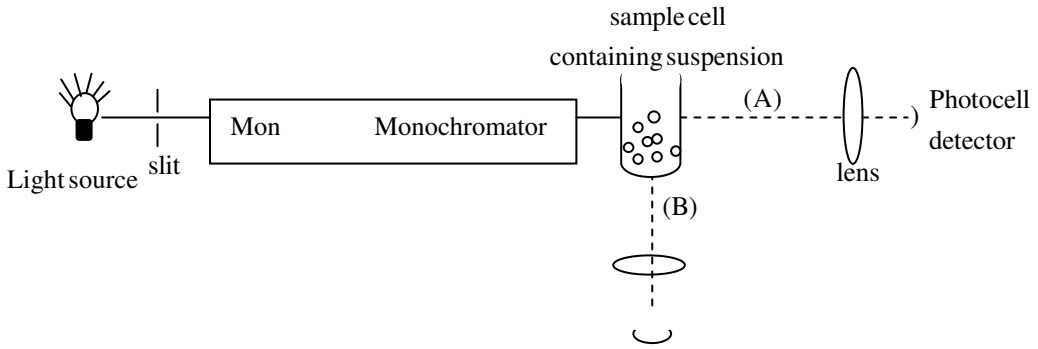
6. Control of purification :- Purity is estimated by checking for absorption of the compound at its λ max. Benzene impurities in commercial absolute alcohol is detected by measuring absorbance at 280nm, while alcohol absorbs at 210nm

Turbidometry and Nephlometry

Particulate matter makes sample turbid, and these suspensions lead to Tyndall effect. They scatter light, while the liquid absorbs it at a particular wavelength. If a radiation which is not absorbed by liquid is taken and if the scattered light by the suspension is measured it will be of less intensity than the incident light. If it is bacteria, the wave length used is 600nm, and a linearity exists between the scattered light and number of particles the scattered light is collected at 90° To the incident light using a photo cell detector (figure 3.13)

In Nephlometry, the transmitted light is measured and not the scattered (figure 3.13)

Figure 3.13: Principle of Turbidometry and Nephlometry



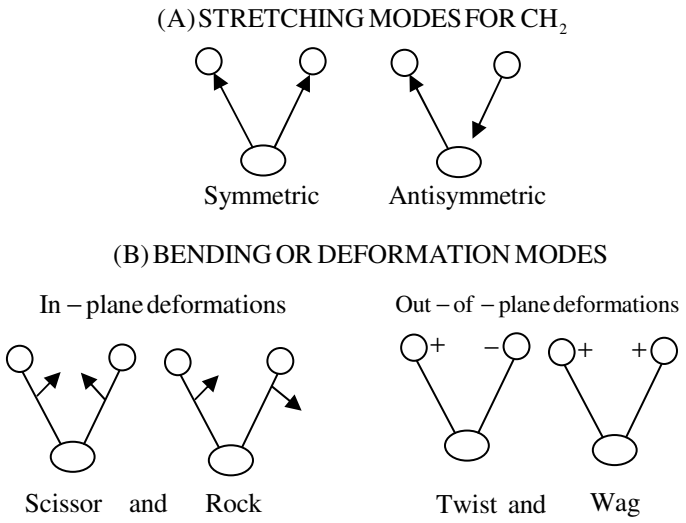
Infrared spectroscopy

IR spectroscopy is not associated with electronic transitions, rather it is associated with vibrational transitions of molecules.

All the molecules are continually vibrating and these are of 2 types, stretching vibrations and bending (figure 3.14). The bond distance between the atoms of a molecule fluctuate to about +0.5A°, this can result in an increase or decrease in bond length about an axis and there are called stretching modes. If vibrations involve changes in the positions of the atoms with respect to original bond axis, these are called bending vibrations and the angle may be about +0.5°.

Both these transitions are law energy transitions and the energy requirement corresponds to the electromagnetic radiation of Infrared region. Consequently the molecule absorbs infrared radiation resulting in variation in transmitted light. The IR graphs are drawn with percent transmission on y-axis and wave length on x-axis.

Figure 3.14: Types of Vibrations : A) Stretching (B) Bending



Calculation of vibrational Frequencies

The vibrational frequency of a bond can be calculated with the help of Hooke's law which correlates frequency with bond strength and atomic masses

$$v \propto \sqrt{\frac{\text{bond strength}}{\text{mass}}} \quad \text{or} \quad v = \frac{1}{2\pi}\sqrt{\frac{K}{m_1 m_2 / (m_1 + m_2)}}$$

Where, v is frequency

K is force constant of the bond

M_1 & m_2 are masses of the two atoms involved in bond formation

Ex: Calculate approximate frequency of C-H stretching vibration given, $K = 5.0 \times 10^5 \, g\, s^{-2}$, mass of carbon $= 20 \times 10^{-24} \, g$ and mass of hydrogen atom $= 1.6 \times 10^{-24} \, g$

$$v = \frac{7}{2 \times 22}\sqrt{\frac{5.0 \times 10^5 \, g\, s^{-1}}{(20 \times 10^{-24} g)(1.6 \times 10^{-24} g)/(20 + 1.6)10^{-24} g}}$$

$= 9.3 \times 10^{13} \, s^{-1}$

This values is divided by speed of light

Then V = 3100 cm^{-1}

Vibrational frequency generally increases with bond strength (as per equation). Double bonds have more vibrational frequency than single bonds (C = C > C – C) and based on bonding atoms also it varies, i.e, C-H bond has higher value than C – C due to mass values of the later.

Modes of vibration

The theory of molecular vibrations, predict that an asymmetrical molecule which has n atoms will have 3n-6 modes of fundamental vibrations. If n = 3, the number of modes of vibration are $(3\times3)-6=3$ fundamental modes of vibration. Methane has $(3\times5)-6=9$ and ethane 18. This is about simple atoms having same type of bonds and this situation is not always true and generally absorption band overlap. These vibrations are called degenerate. There are other frequencies at which bonds appear in an infrared absorption spectrum some of these bonds are called overtone bands. These are generated by modulation of vibrations. A strong absorption at 800cm^{-1}, can lead to weaker absorption at 1600cm^{-1}. Another kind of modulation is at two different frequencies, x and y, which can interact with each other resulting in combinations. These interactions can take place either as x + y or x – y. The resulting weaker absorptions are called beats.

As each molecule has a pattern of arrangement of atoms. The combination patterns thus generated by each macromolecule is unique, or it is a finger print for that molecule in the specified IR region which falls between 900 cm^{-1} to 1400 cm^{-1}. This is called as the finger print region.

Infrared spectroscopy : Mode of operation

The source of light emits radiation, throughout frequency range of equipment IR spectroscopy uses Double beam operator, in which one beam passes through reference while the other passes through the sample.

Sampling Techniques:

This depends on state of the sample. If sample is in vapor phase, it is pumped into gas cell with NaCl window, which is evacuated. Liquids are taken as this films of 0.01 to 0.1 mm diameter. NaCl flats are used throughout IR region. Solid spectra are recorded in 3 different way :- mulls, KBr disks and deposited films.

Applications of IR spectroscopy

1. Identification of compounds based on specific finger print
2. Determine rates of reactions based on changes in bonds
3. Conformation studies
4. To study interaction between molecules.

FOURIER TRANSFORM INFRARED SPECTROSCOPY

FT-IR stands for Fourier Transform Infrared, the preferred method of Infrared Spectroscopy. In Infrared Spectroscopy, IR radiation is passed through a sample, some of the radiation is absorbed by the sample and some of it is transmitted. The resulting spectrum represents the molecular absorption and transmission which is its finger print that is unique for the molecule. In addition, the size of the peaks in the spectrum is a direct indication of the amount of material present. IR is an excellent tool for quantitative analysis too.

The original IR instruments were of dispersive type. These instruments separate the individual frequencies of energy emitted from the infrared source. This is accomplished by the use of prism or grating. An infrared prism works exactly the same way as a visible prism which separates visible light into its colour. A grating separates the IR frequencies in a better way. The detector measures the amount of energy at each frequency which has passed through the sample. This resulting spectrum is a plot of intensity Vs frequency.

FT-IR is preferred over dispersive or filter methods of infrared spectral analysis for several reasons:-

– It is a non-destructive technique
– It provides a precise measurement method which requires no external calibration
– It can increase speed, collecting a scan every second
– It can increase sensitivity one second scans can be co-added together to ratio out random noise.
– It has greater optical throughput.
– It is mechanically simple with only one moving part.

Fourier Transform Infrared Spectrometry:-

This was developed in order to overcome the limitations encountered with dispersive instruments. The main difficulty was the slow scanning process. A method for measuring all of the infrared frequencies simultaneously, rather than individually was needed. A solution to this is the use of a device called an **Interferometer**. The interferometer produces a unique type of signal which has all of the infrared frequencies "encoded" into it. The signal can be measured very quickly, usually of the order of second or so. Thus, the time element per sample is reduced to a matter of a few seconds rather than several minutes.

Most interferometers employ a beam splitter which takes the incoming infrared beam and divides it into two optical beams. One beam reflects off a flat mirror which is fixed in place. The other beam reflects off a flat mirror which is a mechanism which allows this mirror to move a short distance away from beam splitter. The two beams reflect off of their respective mirrors and are recombined when they meet back at the beam splitter. The two beams reflect off of their respective mirrors and are recombined when they meet back at the beam splitter. Because the path that one beam travels is of fixed length and the other is

constantly changing as its mirror moves, the signal which exists the interferometer is the result of these two beams "interfering with each other. The resulting signal is called an

Interferogram. Which has the unique property that every data point (a function of the mirror moving position) which makes up the signal has information about every IR frequency which comes from the source. This means that as the Interferogram is measured, all frequencies are being measured simultaneously. Thus, the use of the interferometer results in extremely fast measurements. The measured interferogram signal Decoding is accomplished via a well known mathematical technique called the Fourier Transformation. This transformation is performed by the computer which then presents the user with the desired spectral information for analysis.

Components of FT-IR spectrometers:

The following are the components of FT–IR spectroscopy (figure 3.15).

a) IR sources:- These use mid and near IR regions of $2-25\mu m$ and $1-2.5\mu m$. The sources of radiation are therefore, silicon carbide element that is heated to around 1200 k and a tungsten halogen lamp respectively.

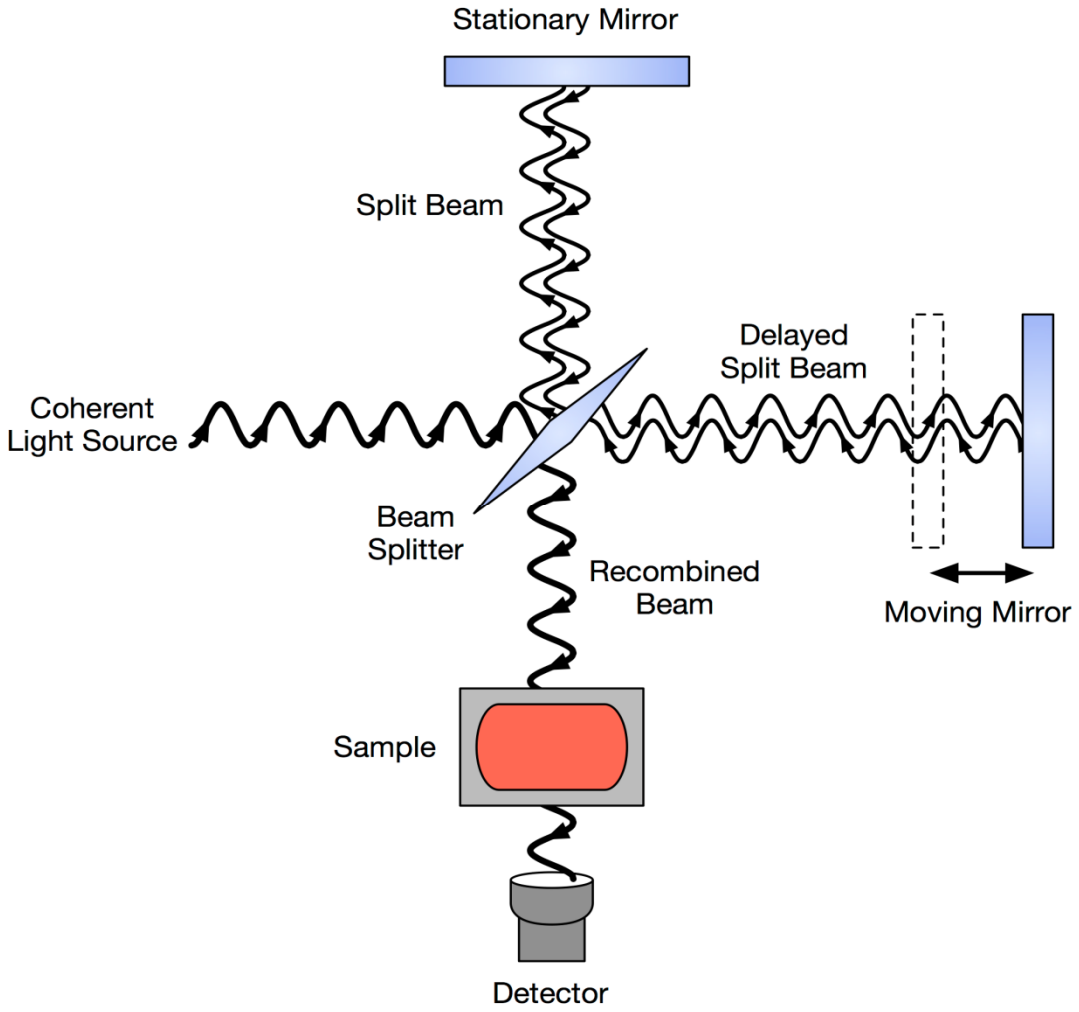
b) Detectors: Mid – IR spectrometers commonly use Pyroelectric detectors that respond to changes in temperature as the intensity of IR radiation falling on them varies. The sensitive elements in these detectors are either Deuterated Triglycine Sulfate (DTGS) or Lithium Tantalate ($LiTaO_3$). Cooled photoelectric detectors are employed for situations requiring higher sensitivity or faster response. Liquid nitrogen cooled mercury Cadmium Telluride (MCT) detectors are the most widely used in the mid infrared. With these detectors an Interferogram can be measured in as little as 10 milliseconds. Uncooled Indium Gallium Arsenide Photodiodes or DTGS are the usual choices in near – IR systems.

c) Beam spilliters: For the mid- IR region, the beam splitter is usually made of KBr with germanium based coating that makes it semi-reflective. ZnSe is an alternative where moisture vapor can be a problem but is limited to about 20 □m ($500cm^{-1}$). CaF_2 is the usual material for the near IR, being both harder and less sensitive to moisture than KBr but cannot be used beyond about 8 □m (1200 Cm^{-1}).

In a simple Michelson interferometer, one beam passed twice through the beam splitter but the other passes through only once. To correct for this an additional compensator plate for equal thickness is incorporated.

d) The computer:- The measured signal is digitalized and sent to the computer where the Fourier Transformation takes place. The final IR spectrum is then presented to the user for interpretation and any further manipulation.

Figure 3.15: Components of FT-IR spectroscopy



Applications:-

i) Compositional analysis of organic, inorganic and polymers

ii) Biological and biomedical fields like detection of water in biological membranes.

iii) Measurement of toxic gas in feels etc.

CIRCULAR DICHRONISM(CD)

The differential absorbance of the circulating polarized light resulting from a differentiation in absorptivity of the two rays is called circular dichroism (CD). It is the difference between two absorption spectra, one obtained using the left while the other using the right circularly polarized component. If this is so, one can apply Beer's law to circular dichroism.

Thus,

$A_L = a_L Cb$

and $A_R = a_R Cb$

Where the two subscripts L and R indicate the results obtained employing the left and right circular components respectively. Taking the difference, we get

$$A_L - A_R = (a_L - a_R)Cb$$

$$\therefore \Delta a = (A_L - A_R)/Cb \qquad (a_L - a_R = \Delta a)$$

Where, Δa means the difference between the two extinction coefficients. The usual representation of CD spectrum is to demonstrate Δa as a function of wavelength. As circular dichroism depends on absorption of light it will be observed only in those spectral regions where absorption occurs and will not be observed in the regions where no absorption occurs. If absorption does not occur, then $a_L - a_R$ is zero.

Differences between CD spectra and absorption spectra -

i) Extinction coefficients are always positive, but Δa can assume +ve or –ve sign based on the values of a_L and a_R. Thus, optical isomers of any given compound at a given wavelength will give rise to CD spectra of opposite signs,

a_d isomer $= -a_i$ isomer

ii) CD spectra and the absorption spectra differ in the relative size of 'a' and the average coefficient, $(a_L + a_R)/2$. Δa for most of the molecules of biological interest are less than 1% of $(a_L + a_R)/2$.

To arrive at relationship between absorbance and transmission, A&T,

$A = -\log T$, then

$$\Delta a = (-\log T_L + \log T_R)/Cb$$

$$= \frac{1}{C} b \log \frac{T_R}{T_L} = \frac{1}{Cb}; \log \frac{I_R / I_0}{I_L / I_0}$$

$$= \frac{1}{Cb} \log \frac{I_R}{I_L}$$

Where I_R & I_L are intensities transmitted. The electrical vector for CD is also expressed in terms of molecular ellipticity (θ).

The units of θ are degree centimeter squared per decimole $(deg - cm^2/dmole)$. Those of a Δa are centimeter squared per millimole $(cm^2/mmole)$.

Relationship between them is-

$$[\theta] = (3300 deg\ mmole/dmole)\Delta a.$$

CD Spectra:- The spectra are represented with wavelength versus extinction coefficient.

Applications:-

1. To determine optical activity of molecules.
2. To deduce conformation of macromolecules.
3. To study conformational changes which resulting from binding of enzyme to substrate or to Ligand binding studies.

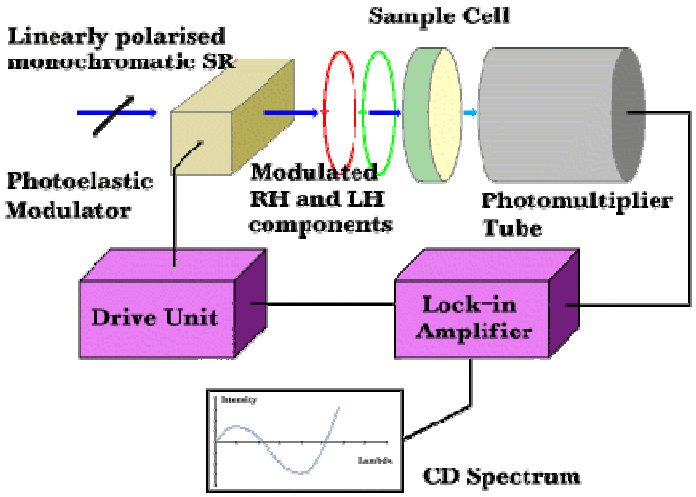
4. To study position of substitutes as in carbohydrates for α or β – glycan derivatives.

5. Forms of DNA and base stacking of nucleic acids are studied using circular Dichroism.

Instrumentation:- These operate in the wavelength range of 185–800 mm. Two light sources may be used to get L and R circular polarized light, while usage of molecular simplifies the equipment (figure 3.16).

The light source can be tungsten filament lamp that has polarizers next to them. The light is then passed through crystal which is subjected to alternating electric field. The crystal is called as electric modulator or a photoelectric modulator and results in L and R components to be transmitted alternately. The output from sample is subjected to photomultiplier which amplifies signals. The resulting electronic signals are processed to get final result.

Figure 3.16: Components of Circular Dichorism



RAMAN SPECTROSCOPY

Introduction: When incident light is scattered by intervening sample molecules, the frequency of light scattered is same as that of incident light. Such oscillations are said to be elastic. However, during scattering of monochromatic light by molecules, a small fraction of the scattered light is observed to have a different frequency from that of the incident photon. This phenomenon is known as the **Raman Effect.** This was discovered in 1928 by Sir C.V Raman. This has become an indispensible tool for the elucidation of molecular structure. It has been of immense use in locating various functional groups or chemical bonds in molecules and also for quantitative analysis of complex molecules.

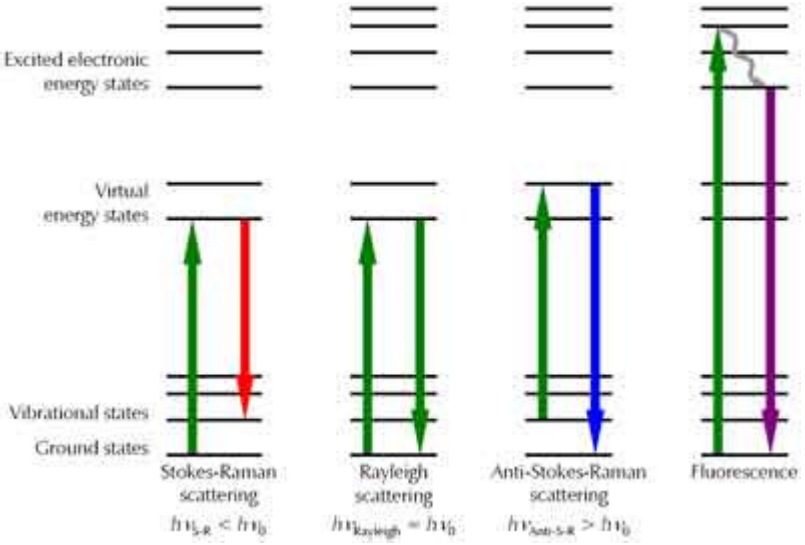
Only those vibrations which cause a change in dipole moment, i.e., change displacements are observed in the IR region. These vibrations are which do not cause a change in dipole moment and are consequently inactive in the infrared region, are observed in Raman Spectra. Thus, although the origin of Raman and Infrared spectra are quite different, the two techniques provide complementary information about frequencies of molecular vibrations and therefore about the structure and conformation of a molecule.

Principle:- Rayleigh scattering results when the sample molecules scatter light which is of the same frequency as that of the incident photon collisions between molecules can lead to energy changes that are in very small proportions (of less than 10^{-6}) and well before scattering takes place collision of these molecules with photon and among themselves does not lift the molecules to any quantized level, rather they are thought to be virtually excited (figure 3.17). While the excited molecules during emission of absorbed energy do not come down to their natural ground state, the emitted radiation has a frequency different from that of the incident one. All these patterns are a part of Raman spectrum.

Several such shifted lines, known as **Stokes lines** are observed in the Raman Spectrum and each corresponds to a different vibration in the molecule. The Raman spectrum thus provides detailed information about the vibrational spectrum of the molecule. These lines are known as **anti-strokes lines**. These arise when some molecules absorb incident radiation when they are in lower state.

Since most of the molecules indulge in **Rayleigh scattering**, radiation due to it is considerably more intense than the radiation due to Raman shifted components. The primary function of Raman spectrometer is to reject totally radiations due to Rayleigh Scattering and to detect the weak Raman - shifted components. The Raman spectrometer has an intense monochromatic light source, sensitive detector and good light gathering power.

Figure 3.17: Transitions of energy for Reyligh and Raman Scattering



MASS SPECTROMETRY (MS)

This is an analytical technique in which the molecules in the test sample are converted to gaseous and which are subsequently separated in a mass spectrometer according to their mass to charge ratio (m/z) and detected. The exact principle involved addition or loss of protons by biomolecules, that will result in variation of mass by 1 Da for each proton. The mass spectrum allows an accurate measure to be made of the relative molecular mass M_Z.

Essential features of mass spectrometers include
– Production of ions in the gas phase
– Acceleration of the ions to a specific velocity in an electric field.
– Separation of the ions in a mass analyzer
– Detection of each species of a particular m/z ratio.
The instruments are calibrated with standard components of known values. Carbon scale is used in MS, as it has a mass of $^{12}C = 12.000$.

The equipment separates ions by use of either a magnetic or electric field. Alternatively the time taken for ions of different masses to travel a given distance in space is measured accurately in the time of flight (TOP) by mass spectrometer.

Components of Mass spectrometer

It consists of the following components (figure3.18)

1. A high vacuum system $(10^{-6}$ tonnor) μ torr):

These include turbo molecular pumps, diffusion pumps and rotary vane pumps.

2. A sample inlet: This comprises a sample or a target plate; a high performance liquid chromatography (HPLC), gas chromatography (GC) or capillary electrophoresis system; Solid probe; Electron impact or direct chemical ionization chamber.

3. An ion source (to convert molecules into gas phase ions): This can be MALDI, ESI, fast atom bombardment (FAB), electron impact or direct chemical ionization.

4. A mass filter/analyper; This can be TOF; Quardrupole – ion trap; magnetic sector or ion cyclotron Fourier transform.

5. A detector : This can be a conversion dynode; electron multiplier; micro channel plate or array detector.

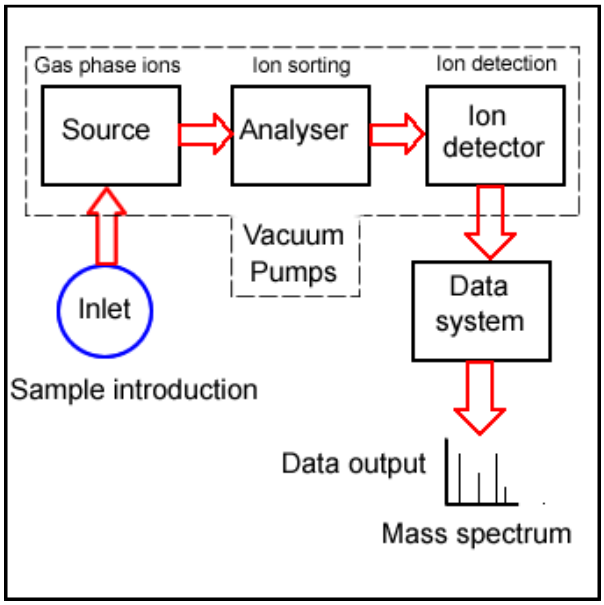


Figure 3.18: Components of Mass Spectroscopy

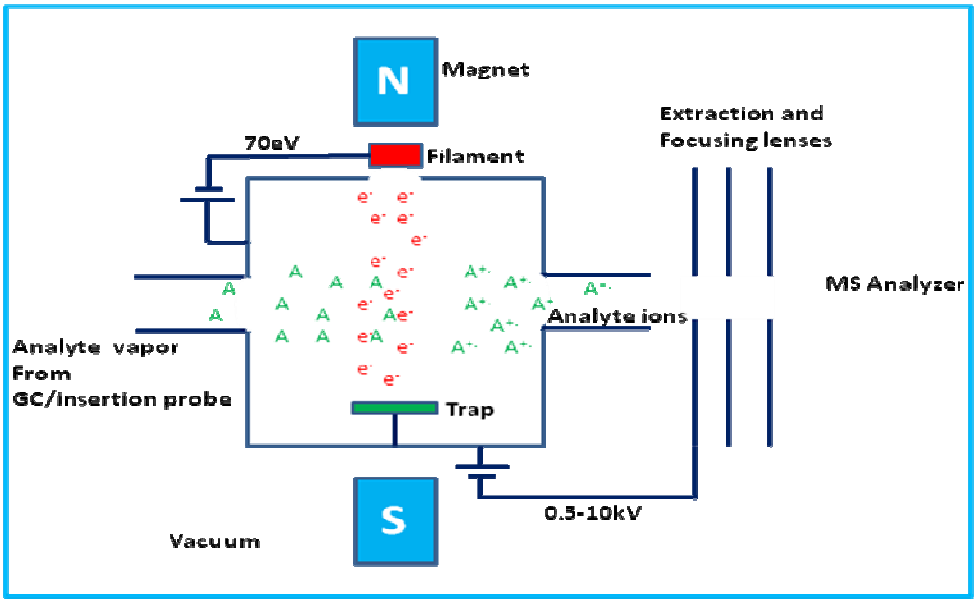
1. Vacuum system: Mass analyzers operate under vacuum to minimize collisions between ions and air molecules. Rotary vane pump and turbomolecular pumps are used. The later provides a working high vacuum.
2. Ionization: Ions are produced from neutral molecules by removing an electron to produce a positively charged cation or by adding an electron to form an anion. Methods used for ionization include:

a) Electron Input ionization (EI):

Widely used for analyzing of metabolic, pollutants and pharmaceutical compounds.

In this, a stream of electrons from a heated metal filament is accelerated to 70eV potential. Sample ionization occurs when the analyte vapours are allowed to diffuse into the chamber of electron stream (figure 3.19). This can lead to loss of electrons by the molecule due to loss of electrons by the molecule due to electron bombardment or gain of electron giving rise to cations (M^+) or anions (M^-). These daughter ions are recorded as the mass spectrum.

Figure 3.19: Schematic diagram of Electron impact Ionizer



B. Chemical ionization:- Reagents like methane and ammonia are used for initial ionization of molecules. The high gas pressure in the source results in ion-molecule reactions between reagent gas ions (such as NH_3^+ and CH_4^+) some of which react with analyte to produce ions.

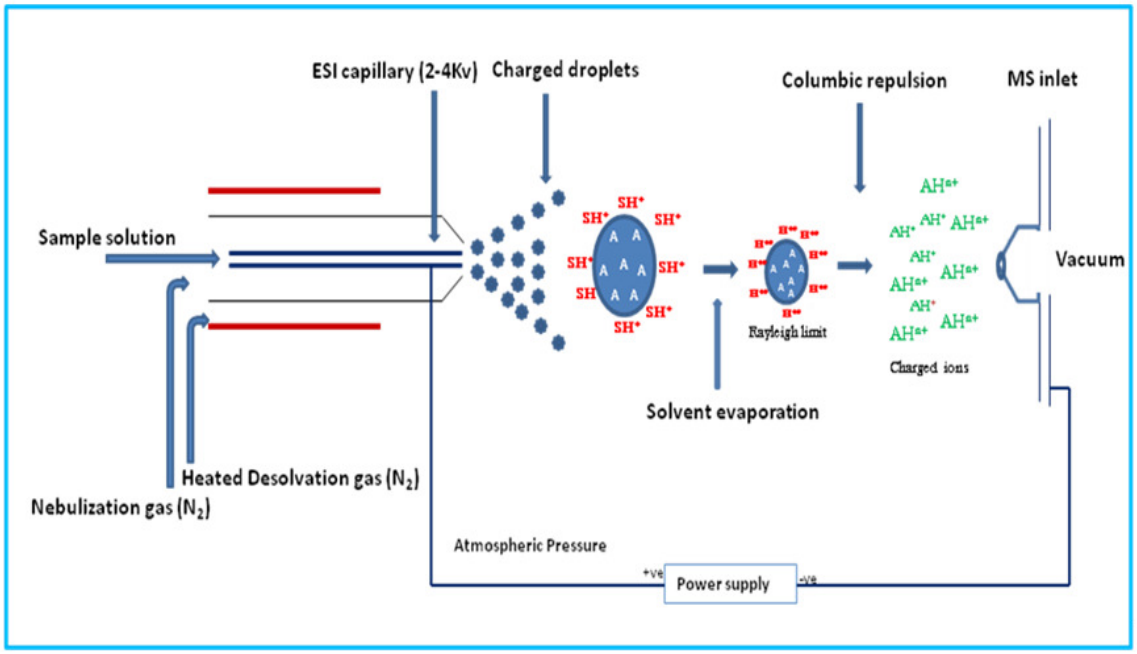
C. Fast atom bombardment:- In this method the sample is mixed with a relatively involatile, viscous matrix such as glycerol, thioglycerol or m-nitrobenzyl alcohol. The mixture is placed on a probe, that is introduced into the source housing and bombarded with an ionizing beam of neutral atoms (Ar, He, Xe) of high velocity.

Later development involved usage of a beam of Caesium ions (Cs+) was introduced. Bombardment will result in ion species which are either protonated or deprotonated

$\left((M+H)^+ \& \left(M-H^-\right)\right)$. These pseudomolecular ion species formed are recorded in mass spectrum.

d) Electronspray ionization (ES): This is a soft ionization technique and enables analysis of large intact biomolecules, such as proteins and DNA. The electrospray (ES) creates very small droplets of solvent containing analyte. The charged liquid droplets are produced by atomization on or nebulisation. In ESI strong electric field is used and as solvent with analyte is subjected to this, the solvent evaporates in the high vacuum region. As the droplet moves, its size decreases finally leaving charged analytes (figure 3.20). The solvents that are used include- 50: 50 of acetonitrile and water, 50:50 of methanol and water; 1% aceticacid or 0.1% formic acid. Ammonium hydroxide, Trifluoracetic acid can also be used.

Figure 3.20: schematic representation of Electron spray ionization



3. Mass analyses: These are involved in separation of ions based on mass to charge ratio. These include

a) Quadropole mass spectrometry:-

This analyser has four parallel cylindrical rods which are applied with DC voltage As ions move down the rods which are applied with varying voltage, the ions are accelerated down based on m/z ratio.

b) Ion trap mass spectrometry:-

This involves usage of Ions traps, that store in them ions of selected m/z ratio. To perform tandem MS a collision gas is introduced that releases fragment ions inturn to generate fragment spectrum.

c) Nanospray and on-line tandem mass spectrometry:

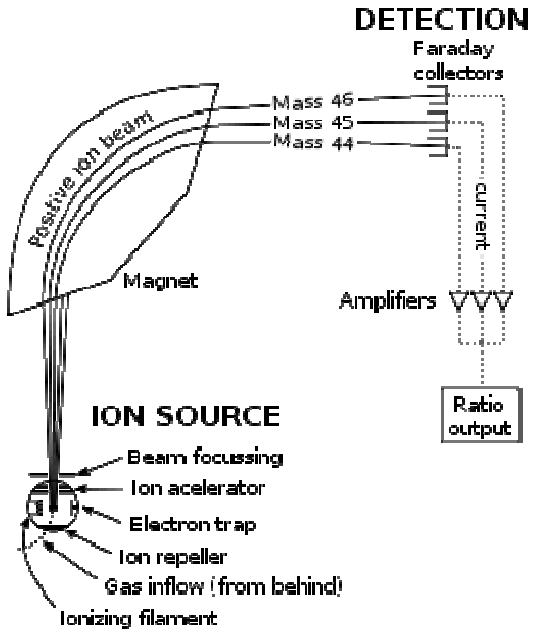
In this nanospray is generated using fine needles that dispose the sample accurately at the entrance of source. At low flow rates and usage of stream splitters device allows good separation of ions.

e) Magnetic sector analysis (figure 3.21)

The electric sector acts as a kinetic energy filter and allows only ions of a particular kinetic energy to pass, irrespective of m/z ratio, increasing resolution.

Ions emerging out of ESA have same velocity. As they enter magnetic sector analyzer, which is a curved trajectory path, depending on m/z ratio and velocity of ions they reach the detection. At a particular point of time, one set of ions reach the detector.

Figure 3.21: Schematic representation of magnetic sector analyzer



f) Plasma Desorption ionization:-

This is first one of a kind of Mass spectrometer, that can be used to analyzer proteins and large biomolecules. In this, source of plasma, is ratio active californium, ^{252}Cf that emits nuclei Ba^{20+} and Tc^{18+}. These are ejected in opposite direction, almost collinearly and with equal velocity. These plasma particles emitted in direction opposite to that passing through the sample triggers a time counter, and the desorbed sample ions are accelerated electrically and detected by a TOF analyser.

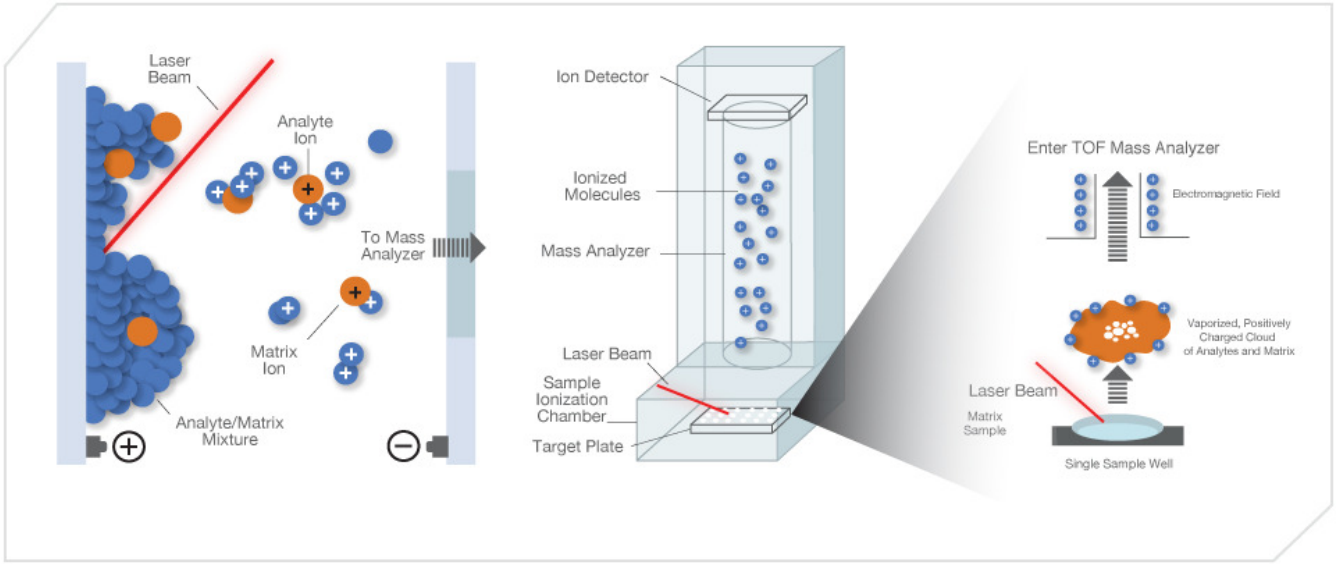
f) MALDI, TOF Mass spectrometry:-

Matrix - assisted laser desorption ionization (MALDI) produces gas phase protonated ions by excitation of sample molecules from the energy of a laser transferred via an ultra violet (UV) light absorbing matrix. The matrix is a conjugated organic compound that is mixed with compound and dried onto the target plate, where sample and matrix co-crystallize upon

drying. Pulses of laser light of few nanoseconds causes drying of rapid excitation and vaporization of crystalline matrix. This later causes ejection of matrix and analyte ions hat are analyzed by TOF analyser (figure 3.22).

Many other modifications of MALDI have come up for better resolution of analyte.

Figure 3.22: MALDI ionization mechanism with TOF analyzer

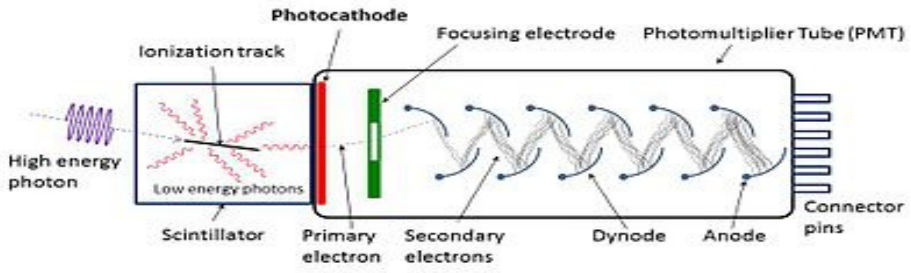


4) Detectors-

The ions from mass analyzer impinges on a surface of a detector where the charge is neutralized wither by collection or donation of electrons. As electron current flows, it is amplified and ultimately converted into a signal that is processed by a computer. The Total Ion Current (TIC) is the sum of the current carried by all the ions being detected at any given moment.

Electron multiplier and conversion dynodes are used in the detectors. The ion beam from the mass analyzer is focused the onto the conversion dynode, which emits electrons in direct proportion to the number of bombarding ions. A positive or a negative ion hitting conversion dynode causes emission of secondary particles which contain secondary ions, electrons and neutral particles (figure 3.23). These secondary particles are accelerated into the dynodes of the electron multiplier. They strike the dynodes with sufficient energy to dislodge electrons, which pass further into the electron multipliers, colliding with the dynodes, producing more and more electrons.

Figure 3.23: Diagram of a Conversion dynode and electron multiplier



NUCLEAR MAGNETIC RESONANCE SPECTROMETRY

NMR is a technique that is used to study the unaltered structure and conformation of macromolecules in solution. Most nuclei, protons and electrons posses inherent magnetic field, that is too small to observe under the ambient & magnetic field of earth. Artificially created intense magnetic field can, however make the nuclei assume specific orientation with corresponding potential energy levels. Nuclear magnetic resonance spectroscopy addresses itself towards detecting the minute amount of energy absorbed or emitted as the nuclei jump from one energy level to another.

Magnetic properties of Nuclei:-

In order to explain the magnetic properties of certain nuclei, it is necessary to assume that the nuclear charge is spinning around an axis. Such nuclei are supposed to posses an angular momentum represented by a spin quantum number, I, Which is assigned half integral values, $0,1/2,1,3/2.....9/2$ depending on the particular nucleus

Nuclei possessing an odd mass number (either the number of neutrons or number of protons must be odd) are assigned half integral spin quantum numbers, for example ^{1}H, $^{31}P\left(\frac{1}{2}\right)$ and $^{11}B\left(\frac{3}{2}\right)$. Figure 3.24 illustrates the principle of NMR. The spinning nucleus or a spinning charge gives rise to a magnetic field. There is an associated nuclear magnetic moment, μ, along the axis of the spin. Nuclei possessing odd numbers of both neutrons and protons have an integral spin number of I=1. Thus the charge on them is distributed non-symmetrically. Nuclei possessing even number of both protons and neutrons are not measured in NMR as their angular momentum I=0 and they do not exhibit magnetic

properties when the nuclei are placed in a powerful uniform magnetic field the spin axis of nuclei having magnetic momentum align themselves in an inclined manner with respect to the field, and like the top of a gyroscope, orient about the field direction in such a way that each pole of the nuclear axis sweep out a circular path in the XY-plane. When a second weaker field of ration frequency (RF) is applied at right angles to the uniform magnetic field, the nuclei may undergo a transition to a high energy level by absorbing the RF energy. Only that RF whose sense of rotation is exactly equal to the rate of precession of nuclear dipole with the absorbed.

The 'I' value determines the quantized levels available for a nucleus, which are given by the series:

I, I-1, I-2,… -I.

For any given nucleus, the possible levels of orientation are 2I+1. Thus for ^{1}H, $^{15}N, ^{19}F and ^{31}P,$ with I value of $\frac{1}{2},$ have only two orientation available. These levels are described as i) Aligned with the applied field (lower energy) and

ii) Anti parallel to the field (higher energy) (figure 3.25). These two levels will have energy of $-\mu H_0 \text{ and } +\mu H_0$ for low and high energy respectively, whose H_0 is the intensity of the applied magnetic field. And μ is the nuclear magnetic moment. The different in energy, $\Delta E,$ is equal to $2\mu H_0.$

The general relationship is as follows:

$$\Delta E = \frac{\mu H_0}{I}$$

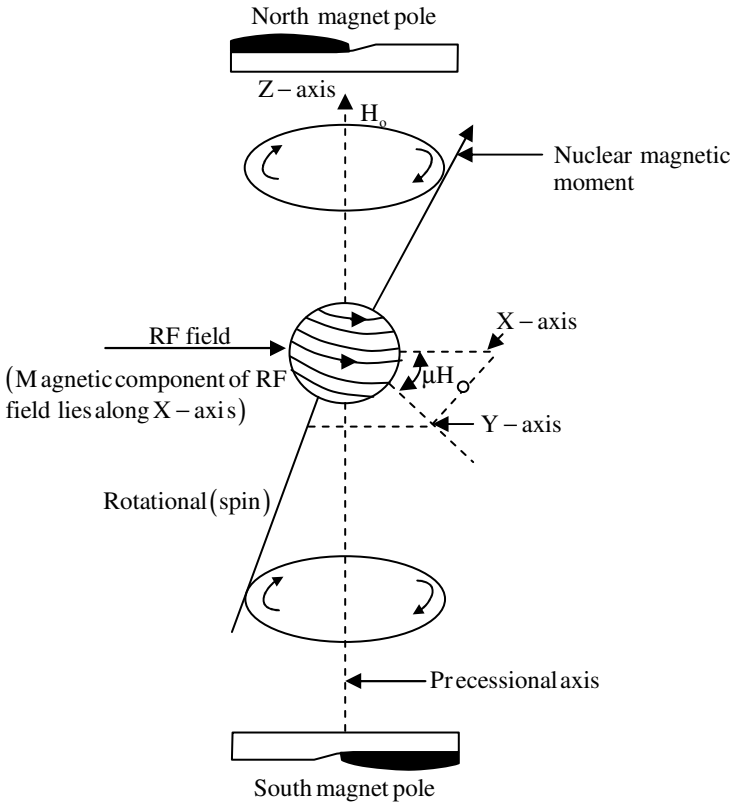
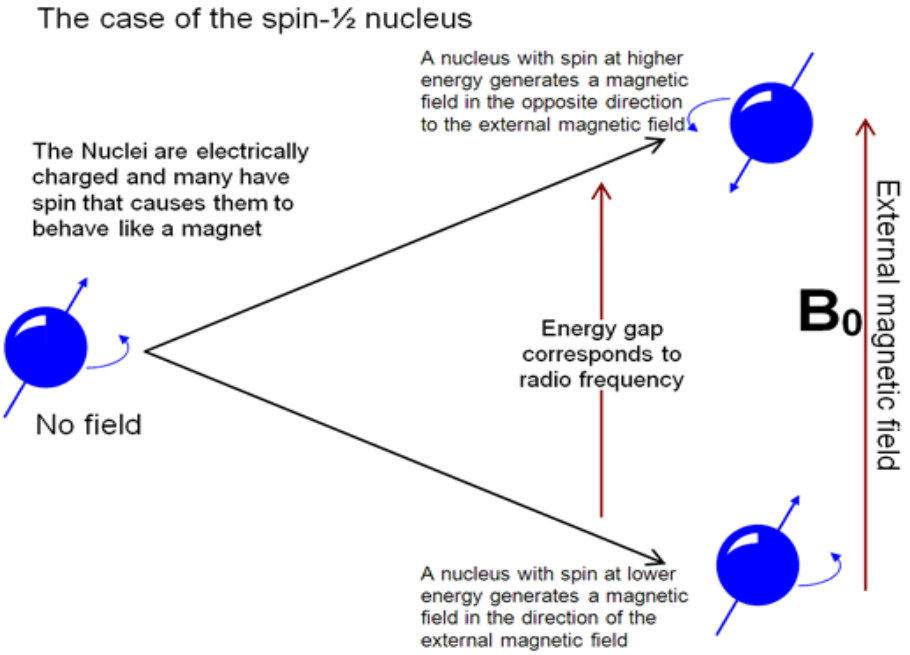


Figure 3.24: Principle representing Nuclear magnetic resonance

Figure 3.25: Energy states of Nuclei in magnetic field



Nuclear Resonance: In order to change from low energy state to high energy state, nuclei must absorb appropriate quantum of energy. In a magnetic field of several hundred millitesla, the nuclei absorb radiation in the radio wave region of the electromagnetic spectrum. This phenomenon is known as nuclear resonance or nuclear magnetic resonance. The above can be thus written as,

$\Delta E = h\nu = 2\mu H_0$

Combining above 2 equations, we get

$$2\pi\gamma = \frac{2\pi\nu H_0}{nl} = \gamma H_0$$

Where 2π serves to convert linear frequency to angular units of frequency.

γ is equal to $2\pi\mu / hI$ is known as gyromagnetic ratio and is a characteristic property of the nucleus. Another relationship of this value is

Gyromagnetic ratio $= \gamma = \frac{2\pi\nu}{H_0}$

This reveals relationship between field & frequency.

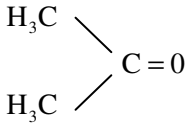
The frequency of ratio wave absorbed during NMR is dictated by

i) The isotope being studied

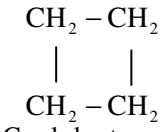
ii) Intensity of magnetic field

Different nuclei require different magnetic field strength to give rise to the same effective magnetic strength which causes absorption. It is therefore a practice that, absorbance of the same at a monochromatic radiowave frequency is set while varying magnetic field intensity. The NMR spectra thus obtained are plot of energy absorbed against the magnetic field strength applied (figure 3.26). The number of signals obtained indicate the different sets of equivalent nuclei protons.

NMR spectrum for methanol resulted in two peaks, one corresponding for CH_3 and the other for $-OH$. Acetone and cyclobutance result in one peak.



Acetone



Cyclobu tan e

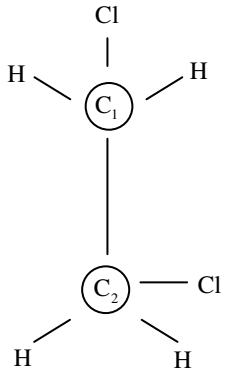
All protons are in same environment.

CH_3CHBr results in two NMR signals (1, 1-dibramomethane).

$CH_3CH_2CAO(propanol)-$ 3 sets of signals, one each for $-CH_3, -CH_2$ & $-CHO$.

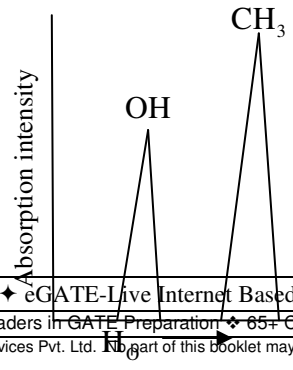
1,2 dicholoropropane is expected to give three signals, but gives four signals .

$CH_3 - CH(Cl) - CH_2Cl$. It stereochemical formula is as follows:



The two protons on C1 do not exist in same environment and hence result in four NMR signals.

Figure 3.26: Energy spectrum of Methanol



Chemical Shifts:

NMR spectra give information about the number of equivalent sets of protons/nuclei that a compound possesses. The position of signals is indicative of the environment surrounding the protons/nuclei (presence of the type of graphs around it electron with drawing or electron releasing). The protons therefore absorb at different applied field strengths based on electronic environment.

The electron involved in covalent bonding are paired and usually do not have a magnetic field. An applied magnetic field, however creates additional modes of circulation of these electrons thereby generating a small localized magnetic field. This magnetic field is proportional to the applied magnetic field. In such nuclei, circulation of electrons around which has given rise to a applied magnetic field, might not resonate. This phenomenon is known as diamagnetic shielding and the nuclei are said to be shielded. Such nuclei are not resonating because rather than experiencing the full magnetic field applied, Ho, they experience a smaller amount of it due to the opposition created by the field of electrons.

$H_{eff} = H_0 - \sigma H_0$

Where σ is shielding constant, whose value depends upon the density of electrons around a proton or nucleus. It is a function of the structure of the compound. To overcome the above situation a higher magnetic field is required to resonate nuclei. Thus, shielding of nucleus shifts the position of the signal i.e., absorption upfield.

Sometimes, the induced field augments the applied field. The nucleus is then said to be deshielded and a smaller applied field is required to make such nuclei to resonate. Thus deshielding shifts the absorption downfield.

To compare changes in n.m.r, absorption arising out of shielding and deshielding with a standard reference the shifts are known as chemical shifts. For proton spectra in non aqueous media, the reference material is Tetramethyl silate $(CH_3)_4 Si,$ abbreviated as TMS. TMS contains 12 protons and all the protons are chemically equivalent. Therefore TMS gives a single sharp signal and hence the difference in the absorption position of the proton with respect to TMS signal is called chemical shift. The magnitude of chemical shift is expressed as $\delta -$ value which is calculated as follows:-

$$\delta = \frac{H_{sample} - H_{TMS}}{\nu_1} \times 10^6$$

Where, H_{sample} and H_{TMS} are the positions

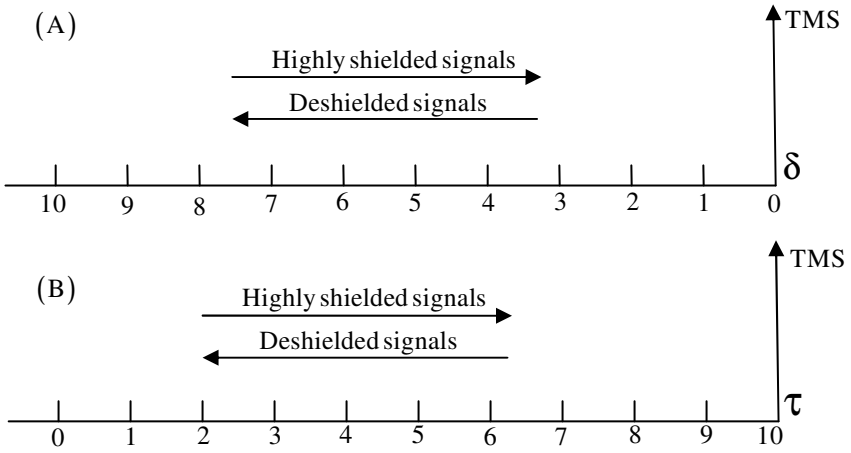
Of the absorption lines for sample and reference respectively and is expressed in frequency units (Hertz) and ν_1 is the operating frequency of the spectrometer, δ is usually expressed in parts per million (ppm). Most decimal shifts have 8 values between 0 to 10.

Another scale used for the measurement of chemical shift is known as the τ Scale.

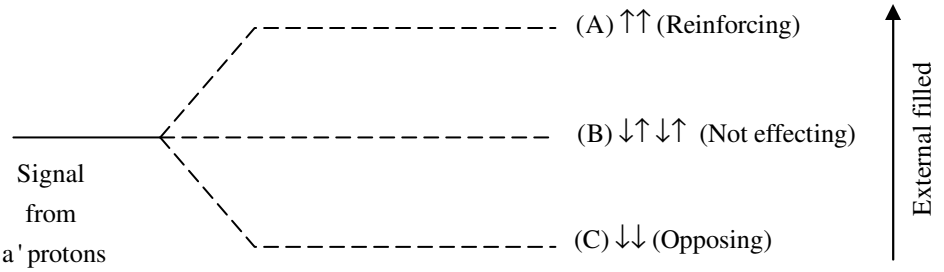
$\tau = 10 - \delta$

It is usual to plot the nmr signals with magnetic field strength increasing in the right TMS is assigned values of 0 ppm and its signal appears to increase to the extreme right (figure 3.27). The protons that experience greater dishielding have larger $\delta-$ values.

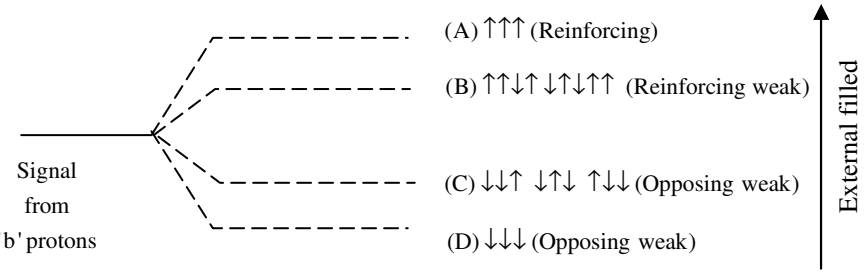
Figure 3.27: Scaless to measure chemical shifts relative to TMS (A) □ value (B) □ value



Hyerfine Splitting:- At times NMR spectra are complicated due to spin-spin coupling. A compound like ethyl bromide CH_3CH_2Br must result in two signals, while the spectrum comprises of multiples of signals. As seen in figure below, the protons of first carbon resulted in three peaks, while the protons of second carbon resulted in four peaks. This is due to coupling of spins between the two groups and theses are of 3 types. For CH_3 group the triplet signals is a result of following spin coupling.



The CH_2 group generates a quartet signal because of coupling with $-CH_3$ group in four ways as shown below.

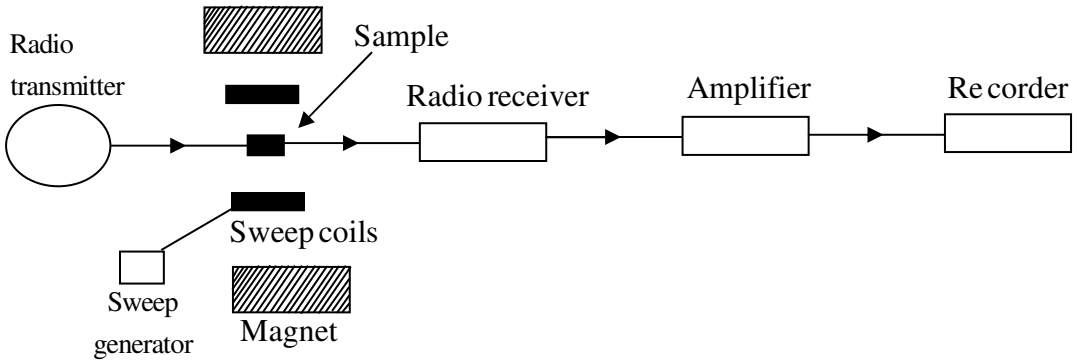


This phenomenon which involves interaction between like and different spins of nearly nuclei is known as spin-spin coupling.

Instrumentation:- It comprises of extremely heavy electromagnets $\left(10^3 - 10^4 \text{kg}\right)$ producing fields ranging from $1-10$ tesla. The newer generation spectrometers, however, use superconducting solenoids which generate fields above 3.5tesla. The magnetic field can be varied by about 10^{-2} tesla. by using an auxiliary sweep generator.

Since the nuclei absorb in the radiowave regions, the source of radiation is a radio frequency transmitter. The sample must be dissolved in a relatively high concentration of solvent which is protons poor like D_2O. The sample is placed in a tube which is rotated at high speed by an air turbine. The absorption signal is then detected by a radio receiver. The signal is amplified and recorded (figure 3.28).

Figure 3.28: Instrumentation of NMR



Applications:-

1. Structured diagnosis of a small organic molecules and small globular proteins.
2. Study of dynamic characteristic of protein structure
3. Studies on complex formation. Like enzyme substrate complex, drug – DNA, Ag-Ab interaction etc.
4. Biological structures and compartments in cells.
5. Quantitative studies.
6. Thermodynamic studies
7. Membrane transport studies.
8. Studies of intact organs etc.

FOURIER TRANSFORM INFRARED SPECTROSCOPY

FT-IR stands for Fourier Transform Infrared, the preferred method of Infrared Spectroscopy. In Infrared Spectroscopy, IR radiation is passed through a sample, some of the radiation is absorbed by the sample and some of it is transmitted. The resulting spectrum represents the molecular absorption and transmission which is its finger print that is unique for the molecule. In addition, the size of the peaks in the spectrum is a direct indication of the amount of material present. IR is an excellent tool for quantitative analysis too.

The original IR instruments were of dispersive type. These instruments separate the individual frequencies of energy emitted from the infrared source. This is accomplished by the use of prism or grating. An infrared prism works exactly the same way as a visible prism which separates visible light into its colour. A grating separates the IR frequencies in a better way. The detector measures the amount of energy at each frequency which has passed through the sample. This resulting spectrum is a plot of intensity Vs frequency.

FT-IR is preferred over dispersive or filter methods of infrared spectral analysis for several reasons:-

– It is a non-destructive technique

– It provides a precise measurement method which requires no external calibration

– It can increase speed, collecting a scan every second

– It can increase sensitivity one second scans can be co-added together to ratio out random noise.

– It has greater optical throughput.

– It is mechanically simple with only one moving part.

Fourier Transform Infrared Spectrometry:-

This was developed in order to overcome the limitations encountered with dispersive instruments. The main difficulty was the slow scanning process. A method for measuring all of the infrared frequencies simultaneously, rather than individually was needed. A solution to this is the use of a device called an **Interferometer**. The interferometer produces a unique type of signal which has all of the infrared frequencies "encoded" into it. The signal can be measured very quickly, usually of the order of second or so. Thus, the time element per sample is reduced to a matter of a few seconds rather than several minutes.

Most interferometers employ a beam splitter which takes the incoming infrared beam and divides it into two optical beams. One beam reflects off a flat mirror which is fixed in place. The other beam reflects off a flat mirror which is a mechanism which allows this mirror to move a short distance away from beam splitter. The two beams reflect off of their respective mirrors and are recombined when they meet back at the beam splitter. The two beams reflect off of their respective mirrors and are recombined when they meet back at the beam splitter. Because the path that one beam travels is of fixed length and the other is constantly changing as its mirror moves, the signal which exists the interferometer is the result of these two beams "interfering with each other. The resulting signal is called an **Interferogram.** Which has the unique property that every data point (a function of the mirror

moving position) which makes up the signal has information about every IR frequency which comes from the source. This means that as the Interferogram is measured, all frequencies are being measured simultaneously. Thus, the use of the interferometer results in extremely fast measurements. The measured interferogram signal Decoding is accomplished via a well known mathematical technique called the Fourier Transformation. This transformation is performed by the computer which then presents the user with the desired spectral information for analysis.

Components of FT-IR spectrometers:

The following are the components of FT–IR spectroscopy (figure 3.16).

g) IR sources:- These use mid and near IR regions of $2-25\mu m$ and $1-2.5\mu m$. The sources of radiation are therefore, silicon carbide element that is heated to around 1200 k and a tungsten halogen lamp respectively.

h) Detectors: Mid – IR spectrometers commonly use Pyroelectric detectors that respond to changes in temperature as the intensity of IR radiation falling on them varies. The sensitive elements in these detectors are either Deuterated Triglycine Sulfate (DTGS) or Lithium Tantalate ($LiTaO_3$). Cooled photoelectric detectors are employed for situations requiring higher sensitivity or faster response. Liquid nitrogen cooled mercury Cadmium Telluride (MCT) detectors are the most widely used in the mid infrared. With these detectors an Interferogram can be measured in as little as 10 milliseconds. Uncooled Indium Gallium Arsenide Photodiodes or DTGS are the usual choices in near – IR systems.

i) Beam spilliters: For the mid- IR region, the beam splitter is usually made of KBr with germanium based coating that makes it semi-reflective. ZnSe is an alternative where moisture vapor can be a problem but is limited to about 20 □m ($500cm^{-1}$). CaF_2 is the usual material for the near IR, being both harder and less sensitive to moisture than KBr but cannot be used beyond about 8 □m (1200 Cm^{-1}).

In a simple Michelson interferometer, one beam passed twice through the beam splitter but the other passes through only once. To correct for this an additional compensator plate for equal thickness is incorporated.

d) The computer:- The measured signal is digitalized and sent to the computer where the Fourier Transformation takes place. The final IR spectrum is then presented to the user for interpretation and any further manipulation.

Applications:-

i) Compositional analysis of organic, inorganic and polymers

ii) Biological and biomedical fields like detection of water in biological membranes.

iii) Measurement of toxic gas in feels etc.

CIRCULAR DICHRONISM(CD)

The differential absorbance of the circulating polarized light resulting from a differentiation in absorptivity of the two rays is called circular dichroism (CD). It is the difference between two absorption spectra, one obtained using the left while th e other using the right circularly polarized component. If this is so, one can apply Beer's law to circular dichroism.

Thus,

$A_L = a_L Cb$

and $A_R = a_R Cb$

Where the two subscripts L and R indicate the results obtained employing the left and right circular components respectively. Taking the difference, we get

$A_L - A_R = (a_L - a_R)Cb$

$\therefore \Delta a = (A_L - A_R)/Cb \qquad (a_L - a_R = \Delta a)$

Where, Δa means the difference between the two extinction coefficients. The usual representation of CD spectrum is to demonstrate Δa as a function of wavelength. As circular dichroism depends on absorption of light it will be observed only in those spectral regions where absorption occurs and will not be observed in the regions where no absorption occurs. If absorption does not occur, then $a_L - a_R$ is zero.

Differences between CD spectra and absorption spectra -

i) Extinction coefficients are always positive, but Δa can assume +ve or –ve sign based on the values of a_L and a_R. Thus, optical isomers of any given compound at a given wavelength will give rise to CD spectra of opposite signs,

a_d isomer $= -a_i$ isomer

ii) CD spectra and the absorption spectra differ in the relative size of 'a' and the average coefficient, $(a_L + a_R)/2$. Δa for most of the molecules of biological interest are less than 1% of $(a_L + a_R)/2$.

To arrive at relationship between absorbance and transmission, A&T,

$A = -\log T$, then

$$\Delta a = (-\log T_L + \log T_R)/Cb$$

$$= \frac{1}{C} b \log \frac{T_R}{T_L} = \frac{1}{Cb}; \log \frac{I_R / I_0}{I_L / I_0}$$

$$= \frac{1}{Cb} \log \frac{I_R}{I_L}$$

Where I_R & I_L are intensities transmitted. The electrical vector for CD is also expressed in terms of molecular ellipticity (θ).

The units of θ are degree centimeter squared per decimole $(deg-cm^2/dmole)$. Those of a Δa are centimeter squared per millimole $(cm^2/mmole)$.

Relationship between them is-

$$[\theta] = (3300 deg\ mmole/dmole)\Delta a.$$

CD Spectra:- The spectra are represented with wavelength versus extinction coefficient.

Applications:-

1. To determine optical activity of molecules.
2. To deduce conformation of macromolecules.
3. To study conformational changes which resulting from binding of enzyme to substrate or to Ligand binding studies.
4. To study position of substitutes as in carbohydrates for α or β – glycan derivatives.
5. Forms of DNA and base stacking of nucleic acids are studied using circular Dichroism.

Instrumentation:- These operate in the wavelength range of 185–800 mm. Two light sources may be used to get L and R circular polarized light, while usage of molecular simplifies the equipment (figure 3.17).

The light source can be tungsten filament lamp that has polarizers next to them. The light is then passed through crystal which is subjected to alternating electric field. The crystal is called as electric modulator or a photoelectric modulator and results in L and R components to be transmitted alternately. The output from sample is subjected to photomultiplier which amplifies signals. The resulting electronic signals are processed to get final result.

RAMAN SPECTROSCOPY

Introduction: When incident light is scattered by intervening sample molecules, the frequency of light scattered is same as that of incident light. Such oscillations are said to be elastic. However, during scattering of monochromatic light by molecules, a small fraction of the scattered light is observed to have a different frequency from that of the incident photon. This phenomenon is known as the **Raman Effect.** This was discovered in 1928 by Sir C.V Raman. This has become an indispensible tool for the elucidation of molecular structure. It has been of immense use in locating various functional groups or chemical bonds in molecules and also for quantitative analysis of complex molecules.

Only those vibrations which cause a change in dipole moment, i.e., change displacements are observed in the IR region. These vibrations are which do not cause a change in dipole moment and are consequently inactive in the infrared region, are observed in Raman Spectra. Thus, although the origin of Raman and Infrared spectra are quite different, the two techniques provide complementary information about frequencies of molecular vibrations and therefore about the structure and conformation of a molecule.

Principle:- Rayleigh scattering results when the sample molecules scatter light which is of the same frequency as that of the incident photon collisions between molecules can lead to

energy changes that are in very small proportions (of less than 10^{-6}) and well before scattering takes place collision of these molecules with photon and among themselves does not lift the molecules to any quantized level, rather they are thought to be virtually excited (figure 3.18). While the excited molecules during emission of absorbed energy do not come down to their natural ground state, the emitted radiation has a frequency different from that of the incident one. All these patterns are a part of Raman spectrum.

Several such shifted lines, known as **Stokes lines** are observed in the Raman Spectrum and each corresponds to a different vibration in the molecule. The Raman spectrum thus provides detailed information about the vibrational spectrum of the molecule. These lines are known as **anti-strokes lines**. These arise when some molecules absorb incident radiation when they are in lower state.

Since most of the molecules indulge in **Rayleigh scattering**, radiation due to it is considerably more intense than the radiation due to Raman shifted components. The primary function of Raman spectrometer is to reject totally radiations due to Rayleigh Scattering and to detect the weak Raman - shifted components. The Raman spectrometer has an intense monochromatic light source, sensitive detector and good light gathering power.

MASS SPECTROMETRY (MS)

This is an analytical technique in which the molecules in the test sample are converted to gaseous and which are subsequently separated in a mass spectrometer according to their mass to charge ratio (m/z) and detected. The exact principle involved addition or loss of protons by biomolecules, that will result in variation of mass by 1 Da for each proton. The mass spectrum allows an accurate measure to be made of the relative molecular mass M_Z.

Essential features of mass spectrometers include

– Production of ions in the gas phase

– Acceleration of the ions to a specific velocity in an electric field.

– Separation of the ions in a mass analyzer

– Detection of each species of a particular m/z ratio.

The instruments are calibrated with standard components of known values. Carbon scale is used in MS, as it has a mass of $^{12}C = 12.000$.

The equipment separates ions by use of either a magnetic or electric field. Alternatively the time taken for ions of different masses to travel a given distance in space is measured accurately in the time of flight (TOP) by mass spectrometer.

Components of Mass spectrometer

It consists of the following components (figure3.19)

1. A high vacuum system $(10^{-6}\text{tonn or})\mu\text{ torr})$:

These include turbo molecular pumps, diffusion pumps and rotary vane pumps.

2. A sample inlet: This comprises a sample or a target plate; a high performance liquid chromatography (HPLC), gas chromatography (GC) or capillary electrophoresis system; Solid probe; Electron impact or direct chemical ionization chamber.

3. An ion source (to convert molecules into gas phase ions): This can be MALDI, ESI, fast atom bombardment (FAB), electron impact or direct chemical ionization.

4. A mass filter/analyper; This can be TOF; Quardrupole – ion trap; magnetic sector or ion cyclotron Fourier transform.

5. A detector : This can be a conversion dynode; electron multiplier; micro channel plate or array detector.

1. **Vacuum system:** Mass analyzers operate under vacuum to minimize collisions between ions and air molecules. Rotary vane pump and turbomolecular pumps are used. The later provides a working high vacuum.

2. **Ionization:** Ions are produced from neutral molecules by removing an electron to produce a positively charged cation or by adding an electron to form an anion. Methods used for ionization include:

a) Electron Input ionization (EI):

Widely used for analyzing of metabolic, pollutants and pharmaceutical compounds.

In this, a stream of electrons from a heated metal filament is accelerated to 70eV potential. Sample ionization occurs when the analyte vapours are allowed to diffuse into the chamber of electron stream (figure 3.20). This can lead to loss of electrons by the molecule due to loss of electrons by the molecule due to electron bombardment or gain of electron giving rise to cations (M^+) or anions (M^-). These daughter ions are recorded as the mass spectrum.

B. Chemical ionization:- Reagents like methane and ammonia are used for initial ionization of molecules. The high gas pressure in the source results in ion-molecule reactions between reagent gas ions (such as NH_3^+ and CH_4^+) some of which react with analyte to produce ions.

C. Fast atom bombardment:- In this method the sample is mixed with a relatively involatile, viscous matrix such as glycerol, thioglycerol or m-nitrobenzyl alcohol. The mixture is placed on a probe, that is introduced into the source housing and bombarded with an ionizing beam of neutral atoms (Ar, He, Xe) of high velocity.

Later development involved usage of a beam of Caesium ions (Cs+) was introduced. Bombardment will result in ion species which are either protonated or deprotonated $\left((M+H)^+ \& \left(M-H^-\right)\right)$. These pseudomolecular ion species formed are recorded in mass spectrum.

d) Electronspray ionization (ES): This is a soft ionization technique and enables analysis of large intact biomolecules, such as proteins and DNA. The electrospray (ES) creates very small droplets of solvent containing analyte. The charged liquid droplets are produced by atomization on or nebulisation. In ESI strong electric field is used and as solvent with analyte is subjected to this, the solvent evaporates in the high vacuum region. As the droplet moves, its size decreases finally leaving charged analytes (figure 3.21). The solvents that are used include- 50: 50 of acetonitrile and water, 50:50 of methanol and water; 1% aceticacid or 0.1% formic acid. Ammonium hydroxide, Trifluoracetic acid can also be used.

3. **Mass analyses:** These are involved in separation of ions based on mass to charge ratio. These include

a) Quadropole mass spectrometry:-

This analyser has four parallel cylindrical rods which are applied with DC voltage As ions move down the rods which are applied with varying voltage, the ions are accelerated down based on m/z ratio.

b) Ion trap mass spectrometry:-

This involves usage of Ions traps, that store in them ions of selected m/z ratio. To perform tandem MS a collision gas is introduced that releases fragment ions inturn to generate fragment spectrum.

c) Nanospray and on-line tandem mass spectrometry:

In this nanospray is generated using fine needles that dispose the sample accurately at the entrance of source. At low flow rates and usage of stream splitters device allows good separation of ions.

j) Magnetic sector analysis (figure 3.22)

The electric sector acts as a kinetic energy filter and allows only ions of a particular kinetic energy to pass, irrespective of m/z ratio, increasing resolution.

Ions emerging out of ESA have same velocity. As they enter magnetic sector analyzer, which is a curved trajectory path, depending on m/z ratio and velocity of ions they reach the detection. At a particular point of time, one set of ions reach the detector.

e) Plasma Desorption ionization:-

This is first one of a kind of Mass spectrometer, that can be used to analyzer proteins and large biomolecules. In this, source of plasma, is ratio active californium, ^{252}Cf that emits nuclei Ba^{20+} and Tc^{18+}. These are ejected in opposite direction, almost collinearly and with equal velocity. These plasma particles emitted in direction opposite to that passing through the sample triggers a time counter, and the desorbed sample ions are accelerated electrically and detected by a TOF analyser.

f) MALDI, TOF Mass spectrometry:-

Matrix - assisted laser desorption ionization (MALDI) produces gas phase protonated ions by excitation of sample molecules from the energy of a laser transferred via an ultra violet (UV) light absorbing matrix. The matrix is a conjugated organic compound that is mixed with compound and dried onto the target plate, where sample and matrix co-crystallize upon drying. Pulses of laser light of few nanoseconds causes drying of rapid excitation and vaporization of crystalline matrix. This later causes ejection of matrix and analyte ions hat are analyzed by TOF analyser (figure 3.23).

Many other modifications of MALDI have come up for better resolution of analyte.

4. Detectors-

The ions from mass analyzer impinges on a surface of a detector where the charge is neutralized wither by collection or donation of electrons. As electron current flows, it is amplified and ultimately converted into a signal that is processed by a computer. The Total Ion Current (TIC) is the sum of the current carried by all the ions being detected at any given moment.

Electron multiplier and conversion dynodes are used in the detectors. The ion beam from the mass analyzer is focused the onto the conversion dynode, which emits electrons in direct proportion to the number of bombarding ions. A positive or a negative ion hitting conversion dynode causes emission of secondary particles which contain secondary ions, electrons and neutral particles (figure 3.24). These secondary particles are accelerated into the dynodes of the electron multiplier. They strike the dynodes with sufficient energy to dislodge electrons, which pass further into the electron multipliers, colliding with the dynodes, producing more and more electrons.

PRACTICE QUESTIONS

1. Calculate absorbance of adhesive whose molar extinction coefficient is 13.3×10^{-3} taken in a curette, whose path length is 1cm, prepared at 0.2 m moles with 4 ml volume.
   (A) 0.55 (B) 0.665 (C) 63.55 (D) 6.65

2. The groups which do not impart color by themselves, but their presence bring about a shift of absorption band toward red end of spectrum are called _____.
   (A) Chromophores (B) Fluorophores (C) Auxophores (D) Auxochromes

3. Which among the following are wave length selectors ?
   (A) Filters (B) Gratings (C) Monochromators (D) All the above

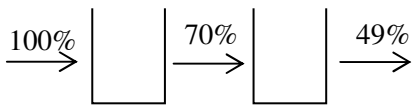
4. The standard reference used to compare the position on of n. m. r absorption arising out of shielding and deshielding is _____.
   (A) Hydrogen (B) Tetramethyl Silane
   (C) Acetylenic phenol (D) Methanol

5. Which among the following statements in true ?
   (A) IR spectrum of no two compounds is alike .
   (B) The numbers of modes of vibrations for a molecules are 3n-6.
   (C) In IR spectroscopy, it comprises of 2 types vibrations
   (D) All of the above

6. Arrange the following transitions in their decreasing order of energies
   (a) $\sigma \rightarrow \sigma^*$ (b) $n \rightarrow \pi^*$ (c) $n \rightarrow \sigma^*$ (d) $\pi \rightarrow \pi^*$

   (A) C>D>A>B (B) D>A>C>B (C) A>C>B>D (D) A>B>C>D

7. Find the intensity light absorption by the absorption medium shown in the following figure.



   (A) 50% (B) 30% (C) 70% (D) 21%

8. The above Phenomenon is explained by

(A) Beer's law
(B) Bauger's law
(C) Lambert's law
(D) Bouger- lambert law

9. Which among the following statement is false ?

(A) Scattered light of different frequency is important in Raman Spectroscopy

(B) Fluorescence microscopy works on the basis of emission spectra

(C) IR spectroscopy results in spectrum that is a finger print to the molecules having similar kinds of groups.

(D) Hypsochromic shift is due to removal of conjugation and a change in the polarity of the solvent due to which the absorption maximum is shifted towards shorter wavelength

10. The differential absorbance of the circularly polarized light resulting from a different in absorption of the two rays is known as _____.

(A) Circular dichrosm
(B) Diffraction
(C) Birefringence
(D) Fluorescence

11. What is the full of MALDI ?

(A) Membrane Associated ligand desorption and Ionization

(B) Matrix- Assisted laser desorption/Ionization

(C) Matrix- Associated high and desorption and Ionization

(D) Molecular – Associated ligand dispersion after Ionization.

ANSWER KEYS

1	B	**2**	D	**3**	D	**4**	B	**5**	D	**6**	C	**7**	B
8	D	**9**	C	**10**	A	11	B						

EXPLANATIONS

1. $A = ambc$
$= 13.3 \times 1 \times 0.05$
$= 0.0665$
The OD of solution is 0.665

2. Auxochromes are groups which by themselves do not act as chromophores but whose presence bring about a shift of the absorption band towards the red end for spectrum.

3. Wave length selectors help to obtain desired wavelength of light. This is by either using filters or monochromators. Gratings are dispensing devices that help to obtain single wave length of light.

4. Tetramenthyl silane (TMS) – $(CH_3)_4Si$, has 12 protons which are chemically equivalent and result in a single sharp peak in n .m . r

   Hence, it is used as reference to represent chemical shifts.

5. All the statements are true regarding IR spectroscopy

   (A) IR spectrum of no two compounds is alike .

   (B) The numbers of modes of vibrations for a molecules are 3n-6.

   (C) IR spectroscopy, it comprises of 2 types vibrations

6. The energy associated with transitions is as follows:

   $\sigma \rightarrow \sigma^* > n \rightarrow \sigma^* > \pi \rightarrow \pi^* > n \rightarrow \pi^*$

7. $I_o = 100$
$I_1 = 70$
$I_o = I_1, = 30 = 30\%$
$= \frac{21}{70} \times 100 = 30\%$

   Hence, the absorption medium has absorbed 30% of the incident beam of light.

8. Banger-Lambert law states that, the amount of light absorbed is proportional to the thickness of the absorbing material and is independent of intensity of incident light.

9. IR spectroscopy result in spectrum which is a finger print to the molecular.

10. The differential absorbance of the circularly polarized light resulting from a different in absorption of the two rays is known as Circular dichroism.

11. MALDI is Matrix assisted Laser Desorption/Ionization : It has the ability to non destructively vaporize and Ionize bimolecules both big and small molecules

5. Chromatography

The word chromatography means "color writing" which is a way that a chemist can test liquid mixtures. While studying the coloring materials in plant life, a Russian botanist invented chromatography in 1903. His name was M.S. Tswett. Chromatography is such an important technique that two nobel prizes have been awarded to chromatographers. Over 60% of chemical analysis worldwide is currently done with chromatography or a variation thereon.

Principles of Chromatography:

Distribution coefficient (Kd):

The Russian Botanist Mikhail Tswett has reported a form of chromatography that he used to separate plant pigments using Calcium carbonate (1903). Modern chromatographic techniques take multiple forms, most of which are automated and adapted to deal with large or very small amounts of substances to be separated and purified.

The basis of all forms of chromatography is the distribution or partition coefficient (Kd), which describes the way in which a compound distributes between two immiscible phases A and B at constant temperature. Now the partition coefficient is expressed as

Concentration in Phase A= Kd

Concentration in Phase B

The term effective distribution coefficient is defines as the total amount, as distinct from the concentration of analyte present in one phase, divided by the total amount present in the other phase.

All chromatographic techniques consists of stationary phase, which may be solid, gel,liquid or a solid/liquid mixture that is immobilized. The mobile phase may be a liquid or a gaseous substance which is passed over stationary phase or through it. Based on distribution coefficient of the analyte, the sample gets separated.

Modes of chromatography:

Chromatographic separations can be carried out in 2 modes.

Column Chromatography : In this the stationary phase is packed into glass or metal column. The mobile phase (eluent) , is passed through the column by gravity feeding or by using peristaltic pump. As eluent flows, components of analyte get separated based upon their partition coefficient.

Thin Layer Chromatography: The stationary phase is coated as thin layer onto a glass, plastic or metal foil plate. The mixture of analytes is applied as a spot or as band near the edge of the coated plate. It migrates by capillary action and during this process, components of analyte migrate to

opposite ends. It finds extensive applications in the field of peptide mapping and natural product research.

Principles of Column chromatography:

Column chromatography is one of the most useful methods for the separation and purification of both solids and liquids when carrying out small-scale experiments. The separation can be liquid/solid (adsorption) or liquid/liquid (partition) in column chromatography. The stationary phase, a solid adsorbent, is usually placed in a vertical glass column and the mobile phase, is added from the top and let flow down through the column by either gravity or external pressure.

Column chromatography is advantageous over most other chromatographic techniques because it can be used in both *analytical* and *preparative* applications. It can be used to determine the number of components of a mixture and as well as the separation and purification of those components.

Column chromatography isolates desired compounds from a mixture in such a way that the mixture is applied from the top of the column. The columns are usually glass or plastic with sinter frits to hold the packing. The liquid solvent (eluent) is passed through the column by gravity or by the application of air pressure. The eluent, instead of rising by capillary action up a thin layer, flows down through the column filled with the adsorbent. Equilibrium is established between the solute adsorbed on the adsorbent and the eluting solvent flowing down through the column. Stationary phases are almost always adsorbents. Adsorbent is a substance that causes passing molecules or ions to adhere to the surface of its particles. The mobile phase is a solvent that flows past the stationary phase, dissolving the molecules of the compounds to be separated some of the time.

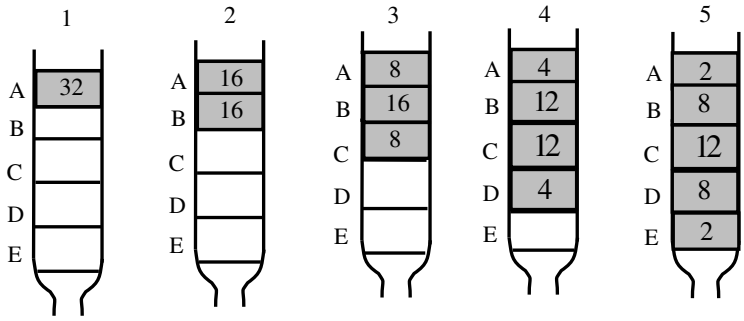
Because the different components in the mixture have different interactions with the stationary and mobile phases, they will be carried along with the mobile phase to varying degrees and a separation will be achieved. The individual components, or elutants, are collected as solvent drips from the bottom of the column.

Many compounds are not visible to the eye when dissolved in a solvent or adsorbed on a adsorbent. Visualization processes make these substances visible. The used techniques for this purpose include UV lights that cause fluorescence or phosphorescence and chemical reactions that give colored compounds.

The principle of column chromatography may beunderstood by illustrating an example of a column packed with solid granular stationary phase and the mobilephase is liquid. Say the sationary phase if packed with material upto 5cm and the mobilephase volume per cm of columnis 1ml. Assuming an analyteof ceoncentration32micrograms tobe added to thecolumn, and allowed it to diffuse for 1cm of stationaryphase. İn the column if the 5cm area is divided into zones A to E, zone A has sample. Nowif 1ml of mobile phase is added and assuming the parition coefficient of sample to be 1, the as the mobile phase moves, the solute gets distributed between stationary phase and mobile phase. So in the zones A and B if concetrations were observed it will be 16 and 16microlitres. İf one more ml of mobile phase is added, the sequence of events re represented in third column of figure 5.1 C. It is apparent that after relatively small number of equilibrations, the analyte distributes itself symmetrically within a band. İn the same way if two analytes are added, of

which one is having Kd value of 1 and the other 100. This indictes that, the analyte having Kd value of 100 get easily separated from the other and both analytes can be obtained as distinct bands.

Figure 5.1: Principle of Column Chromatography Separation



Basics of column Chromatography:

A typical column chromatography having a gas or liquid as mobile phase has following components:

Stationary phase: It is chosen based on analyte under study

A column: This my be conventional type or may be filled with matrix, or the matrix may be coated on the wall of the column.

A mobile phase and delivery system: This is chosen to complement the stationary phaase. This has to helpto seperate the analyte and must be delivered at constant flow rate into the column.

An injector system: To deliver test sample on the top of column in a reproducible manner

A detector and chart recorder: to give continuous record of the presence of analytes in theeluate as it emergesformthecolumn. Detection is generally based on physical parameters such as Visible or UV abosprtion or fluorescence. Peak in the recorded chart indicates presence of analyte.

A fraction collector: For Collecting the analyte for further biochemical studies.

Selection of Stationry phase: Selection of correct stationry phase and mobile phase dictates the extent of successull sepertion of analytes. This maybe achieved by setting up one of the following equilibria.

- Adsorption equilibrium
- Partition equilibrium
- İon – Exchange equilibrium
- Exclusion equilibrium
- Binding equilibrium
-

Chromtographic performance parameters:

1) Retention time and elution volume:

A chromtograph is the pictorial record of the detector response as a function of elution volume or retention time. The retention time t_R for each analyte has two components. First is the time it takes the analyte molecule to pass through the free spaces between the particles of the matrix coated with the stationary phase. Here it is called as the dead time, t_M. The volume of free space is referred to as the Void volume V_o. So, here t_M is time taken by analyte to pass through the

Void volume. The second component is the time the stationary phase retains the analyte and is referred to as the adjusted retention time t^{1}_{R}. This is characteristic of an analyte and is the difference between observed retention time and dead time.

$T^{1}_{R} = t_R - t_M$

2) Capacity factor (Retention factor):-

This is one of the most important parameter in chromatography, represented as K'. It is the additional time that the analyte takes to elute from the column relative to an unretained or excluded analyte that does not interact with the stationary phase and which, by definition has a K' value of O. Thus

$$K' = \frac{t_R - t_M}{t_M} = \frac{t'_R}{t_M}$$

If an analyte spends equal time in stationary phase as well as in mobile phase the K' is equal to 1. K' is therefore also related to distribution coefficient which is defined as the relative concentration of analyte between two phases.

The above equation may be rewritten as,

$$K' = \frac{t'_R}{t_M} = \frac{M_S}{M_M} = K_d \text{ x } \frac{V_S}{V_M}$$

Where,

M_S = mass of analyte in stationary phase

M_M = mass of analyte in mobile phase

V_S = volume of stationary phase

V_M = volume of mobile phase

The ratio $\frac{V_S}{V_M}$ is referred to as the volumetric phase ratio, β. Hence,

$$K' = K_d \beta$$

3) Plate height and Resolution:

Chromatography columns are considered to consist of a number of adjacent zones in each of which there is sufficient space for the analyte to completely equilibrate between the two phases. Each zone is called a **theoretical plate.** The length of the column containing one theoretical plate is referred to as **plate height**, **H**, which has its limits of length in micrometers. The numerical value of both N and H for particular column is expressed with reference to a particular analyte. Plate height is simply related to the width of the analyte peak and is expressed in terms of its **standard deviation** σ**,** and the distance travelled within the column, x , specifically

$$H = \frac{\sigma^2}{x}$$

For symmetrical Gaussian peaks, the base width is equal to 4σ and the peak width at the point of inflection, ω_i is equal to 2σ. Hence the value of H can be calculated from the chromatography by measuring the peak width. The number of theoretical plates in the whole column of length L is equal to L divided by the plate height.

$$N = \frac{L}{H} = \frac{L_x}{\sigma^2}$$

The value of N, which has no units, can be as high as 50,000 to 1 lakh per meter, for efficient columns and the corresponding values of H can be as little as few micrometers. The smaller the plate height, the narrower is the analyte peak.

4) Peak broadening: A number of factors oppose the formation of a narrow analytical peak thereby increasing plate height. Few of these include:

-Simultaneous application & migration by sample

-Diffusion of analyte

-The rate flow of mobile phase has to be is such a way that it prevents the diffusion of analyte.

5) Resolution : The success of a chromatography separation is judged by the ability of the system to resolve one analyte peak from another. Resolution (R_o) is defined as the ratio of the difference in retention time (Δt_R) between the two peaks $(t_{RA} \& t_{RB})$ to the mean $(\omega_{a\Delta})$ of their base widths $(\omega_A \text{ and } \omega_B)$:

$$R_S = \frac{\Delta t_g}{\omega_{av}} = 2\frac{(t_{RA} - t_{RB})}{\omega_A + \omega_B}$$

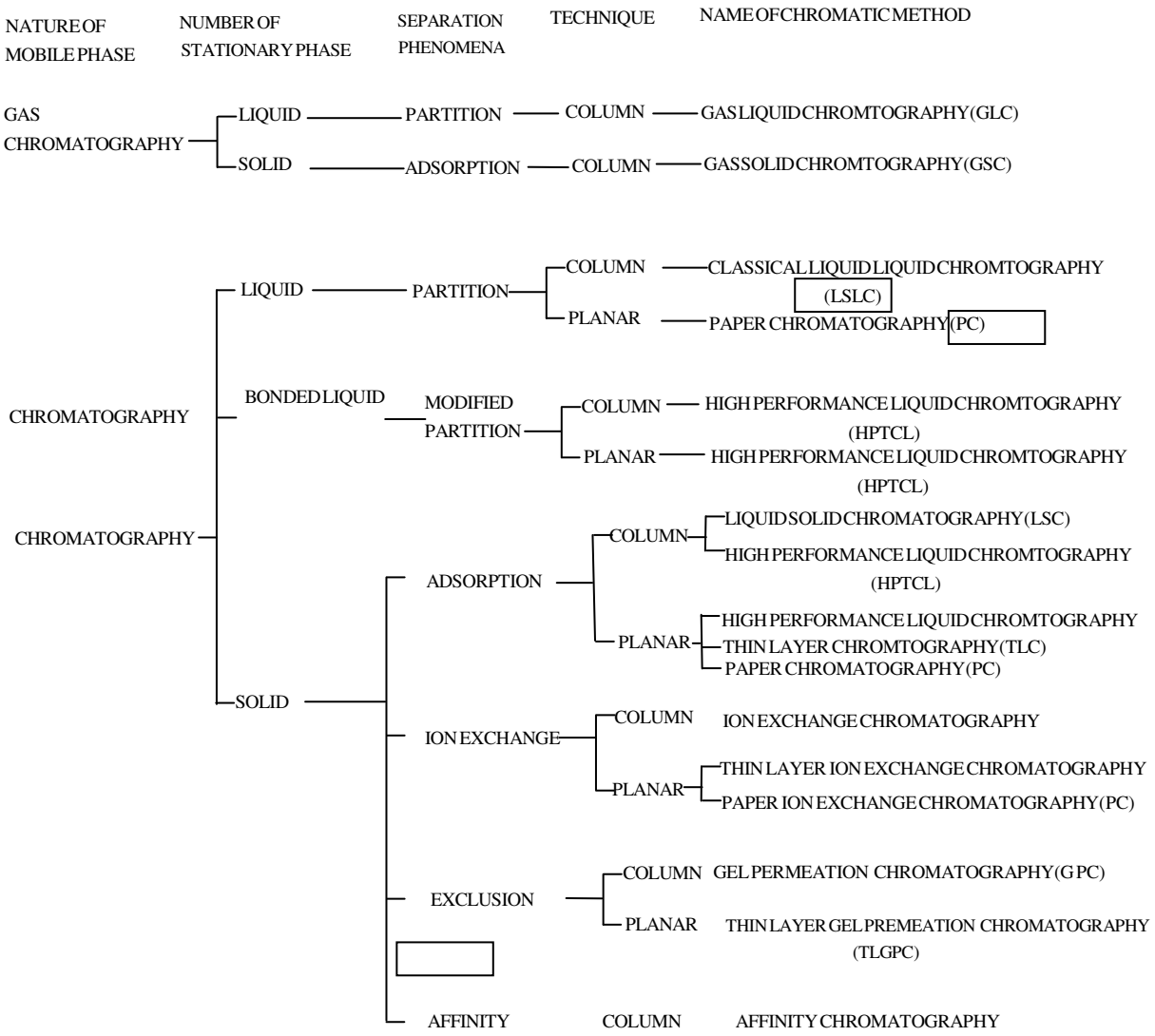
If R_s = 1.0, the separation of two peaks is 97.7% complete.

Unresolved peaks are referred to as fused peaks and Resolution is influenced by column efficiency.

Techniques of chromatography:-

Based upon the choice of stationary phase and mobile phase, based on principle involved and the technique adopted (planer/column) several chromatography techniques have evolved (figure 5.2)

Figure 5.2: Classification of Chromatographic systems



PAPER CHROMATOGRAPHY

Nature of paper: The paper used in this planer chromatography is made up of highly purified cellulose that is washed with 0.1N HCl to get rid of impurities. Cellulose fibres in the paper hold moisture tightly through formation of hydrogen bonds. The cellulose takes up a light negative charge in association with water and thereby exhibits weak ion exchange and adsorptive properties.

Apparatus and paper development:-The apparatus required for paper chromatography comprises of a support for paper, a solvent trough and a air tight chamber (figure 5.3) .

The paper of specific dimensions is taken and a base line is drawn towards its one end on to which the samples are applied. Sample volume is very low and is applied as a spot on the base line using a metal loop or a capillary tube or micropipette tip. This is hen placed in the chamber, keeping the base line above the level of solvent. As the solvent migrates through the capillaries of the paper, based on the partition coefficient/adsorption properties, the analyte mixture gets resolved.

There are two main techniques of paper chromatography – ascending or descending chromatographic techniques. As indicated in the figure, the presence of solvent chamber in the closed jar varies. In ascending chromatography, the glass jar has the solvent, while in descending chromatography a solvent trough is present below lid, that holds the paper hanging down into the jar. In ascending chromatography as the solvent with analyte moves on the paper, it moves against gravity, i.e., the solute experiences both capillary force and an opposing gravitational force. In the descending chromatography both forces act in the same direction. Hence resolution is better in ascending chromatography. The advantage with descending chromatography is that it takes less time.

Radial development: This is another way of sample development in paper chromatography. The paper is cut into circular shape, sample is applied as a spot at the centre of the disc. This is connected to the solvent system using a paper wick. The sample components move outwards, radially and form concentric circles of increasing diameters. The resolution is much sharper in this chromatography.

Two dimensional chromatography: In this technique, sequential development is done in two directions using two different solvent systems (figure 5.4). The sample is applied as a spot at one end of the paper along the base line and is subjected to either ascending or descending chromatography. Ones the solvent has reached 3/4 height of paper, it is removed, dried and placed in another solvent by tilting it by 90^{o}. Since the two solvents used have different eluting properties, the distribution coefficients of sample component differ, resulting in better separation. This technique is used for separation of complex mixtures.

Figure 5.3 Methods of Paper Chromatography

A) Descending B)Ascending

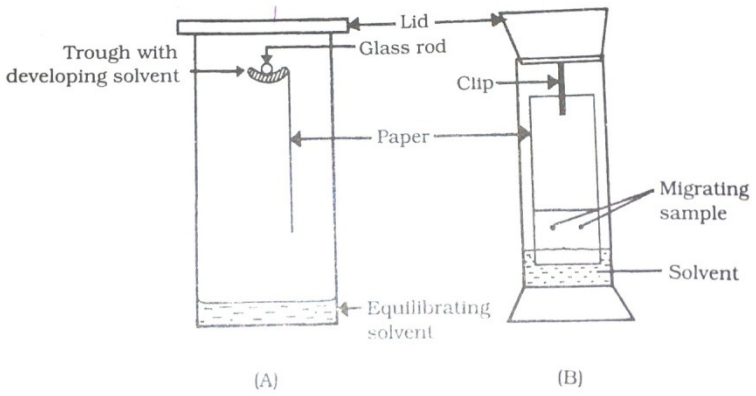
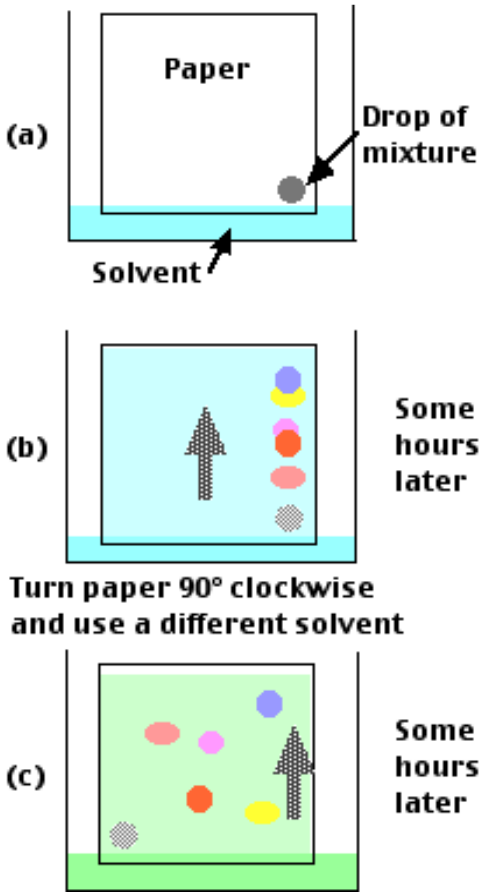


Figure 5.4: Two dimensional Chromtography



Choice of solvent system:

In paper chromatography, water acts as the stationary phase as it gets absorbed by the cellulose paper. The mobile phase is usually a mixture of alcohols, acids, esters, ketones, phenols, amines, hydrocarbons, etc. Ideally the solvents must be immiscible and allows better resolution of the sample mixture.

The solvent system must offer a k_d value between the range 1-100. It must be removable from paper, by simple methods, and the solvent must be stable, i.e. it must not undergo any reaction with sample.

Few solvent systems used in paper chromatography are as follows:

Solvent System	Ratio	Compound
Butan-1-ol/acetic acid/water	40:10:50	Amino acids
Butan-1-ol/pyridine /water	33:33:33	Amino acids
Mathanol/pyridine/water	25:12:63	Amino acids
Butan-1-	50:28:22	Mono & disaccharides

ol/pyridine/water		
Propan-1-ol/petroleum ether	4:96	Plant pigments
Chloroform/petroleum ether	30:70	Plant pigments

Detection :- Various methods of detection are available for detecting sample components. As the molecules are colorless, the paper is sprayed with a suitable color developing agent.
Eg:- Ninhydrin is used for amino acids and it results in purple spots. Other methods like fluorescence, radio labeling can also be done to visualize the samples.

Retardation factor or Relative flow (R_f):-

For a compound, the R_f value is constant. It is determined by measuring the distance migrated by solvent (solvent front) and the distance migrated by solute from the base line. It is represented as

$$R_f = \frac{\text{the distance moved by the solute}}{\text{distance moved by solvent front}}$$

Applications:- Paper chromatography has revolutionized the field of Biochemistry. It is used to check the purity of pharmaceuticals, for the detection of adulterants and contaminants in foods and drinks, to study fruit ripening, in fermentations, detection of drugs and dopes, analysis of cosmetics, monitoring biochemical reactions, etc.

THIN LAYER CHROMATOGRAPHY

In this technique, a thin layer of a finely divided substance is deposited on a flat glass plate/metal plate/plastic sheet. Thin layer chromatography may be either carried out by the adsorption principle (when kieselghur or alumina are used) or by the partition principle (when silica gel which can hold water is used).

Preparation of the layer:
The glass plate on which thin layer is to be prepared is washed and dried the material for preparation of thin layer is made into slurry (thick suspension). This is applied on glass plate using an adjustable spreads, using which desired thickness can be maintained. For analytical separations, the thickness of the layer is kept at 0.25mm, while for preparative purposes, thickness of around 5mm is maintained. Although thin layer technique can be used for many different types of chromatographic separations such as adsorption, gel filtration, ion exchange etc, adsorption remain the most commonly used one.
To keep the stationary phase attached to the plate, a binding material like $CaSO_4$ is mixed with slurry. Once the layer is applied, the plates are kept in oven at 110° C for activation.

Sample application:- This is same as paper chromatography. Rather a grid plate is provided to properly place the samples. During sample application care must be taken not to scrape the thin layer.

Plate Development: The choice of a solvent system depends on the compound and the principle used. Few solvent systems used are mentioned in table below.

Table- certain solvent system and absorbents used in Thin layer chromatography:

Compounds	Adsorbents	Solvent system
Mono & disaccharides	Kiesulguhr G (Na. acetate)	Ethylacetate / Propan-1-ol (65:35)
	Kiesulguhr G (Na.phosphate, pH 5)	Butanol-1-ol/ acetone/Phosphate buffer pH 5 (40:50:10)
Triglycerides	Silico Gel G	petroleum ether/diethyl ether /acetone (90:10:1)
Phospholipids	Silico Gel G	Chloroform/methanol/water (65:25:4)
Cholestrol esters	Silico Gel G	Carbon tetrachloride/ chloroform (95:5)
Amino acids	Silico Gel G	90% ethanol/water- (70:30) Button-1-d/acetic acid/water (80:20:20)
Plants pigments	Kiesulguhr G	Petroleum ether/prop-1-ol (99:1)

Before conducting the chromatography, suitable solvent is added to the TLC chamber and it is saturated with solvent vapour. TLC allows Ascending chromatography and Two dimensional chromatography. The greatest advantage of TLC is the speed at which separation is achieved (10 to 30 minutes).

Detection:- Most of them are same as paper chromatography.

Others specific to TLC include:

-Spraying the plate with 25 to 50% sulphuric acid in ethanol and heating. This results in charring of most of the compounds, which show up as brown spots.

-Iodine vapours is used extensively as a universal reagent for organic compounds. The iodine spots disappear rapidly but can be made permanent by spraying 0.5% benzidine solution in absolute ethanol. Iodine vapour forms a concentrated cloud over the region of the component.

Quantification can be done using densitometers. The other method is to scrap the material having coloured spots, elute into suitable solvent and then optical density is taken of the centrifugal sample using ultra violet or visible spectrophotometry.

Advantages and Applications:-

TLC is a versatile, faster and easily adaptable technique. It is used to identify drugs, contaminants and adultrants. It is also used to resolve plant extracts and many other biochemical preparations.

COLUMN CHROMATOGRAPHY

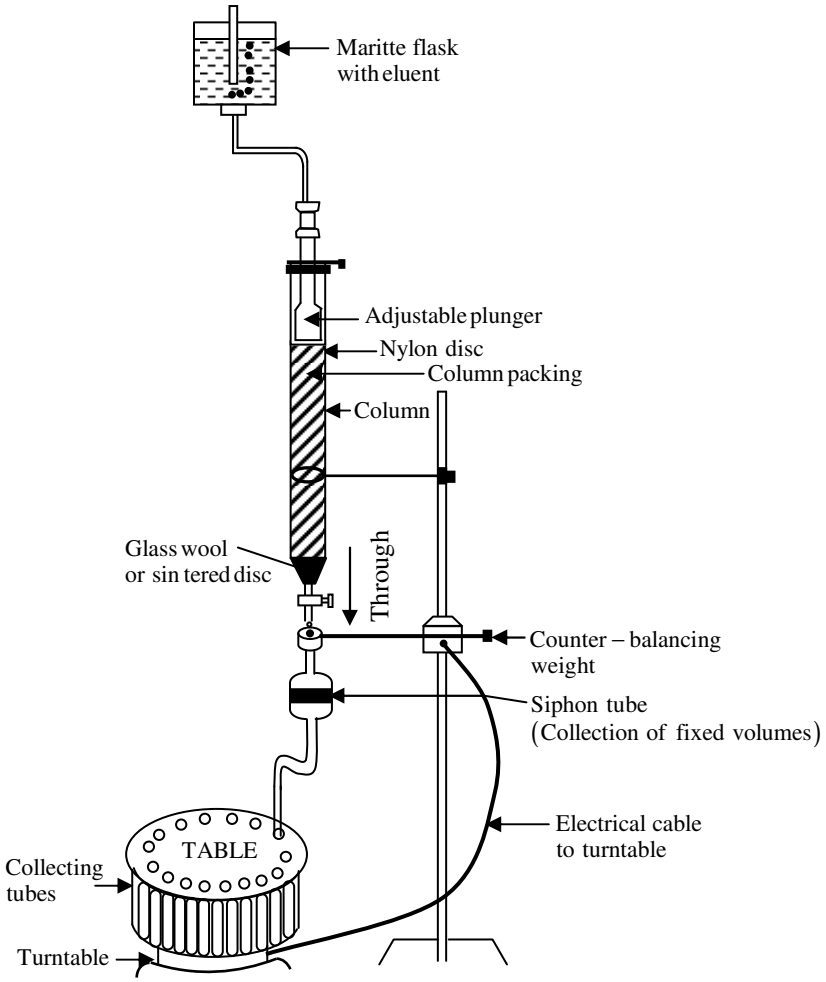
The features and general procedures involved in column chromatography are:

Columns:- They are made up of glass or polyacrylate plate. Different columns differ in their dimensions. Laboratory columns usually have a diameter of 2-70mm and a length of 15-150cm. The ratio of diameter to length ratio varies from 1:10 to 1:100.

Glass columns are generally used and have a sintered glass disc at the bottom to support the stationary phase, while some columns have a replaceable support like nylon mesh. A cheaper alternate is to use a plug of glass wool with a small amount of quartz sand and glass beads.

Temperature fluctuations may be harmful for certain chromatographic separations. These columns are provided with a thermostat jacket. Liquid from a thermostatically controlled bath set at the required working temperature is circulated through the jacket continuously for the length of the experiment to maintain the column interior at a constant temperature. The columns are provided with an inlet and an outlet. The inlet, which may be simple or fitted with a ground glass adaptor that allows eluting solvent to enter in the column without disturbing its surface. The effluent from the column is collected into test tubes continuously by using the collecting system (figure. 5.5)

Figure 5.5: Equipment for column chromatography with fraction collector



Packing the column :- This is very important in achieving satisfactory separations. The column is fitted in upright position and its bottom is sealed with glass wool or a similar support. The column is now filled to about one-third of its height with the mobile phase. A thick suspension called slurry, of the degassed stationary phase (gel, adsorbent or resin) is gently poured into the column with its outlet closed. The upper part of column is stirred all the while to ensure even packing and to avoid air bubbles. The slurry is added till $3/4^{th}$ of column is full. The outlet is now opened and the column is stabilized, by washing it with mobile phase. A filter paper disc or nylon gauze is placed on the surface of the column to prevent disturbance during addition of mobile phase or the sample.

Introduction of sample:-

The top layer of solvent is slowly removed until a thin layer of it is retained on the surface of the packing material. The sample is then slowly added and allowed to start moving into column. As this process is on, solvent is slowly released from the buffer reservoir, making sure that the sample initially moves in the column and does not diffuse back into the solvent. The sample may also be mixed with sucrose or ficoll such that it remains next to the surface of packed material. Later the solvent is added in the column and is maintained to a height of 5-10cm. A tracking dye bromophenol blue can be added along with sample to monitor the progress of sample separation.

Techniques of elution:- There are two main techniques of elution.

i) Isocratic elution and ii) Gradient elution.

When a single solvent is used as an eluant during development, the process is known as isocratic separation. At times, usage of single solvent may not be sufficient. In this situation pH, ionic strength or polarity of eluant is changed with respect to time. The process where the composition of the mobile phase is changed giving rise to a gradient is known as gradient elution. The partitioning behavior of solute mixture changes leading sharp peaks and better resolution.

Gradient is generated by mixing solvents with varying pH or ionic strength steadily. Say pH 2 is initially allowed into column and the tank having pH -2 buffer is mixed slowly with a much basic buffer. A slow increase in pH will alter partitioning behaviour of solutes.

Flow Rates (F_c):- It is expressed as volume of mobile phase added per unit volume. For a satisfactory resolution, eluant flow is to be maintained at a stable rate, which is done by peristaltic pump (30-120ml/hr cm^2)

Analysis and collecting of Effluent:

The effluent, as it emerges from column outlet is to be analyzed. The properties of compound are used for analysis by ultraviolet absorption, visible or fluorescence spectrophotometry. A wide variety of monitoring equipment are also available to monitor process of migration of sample to analysis fractions. Few examples include fluorescence detectors, polarimeters, voltmeters, refractometric detectors and conductivity detectors.

The electrical signal generated by detector is displayed on the chart paper. The area under the peak is proportional to the amount of compound present. This area under peak may be determined by measuring the height of the peak and its width at half the height.

Fractional collectors enable easy collection of fractions based on the input given, i.e. drop wise collection or volume based or time based collection. Latest devices have detector systems attached with them that allow no time delay in analysis.

GEL PERMEATION CHROMATOGRAPHY

Gel permeation chromatography is a separation method depending upon molecular size. This method is also known as molecular sieve, gel filtration or molecular exclusion chromatography.

The advantages with this method are:

1) Gentleness of technique permitting separation of labile species
2) 100% solute recovery.
3) Excellent reproducibility and.
4) Comparatively short time and relatively in expressive equipment needed for its operation.

Principle :- In gel permeating chromatography, the column is packed with beads or porous glass granules which are equilibrated with a suitable solvent system. If the mixture of molecules of different size is placed on the top of such an equilibrated column the larger molecules pass through the interstitial spaces between the beads this is because the pores of the gel have smaller diameter than what is needed for large molecules to enter. Large molecular therefore move down the column with little resistance. The small molecules however can enter the pores and effectively move down from the stream of eluting solvent (figure 5.6). These molecular are thus retarded. The degree of retardation of a molecule is proportional to the time it spends inside the gel poles ; which is a function of its molecular size and the pore diameter.

The molecular with stokes radio equal to or exceeding pore diameter, do not enter the gel and are said to be excluded. Therefore, exclusion limit of a gel is the molecular weight of the smallest molecule that is incapable of entering the gel pores.

For a given gel, the distribution of a solute particle between the inner and outer solvent (Solvent present around beads) is defined by the term distribution coefficient, K_d, which is a function of its molecular size. $K_d = 0$ when the solute molecules are large and are completely from the inner solvent. If $K_d = 1$, the solute is small enough to penetrate through the inner pores of gel beads and diffuse into inner solvent. The K_d value for intermediary size particles varies from 0 to 1.

The volume of outer solvent i.e, the solvent surrounding the gel beads is indicated as V_o, or the void volume the volume of solvent inside gel, i.e, the inner solvent is indicated by V_i . If distribution coefficient is K_d, them the effective volume V_e is represented as $V_e = V_o + K_d V_i$

Figure 5.7 indicates the terms with respect to solvent. The volume of inner solvent V_i, can be calculated if the dry weight of gel employed and the water regain value of the particular gel is known. Thus,

$$V_i = a\ W\ r$$

Where, a = the dry weight of the gel

Wr = water regain value

The value of K_d is characteristic of a given molecule and does not vary with the geometry of the gel bed. The value V_e of gel depends on the size of the column.

For two substances possessing different molecular weights, and therefore, different distribution coefficients (K_{d1} & K_{d2}). The difference in their effluent volumes Vs is given by

$V_s = V_{ei}, - V_{e2} = (V_o + K_{d1}V_1) – (V_o + K_{d2}V_1)$

Therefore $V_s = (K_{d1} – K_{d2})V_1$

Thus, the sample volume applied for complete separation of two substances should not exceed V_o.

Types of Gels:-

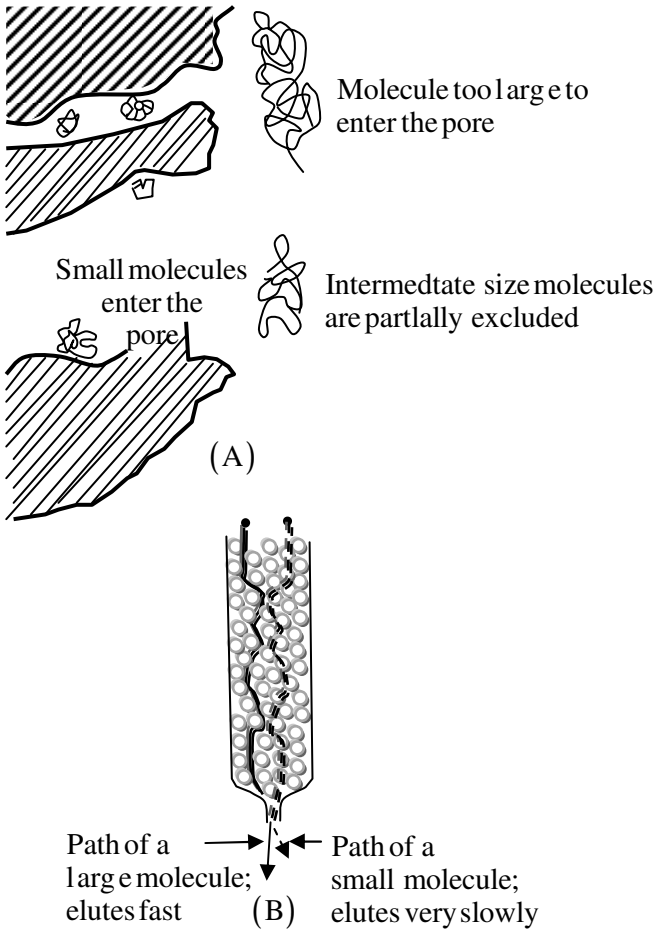
The gel medium should have the following characteristics.

i) The gel material should be chemically inert.
ii) It should preferably contain vanishingly small number of ionic groups.
iii) Gel material should provide a wide choice of pore and particle sizes.
iv) A given gel should have uniform particle and particle sizes.
v) The gel matrix should have mechanical rigidity size.

Few materials used in gel chromatography are as follows:

(a) Sephadex : It is used for separation of proteins and most biomolecules. It is prepared from dextran by cross linking with epichlorohydrin (figure 5.7). Pore size of sephadex is controlled by molecular weight of dextran and the amount of epichlorohydrin used in the preparation. Gels with exclusion limits varying from 1 to 2,00,000 Daltons can be produced. The gels are identified by numbers such as G-10 or G-200, which refers to the water regain value. It is the volume of water taken by gel to get swollen and the numerical value must be multiplied by 10. Say for G -10, 1 gram of substance takes up 10×10 = 100 grams of water to completely swell up. Other materials that can be used to swell up sephadex gels are glycol, dimethylsalfoxide and formamide.

Figure 5.6: Principle of Gel permeation Chromatography
A) Schematic representation of exclusion of large molecules
B) Effect of particle size on their elution rates



The following table represents different sephadex gel and their features.

Type	**Water regain (g/g dry sephadex)**	**Exclusion limit Mol wt**	**Fraction range Mol. Wt limits**
G-25	2.5	5,000	100-5,000
G-50	5.0	10,000	500-10,000
G-75	7.5	50,000	1000-50,000
G-100	10.0	100,000	5000-100,000
G-200	20.0	200,00	5000-200,000

b) Polyacrylamide gels :- There are very popular and the preparation in mentioned in the chapter electrophoresis. Polyacrylamide gels are identified by numbers such as P-10 or P-100 which if multiplied by a factor of 1000 will indicate the exclusion limit of the gel in thousands of Daltons. Polyacrylamide gels can be used to separate moleculas of upto 300,000 daltons. However, the large pored gels lack mechanical rigidity and can become compressed in the column. Polyacrylamide is insoluble in water and organic solvents between the P^H range 2-11 and can be used to swell the gel beads. These are however unstable to bases.

(c) Agarose :- Agarose gel is produced from agar. They are linear polysaccharides of alternating residues of D-glucose and 3,6-anhydro – L-galactose units and owe their gelling properties to hydrogen bonding of both inner – and intra – molecular type. These gels are hydrophilic and are almost completely free of charged groups, hence are inert to solute molecules inside. By virtue, they are of high porosity and can be used to separate molecules and particles of several million Daltons. These gels can be used to study virus, nucleic acids and polysaccharides.

These gels are compatible with all aqueous buffers and are completely stable within the pH range of 4 – 10. These gels can be used even if pH is 13. High salt concentrations do not effect these gels. However, these gels are used between temperatures 0° to 30°C as agarose gels are sensitive to high temperatures.

(d) Styragels :- These are polystyrene gels which offer range of porosity. These are non aqueous gels that can withstand temperatures of 150°C. These gels can be used in solvents such as Tetrahydrofuran, Benzene, Trichlorobenzene, Perchloroethylene, cresol, Dimethyl sulphoxide, Chloroform, Carbon tetrachloride etc. However acetone, alcohols and water cannot be used.

(e) Controlled pore glass beads: These are fine glass spheres made up of borosilicate glass and have large number of pores within a very narrow size distribution. To prevent adsorption of proteins, the glass beads are treated with hexamethyl disilazine. These glass spheres have a molecular exclusion limit ranging from 3000 to 9 million Daltons.

Gel Permeation column Preparation

The column preparation protocol is same as that mentioned earlier. The gel is initially swollen overnight so that the beads completely open up to their maximum extent in sufficient volume of solvent. Swelling time can be reduced by adding the dry gel beads to water and placing the slurry in boiling water. On the other hand porous glass beads do not require such treatment.

Gel bed preparation, Sample preparation is same as mentioned earlier. The volume of sample varies with column size. Blue dextran is added to find out void volume as it moves out through outer spaces due to its high molecular weight.

Detection :- This is done by utilizing the properties of analyte like its absorption properties or by adding detectable ligands.

Applications :-

1. Gel permeation chromatography is used for separation of biomolecules based on their size or for their purification. Proteins, enzymes, hormones, antibodies, nucleic acids, polysaccharides and even viruses and are separated by using different types of gels or glass granules. These can also be used to separate low molecular weight compounds such as amino acids, small peptides and oligonucleotides. This can also be used for separation of 45 and 5S oligo nucleotides.
2. Used to remove salts and small molecules from macromolecules. Salts being small molecules penetrate through all pores of gel beads and its movement is retarted, while large macromolecules are eluted out first as they remain in void volume.
3. Gel beads can be used to concentrate dilute solutions of biomolecules. If Sephadex G-200 in added, it will absorb 20 times its water content and Sephdex G-25 will absorb 250 gm of water. If these beads are placed in dilute solution, gel absorbs water there by concentrating the solution.
4. Determination of molecular weight of macromolecules. Marker proteins of known molecular weight are initially eluted. A graph with elution volume verses log of molecular weight is prepared. The column in completely washed, equilibrated with eluant and then test sample is added. By comparing the elution volume in which the test solute elutes with the graph, molecular weight of unknown sample can be obtained.

ION – EXCHANGE CHROMATOGRAPHY

Principle :-

Ion - exchange may be defined as the reversible exchange of ions in solution with ions electrostatically bound to inert support medium. The governing factor in ion – exchange is the electrostatic force of attraction, which inturn depends upon, relative charge, radius of the hydrating ions and the degree of non-bonding interactions. Ion exchange separations are carried out usually in packed columns having ion exchangers.

Ion exchangers are of two types- Anion exchanges and Cation exchanges. These are inert and insoluble support media which may be covalently bound to positive (anion exchange) or negative (cation exchanges) functional groups. The ions bound electrostatically to the exchanger are called counter ions. This technique is extremely useful in the separation of charged compounds. The value of this technique lies in the fact that conditions can be manipulated such that molecules bind to Ion exchanges.

The process of Ion exchange is represented in the figure 5.7. The exchanges is fully charged as in figure 5.7 A. The sample containing the ionic species to be separated is allowed to percolate through the exchanger for such a length of time that will be sufficient for the equilibrium to be achieved (figure 5.7B). Exchange of ions occurs as follows:

$$E^-Y^+ + X^+ \rightleftharpoons E^-X^+ + Y^+$$

Where E^- is the charged cationic exchanges, Y^+ is the counter ion and X^+ is charged molecule. Elution of bound X^+ can be done in two ways - change in pH that will alter binding properties of X^+ there by eluting the molecule (figure 5.7 C) or by adding increase concentration of Y^+ in the solvent, eluting X^+ ions (figure 5.7D)

The sequential usage of anionic as well as cationic exchangers can be done for best separation of proteins which is indicated in the figure 5.8.

Figure 5.7: Basic Process of Ion exchange using Cation Exchanger

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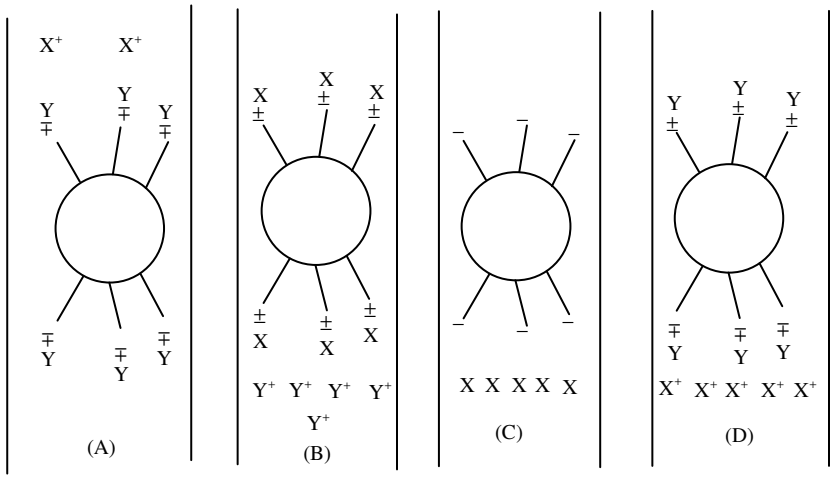
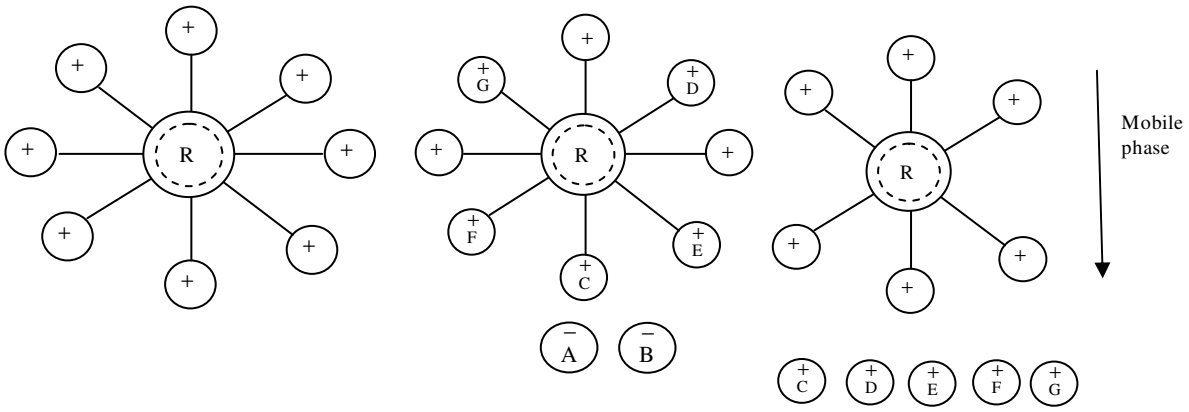
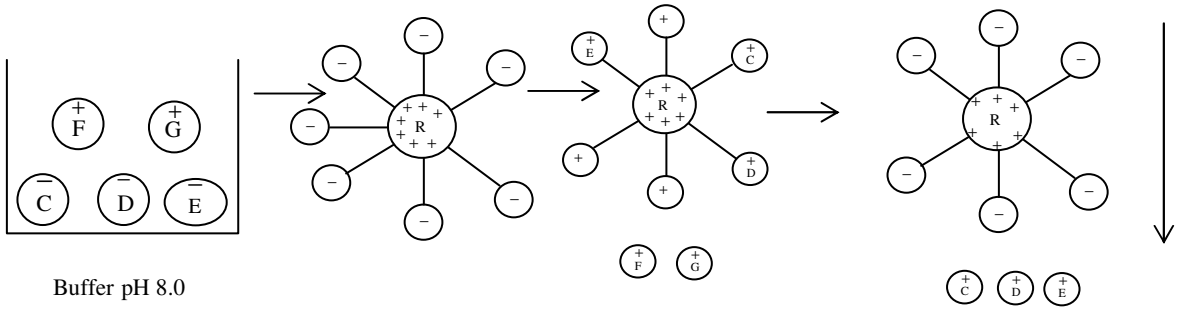


Figure 5.8: Sequential use of Cation and Anion exchange Chromatography resulting high degree of purification of desired protein



If the pH of the protein suspension is changed to 6.5 proteins (A) & (B) will posses net negative charge because their isoelectic pH is below the pH of the medium. Other proteins will be positively charged.

Performing cation exc hange chromatography (only one resin bead with positively charged cations is shown). (A) & (B) cannot be exchanged and so elute out quickly. (C)* (D)8 (E*)(F*) & (G*) blind to the bead and are retarded. these can be later and collected. two unwanted proteins have been removed and we have a mixture of five proteins



Buffer pH 8.0

Changing the pH of the protien solution to 8.0 F & G become positively charged while (C) (D)& (E) assume negative charge.

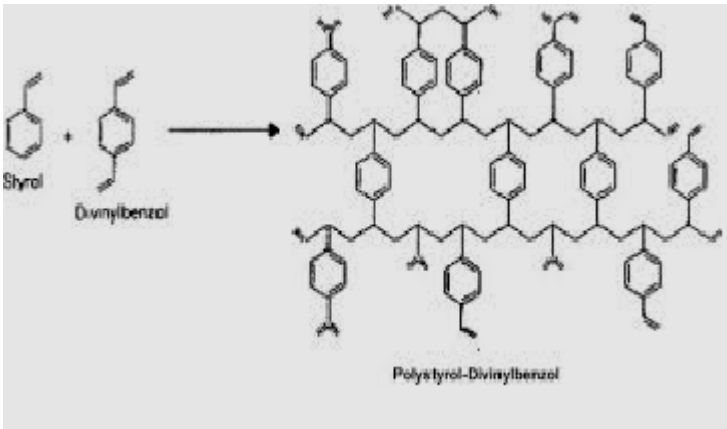
Performing anion- exchange chromatograph. (F*) & (G*) are not exchanged elute quickly and are discarded. (C*), (D*) & (E*) bind to beads and can be elute later, the desired protien (D) is now in an mixture of only three protiens. thus protein solution has been enriched for (D).

Types of Ion Exchange Resins :-

Two main groups of materials are used in the preparation of Ion exchange resins: polystyrene and cellulose. Selection of material depends on type of compound to be separated. Figure 5.9 indicates the formation of polystyrene 3-dimensional structure by cross linking with divinyl benzene. High ratio of divinyl benzene to styrene increases rigidity, reduce swelling, reduce porosity and solubility of polymeric structure.

Acidic functional groups are added by treating resin beads with sulphuric acid. These are strong cationic exchangers, as they can readily loose protons. To prepare weak cationic exchangers, carboxylate groups are attached. In the same way strong anion exchanges are prepared with a tertiary amine yielding a strongly basic quaternary ammonium group. Weak anionic exchanges can be prepared with secondary anion yielding a weakly basic tertiary amine.

Figure 5.9- Cross linking of Styrene with Divinyl Benzene



The following table lists various ionic exchange resius and their nature.

Type	Nature
Strong cation	Sulphonated Polystyrene Sulphopropyl cellulose
Weak cation	Condensed acrylic acid Carboxy methyl cellulose
Strong anion	Polystyrene with – CH_2NMe_3C Diethyl (2 hydroxy propyl) quarternary amino cellulose
Weak anion	Polystyrene with secondary amine Diethyl amino ethyl cellulose Diethyl amino ethyl agarose

Preparation of the exchange medium

Conversion of the exchanger from the form in which it is supplied to the form in which it is to be used is known as preparation of exchanger. It involves removing impurities, swelling and making the functional groups charged. Swelling of anion exchanger is usually carried out by treating first with an acid (0.5 N HCl) and then with base (0.5 N NaOH). The sequence is reverse with cationic exchangers. This is followed by removal of fines. Now the exchangers are equilibrated with suitable counter ions.

Choice of Buffers :-

The choice of buffers depends on the compound of interest and the type of ion-exchanger. Anion exchange chromatography should be carried out with cationic buffers. Reverse is true for cationic exchangers. The pK of the buffer should be as near as possible to the pH at which the system is buffered.

Practical Procedure:- To the packed column, sample component prepared in the same buffer is added. The column is allowed to stand with sample for some duration, which allows its exchange with counter ions. The column is now washed with buffer that removes unbound ions. Elution of bound sample is done by gradient elution method by varying pH or by adding increased concentration of counter ions.

Applications :-

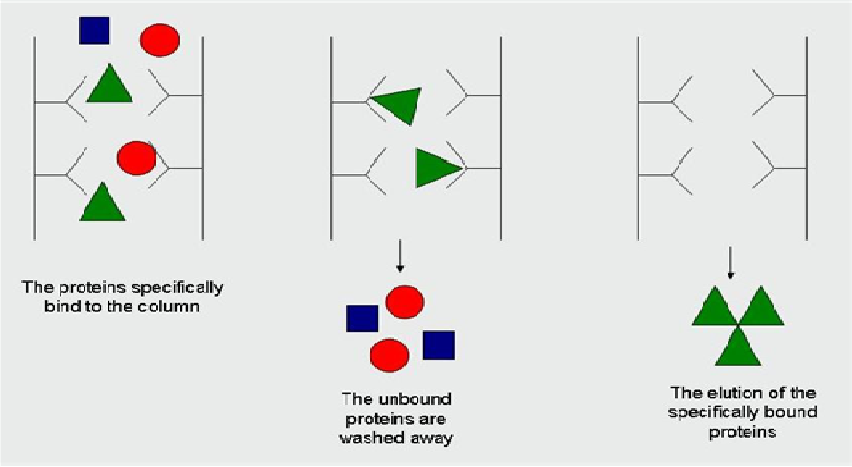
Ion exchange chromatography has several applications which include :

1. The most important used is in the analysis of amino acids Auto analyzer, that reveals amino acid composition of a protein and it works on ion - exchange principle.
2. Used in determination of nitrogen base composition of nucleic acids.
3. Most often used in deionization of water.
4. Many biological applications require ultrapure, metal ion free reagents. Such commercial preparations use Ion exchange chromatography.
5. Preparation of biological sample for atomic emission studies.
6. For separation of vitamins, biological amines, organic acids and bases.

AFFINITY CHROMATOGRAPHY

Affinity chromatography exploits the capacity of bimoleculas for specific non-covalent binding of other molecule called ligands. (figure 5.10) Biomolecules have this amazing property to bind specifically to certain molecules, like enzymes to substrates, hormones to receptors etc. When a mixture of proteins having an enzyme is available, to purify enzyme a specific subtract is immobilized as ligand to a matrix. The entire column is washed to get rid of impurities. The bound enzyme can be purified by applying several methods – like addition of a molecule that has more affinity for enzyme, or by adding another molecule that can displace enzyme, by altering pH that changes ligand binding characteristics of enzyme.

Figure 5.10: Principle of Affinity chromatography



This is the best method that allows single step purification process for a biomolecule. Few separations that can be carried out using affinity chromatography are listed in the following table. Group specific ligands are commonly used in affinity chromatography :-

Micro Molecule / cell	**Ligand**
Avidin	Biocytic
α-Chymotrypsin	Tryptophan
Thrombin	Benzamidine
Coagulation factor	Heparin
Interferon	Antibody
Poly (A) messenger RNA	Poly (U) or polydT
Ribosomal RNA	Lysine
Glycoproteins, Glycolipids	Concanavalin A
Fat cells	Inulin, Concavalin A

The variables that need to be considered before initiating affinity chromatography are,

i) The type of matrix to be used.

ii) Selection of the ligand, its nature and the means of covalently binding it to the matrix, and

iii) The conditions exploited to bind and dissociate (elute) the macromolecule from the column.

Supporting matrix :- Characteristics required for an ideal insoluble matrix include :

i) The matrix must be inert

ii) It should posses good flow properties.

iii) It must be chemically and mechanically stable to varying pH, ionic strength and the denaturing conditions employed for binding and elution.

iv) It should have large number of suitable chemical groups for the binding of ligand.

v) It must be highly porous to provide enough surface area.

Theses particles are generally spherical and rigid. The matrix is made up to agarose, polyacrylamide or controlled porous glass beads.

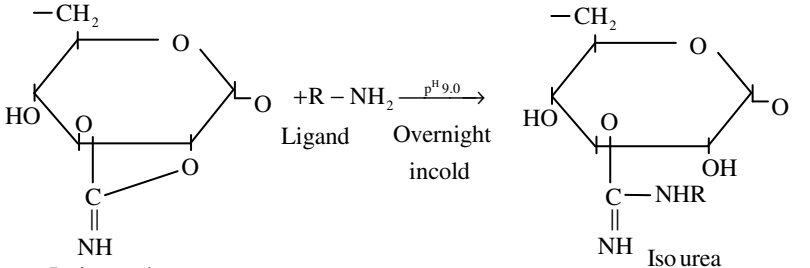
Ligand selection :- The ligand used in the construction of affinity column can be substrate analogues, effectors, enzyme cofactors, receptor antagonists, synthetic antagonists, Epitopes of antigens etc. However, the Ligand chosen must not bind strongly to the molecule, but must be dissociable. It must have chemical groups that allow its linkage to the matrix.

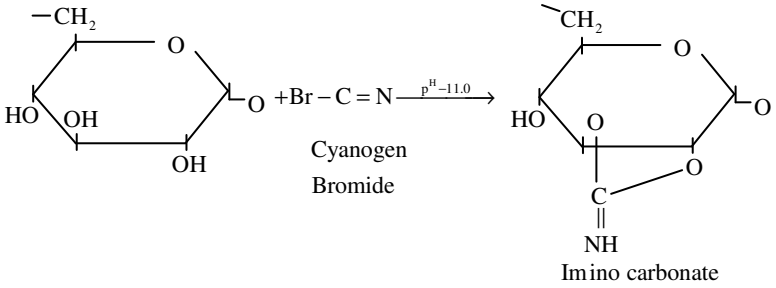
Ligand attachment :-Covalent coupling of ligand to matrix involves the following steps :

i) Activation of matrix functional group.

ii) Covalent attachment of ligand to activated groups.

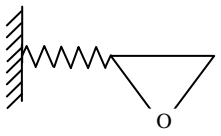
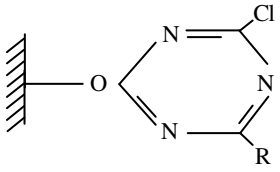
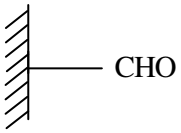
The most common method of activation agarose (or a polysaccharide) involves treatment with cyanogens bromide at alkaline pH of 11.0. The exothermic reaction involves changes in pH and temperature which is to be maintained over night. The intermediates involved in the reaction are as follows.





Alternative methods of binding ligands is shown in following table.

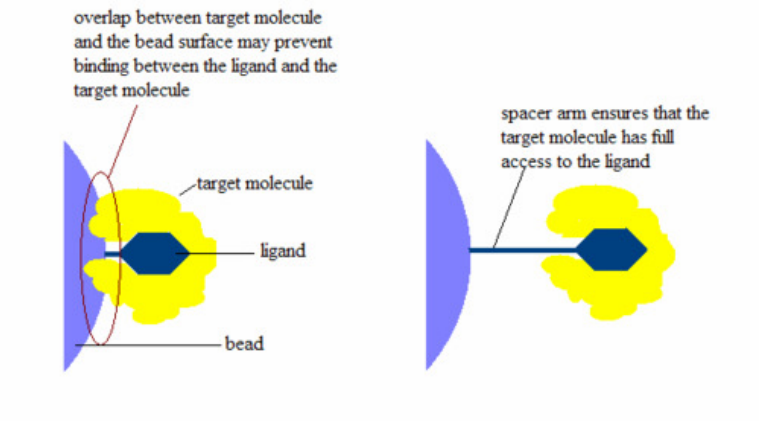
Table :- Alternative methods of activation of polysaccharide supports.

Method	Active Functionality for Ligand coupling	Reaction with type of ligand
Epoxides		Reacts with amuses and other mucleophiles
Triazines		Reacts with amino groups and other nucleophiles
Periodate		Reacts with amines or hydrazines

The most preferred method is cyanogens bromide treatment method.

The arm :- If ligand is directly attached to activated groups of the support, the macromolecule they may encounter steric restrictions due to which they may not become absorbed to the matrix. To avoid this spacers are introduced between support and the Ligand. The space is also called arm (Figure 5.11). Examples of spacer include- hexamethylene diamine, $3,3^1$–diamino dipropylamine, 1,6–diaminohexane, 6–aminohexanoic acid, and 1,4–bis–(2,3– epoxy propoxy) butane.

Figure 5.11: Need of a spacer arm in affinity chromatography



Application :-

1. Affinity chromatography is used to purity a large variety of macromolecules such as enzymes, immunoglobulins, membrane receptors, nucleic acids and even polysaccharides.
2. In whole cell purification. To isolate specific T & B lymphocytes.
3. To Purify metal binding proteins.
4. Usage of magnetic gel beads for easy purification of proteins. In this produce polyacrylamide or agarose gel beads having Fe_3O_4 core are coated with ligand. These are coupled with ligand. Addition of protein mixture will enable binding of ligand with specific protein. The beads are washed with buffer and can be recovered by applying magnetic field.

GAS CHROMATOGRAPHY

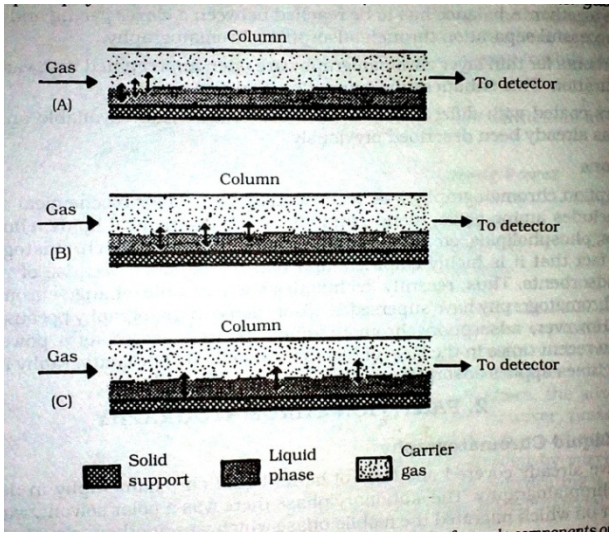
In this technique, gas is the mobile phase which is used for separation of mixtures. This technique is of 2 types – Gas solid chromatography & Gas liquid chromatography.

Principle :- In Gas solid chromatography, solid phase is coated in the interior of column. When sample is added, it is carried by gas by exerting adsorption forces on to solid phase. The sample components therefore become distributed between the gas and the solid surface on the basis of difference in the adsorption - desorption behavior on the solid surface. This eventually leads to their separation from each other.

In Gas liquid chromatography (figure 5.12) the stationary phase is a non-volatile liquid. This liquid phase is dispersed over an inert solid support which is coated inside a column.

When sample is added and carrier gas is allowed to run based on the distribution coefficient of sample components between liquid and the gas phase, the components get separated.

Figure 5.12: Schematic representation of gas chromatographic separation of sample components on the basis of their partition coefficient

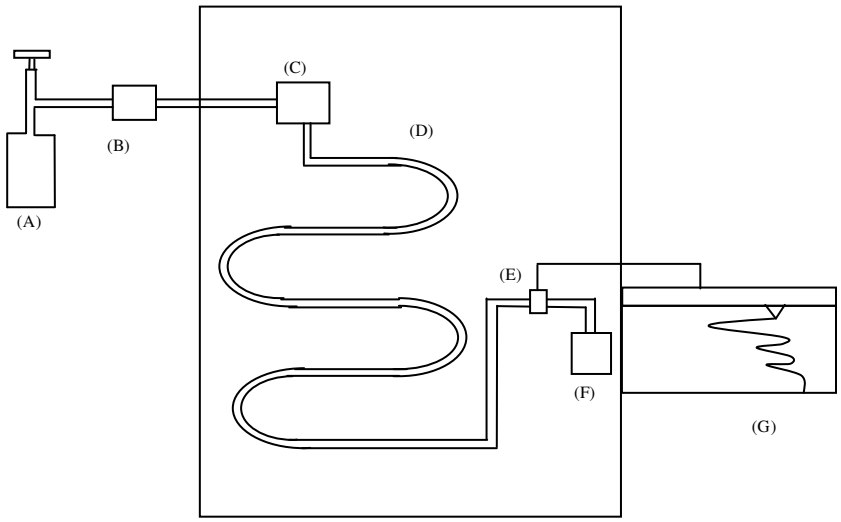


Components of Gas Chromatography :- (figure 5.13)

The essential components of Gas are

1. Carrier gas tank
2. Pressure regulator
3. Sample injection chamber
4. Column.
5. Detector
6. Fraction collector
7. Recorder

Figure 5.13 : Schematic representation of gas chromatography A) Carrier gas tank B)



Carrier gas is allowed to flow at study rate. The components from sample injection chamber, column and detector are incubated and maintained at high temperatures.

Carrier gas :- Carrier gas is the mobile phase. The gas is chemically inert, pure and has high density that allows good separation of components. Commonly used gases are Nitrogen and Argon, Helium. Hydrogen and Carbon dioxide are also used as carrier gas.

The carrier gas must be free of impurities and water vapour. The flow rate of gas in the column is 40 – 80 cm^3/minute.

Columns :- Two distinct types of columns are used packed and open tubular types. The open tubular type is also Known as capillary columns. The packed columns are made up of stainless steel, Copper or glass tubing of 1.6, 3.2, 6.4 or 9.5mm bore and length between 1-15 meters. Open tubular or capillary column have an inside diameter of 0.25mm and length of 15 - 30 meters length. The stationary phase can be coated with liquid phase of 1 μm thickness.

The open tubular columns are of two types, Wall-coated open tubular column (W C O T) in which liquid is coated on the column wall. However, these columns are suitable only for analytical purposes and not suitable for preparative purposes.

The second type of columns are, support coated open tubular columns (SCOT). They have porous layer coating of solid phase that can also hold liquid phase. These have high sample capacity.

Solid support :- This is chemically inert, wettable fine material that offer high specific surface of $\tilde{}1m^2/g$. These must also be thermally stable and mechanically strong. The materials used are diatomaceous earth treated with hexa methyl disilazane or Teflon. Other examples include crushed firebrick, Glass microsphere, poly tetra fluoroethylene etc.

Liquid phase :- A good liquid phase should offer good solubility to the sample. A liquid phase to be used in GC must have following characteristics :

- It must be non - volatile as the produce uses high temperatures
- It must be thermally stable
- Provide appropriate partition coefficient to the sample and
- It should be inert towards the solutes.

The following table indicates few liquid phases and samples that can be separated by GLC.

Stationary phase	Typical samples
Silicon rubber gum (SE-30)	Steroid, alkaloids, pesticides
Apliezon L	Esters, Ethers and high boiling hydro
Carbowax 20M (PEG)	carbons.
Silicon oil (DC-550)	Amines, Ketenes, alcohols
Dinomyl phthalate	All types
Equalane	All types
Hexa decane	Hydrocarbons
Silver nitrate in propylene glycol	Hydrocarbons, fluorides
	Olefins cyclic hydrocarbons

Coating the support – The liquid phase is solubilized in a solvent by gently heating it and the solvent is removed as liquid phase liquefies. This is applied inside the column, by pumping it into column, followed by its rotation and plugging column at both the ends. A thin layer may be obtained by pumping diluted solution and allowing excess to run off, followed by application of hot carrier gas. This leaves a thin film of liquid phase.

Sample application :- In this technique, the sample is introduced in column in vapour form. It should be non – polar or of very low polarity, else it will leave tailing. The volume of sample depends on the column being used. For analytical purposes 10^{-3} to 10^{-2} ml sample is used while for a column of bore size 9.4mm, 1 to 50 ml sample volume is used. Sample injection is done using special syringes into sample injection port.

Detectors :- These are located at the exit end of the column. The most commonly used detectors are

1) Flame ionization detector
2) Electron capture detector and
3) Thermionic emission detector.

1. Flame ionization detector :- These are most widely used detectors. They are used to measure organic compounds and they can detect compounds as low as one nanogram. Hydrogen is used as carrier gas.

   Hydrogen when burnt gives a colorless flame, the jet of which forms one electrode. The other electrode is mounted just near the tip of the flame and consists of a platinum wire (figure 5.14) The flame changes the color, the moment a separate compound enters it. If sample components enter, they become ionized in the flame and give rise to a current between the two electrodes. As the resistance of the flame is very high and the current generated is weak. The associated electronics are complicated and moderately expensive.

2. Electron capture detector :- This detector has radio active source (^{63}Ni) Which ionizes the carrier gas coming out of the column. The electrons produced give rise to a current across the electrodes to which suitable voltage is applied (figure 5.15). When a sample component, which has the ability to capture electrons, comes out of the column, it captures the ionized electrons thereby causing a drop in the current. This change in the current is measured and recorded. The detector is used to measure poly halogenated compounds, mainly pesticides such as DDT, dieldron and aldrine. The sensitivity of this detector is very high and can measure as little as one picogram of compound.

3. Thermionic emission detector: The detector employs fuel – pool hydrogen plasma. This low temperature source suppresses the normal flame ionization response of the compounds not containing nitrogen or phosphorus. A non-volatile rubidium silicate bead is centered about 1.25cm above the plasma jet. This bead is electrically heated to about 600-800°C. This design allows adjustment of the bead temperature independent of the plasma as a source of thermal energy. With vanishingly small hydrogen flow, the detector responds to both nitrogen and phosphorus containing compounds by greatly enhancing the ionic dissociation of rubidium chloride. It can be used to measure organophosphorus compounds, and sensitivity is less than one picogram of the compound.

Figure 5.14: Flame Ionization detector

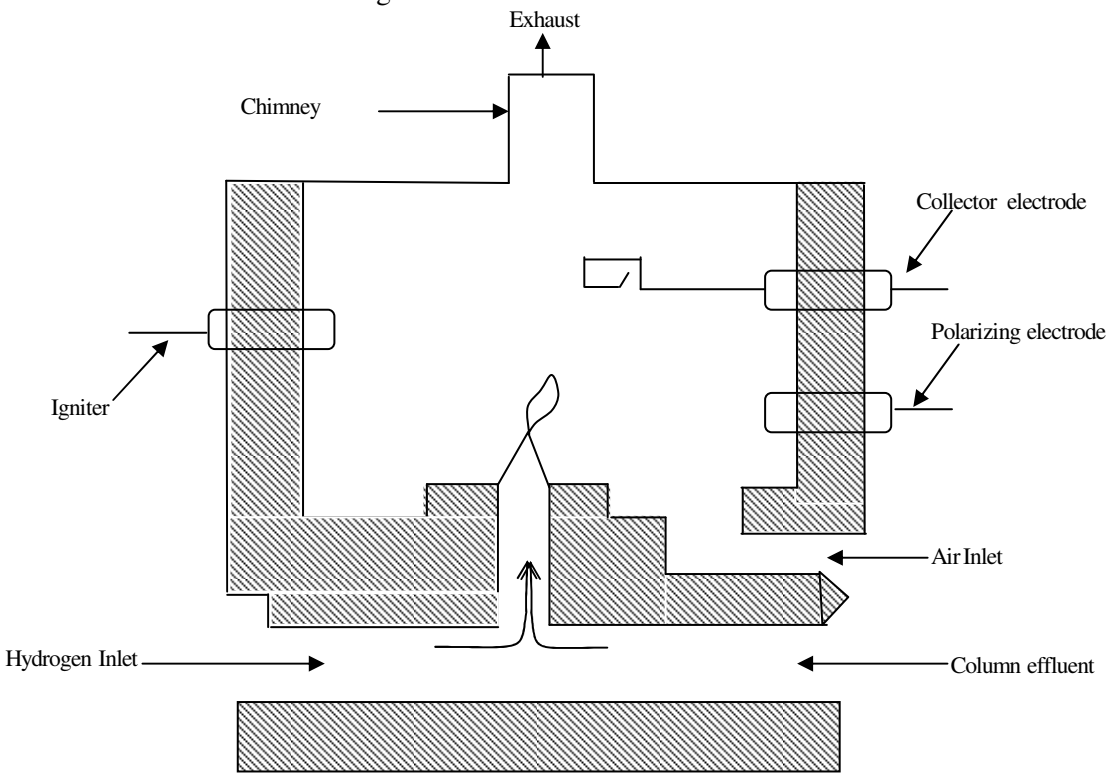
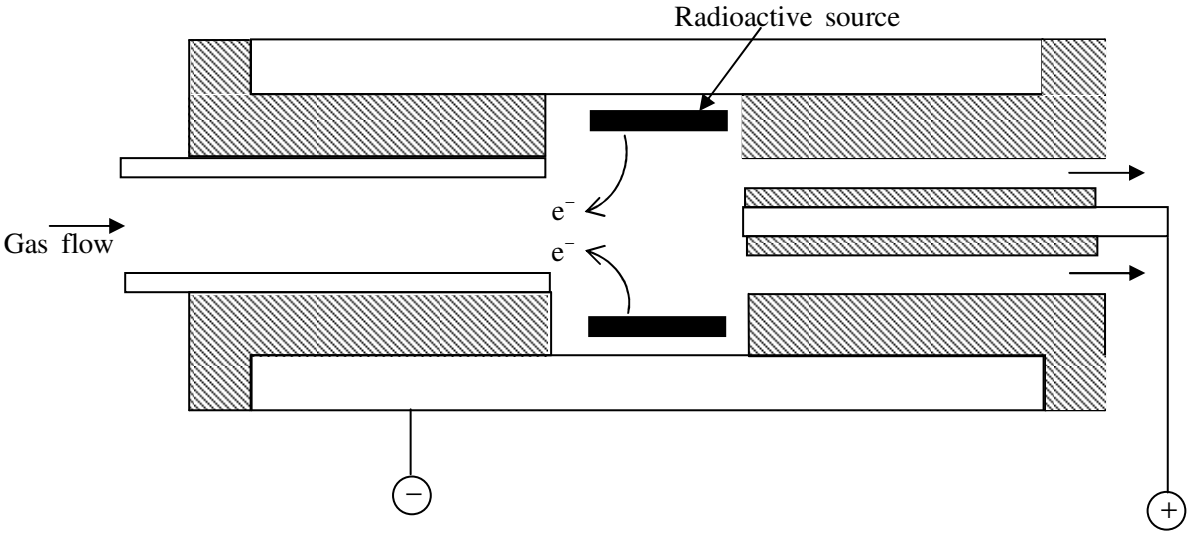
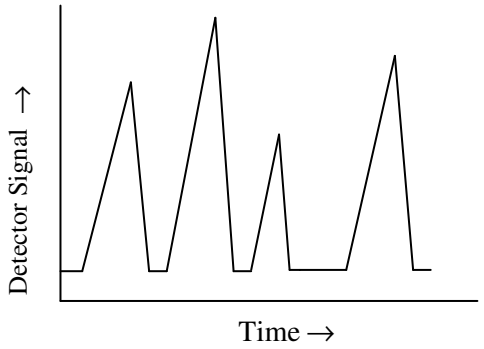


Figure 5.15 : Electron capture detector



Chromatogram :-The detector output is suitably amplified and is traced on a strip chart recorder. The resulting chromatogram (as shown in the figure below) has sample concentration versus time.



Applications :- GC finds its application in separation of components of tobacco smoke, atmospheric pollutants, solvents, plant extracts, essential oils, volatile vegetable oils, organic acids etc. It can be used to study reaction rates, energies and mechanisms of reactions.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

High Performance Liquid chromatography (HPLC) is a chromatographic technique that uses extremely high pressures (upto 8000 psi), and high flow rates. This process reduces the experiment time without loss of efficiency. It requires vanishingly small amounts of sample (pico or even femtogram). This is primarily an analytical technique but can also be used for preparative purposes. HPLC is primary used for the separation of polar compounds such as durg metabolites. The advantage with HPLC is that it may employ the principles of adsorption, partition, ion exchange, exclusion and affinity chromatography. This makes it a special technique. The differences between HPLC and conventional chromatography are as follows :-

Characteristics	Conventional chromatography	H P L C
Number of plates / sec	0.02	5
Pressure	Negligible	upto 8000 psi
Flow rate (mm/min)	5-50	600
Time of experiment	Hours to days	Minutes to hours
Equipment	simple, column &	Integrated

	accessories	chromatography
Purpose	Predominantly	predominantly
Quantity chromatographed	preparative	analytical
	mg to Kg	ng to pg

Components of HPLC :-

The major components are HPLC are (figure 5.16)

i) A solvent reservoir to store mobile phase.

ii) High pressure pump to push the mobile phase through the column.

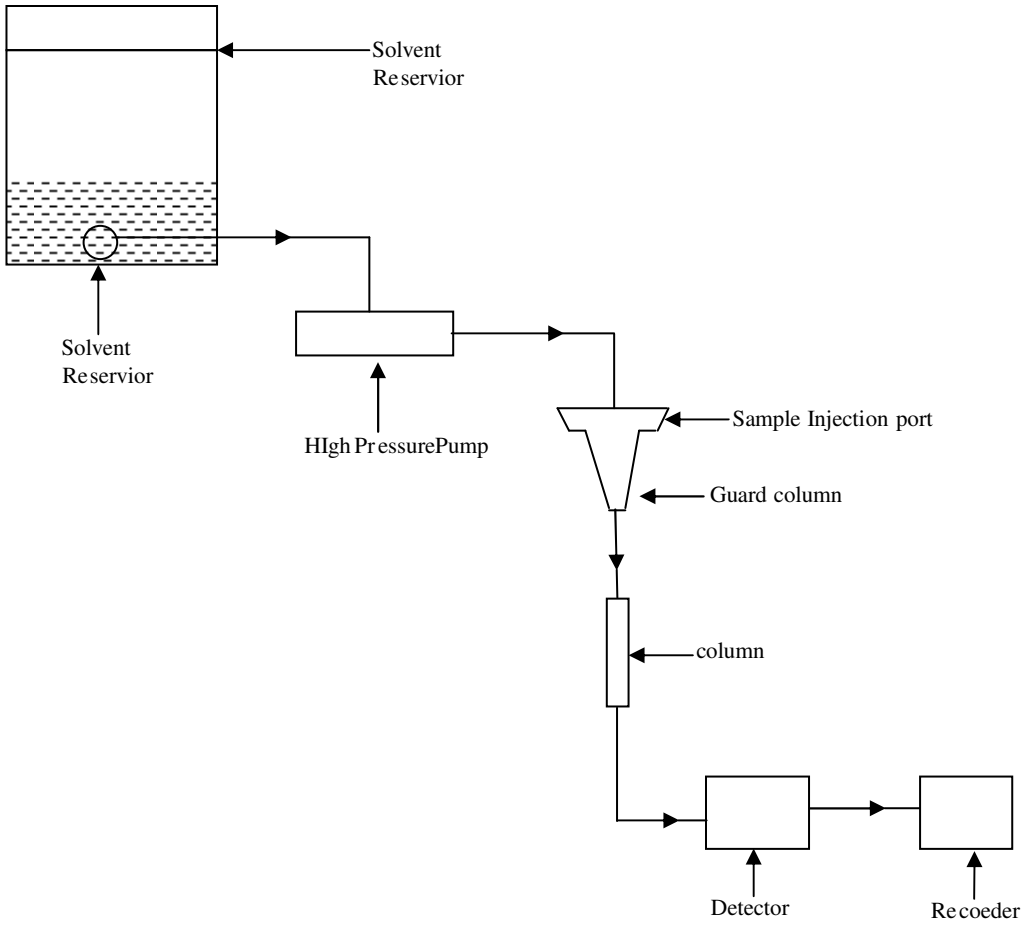
iii) A device to inject the sample into the mobile phase.

iv) A column in which the separation will take place.

v) A detector used in detecting the concentration of the sample components as they come out of the column.

vi) A potentiometric recorder to produce a chromatogram.

Figure 5.16: components of HPLC



Solvent Reservoir and the solvent: The solvent reservoir must be having a capacity to accommodate volume for repetitive analysis. It should have provision for degassing, as solvent used in HPLC must not have dissolved gases. The vessel must be made up of material which is inert. Hence it is made up of material which is inert and they are usually glass or steel containers of 0.5 to 2.0 liters capacity. They are also equipped with micro filter, to remove fine contaminants or suspensions.

Pumping system :- It is considered to be the heart of HPLC. These pumps must allow uniform flow rate throughout experimentation else it will introduce noise in recording. They must pump solvent with 0.5 to 10ml / min flow rate. They must also adapt to gradient operation.

Various pumping systems available for HPLC are

- Holding coil
- Pneumatic amplifier
- Piston / diaphragm driven by a moving fluid
- Reciprocating pump
- Syringe pumps

Sample injection :- There are 2 ways of sample injection the first is by using micro syringe that can with stand high pressures. The sample is either directly introduced into column or on to a material in the sample injection port by pressure drop at that moment. The second is by using small volume metal loop that allows release of sample into column.

The column :- The columns for HPLC are usually made up of stainless steel, glass, aluminum, copper or PTFE. Stainless steel is preferred as it can with stand pressures of upto 8000 psi. Straight columns of 20-50 cm height are used having inner diameter of 1-4mm. They have mirror finishing inside which allows efficient packing of material. One end of the column is generally plugged with Teflon / disc.

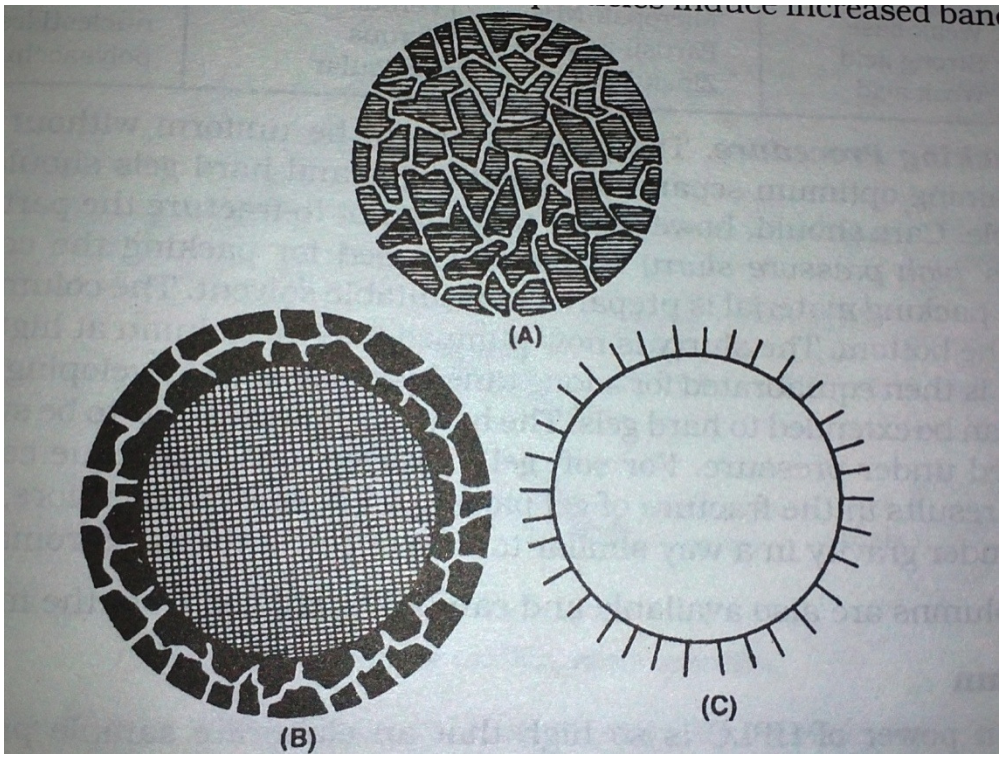
Column Packing :- Maximal separation without or with minimal band broadening is a function of stationary and mobile phase chosen. Usage of coarser particles will lead to band broadening. HPLC uses particles which are fine and uniform and they do not resist flow of solvent. There are three forms of column packing material available:

i) Microporous supports where micropores ramify through the particles. These particles are generally 5 – 10 mm in diameter (figure 5.17A).

ii) Pellicular supports consists of a solid inert core onto which are coated several porous particles. These supports are therefore superficially porous (figure 5.17B).

iii) Bonded phases where the stationary phase is chemically bonded to an inert support (Figure 5. 17C).

Figure 5.17 : Scematic representation of the three types of supports used in HPLC.
A)Microporous type B) Pellicular and C) Bonded phase



Individual stationary supports for different modes of HPLC are shown in follow table.
Table showing List of stationary phases used in various modes of HPLC

Mode	**Nature**	**Commercial Name**	**Type of support**	**Applications**
Adsorption	Silica Alumina Silica Alumina	Corasil Pellumina Partisil micropak A1	Pellicular Pellicular Microporous	Steroids, vitamins,Chlorinatepestices polar herbicides, triglycerides
Partition	Octadecysilane Octadecylsilane Alkylamine	Bondapak C_{18} μ Bondapac kC μ Bondapak C-NH_2	Pellicular Porous porous	Dansylated amino acids, Drugs, pesticides, Aflatoxins, fatty acids.

Exclusion	Glass Polydtyrene Divinybenz ene	Bio-Glass Stragel	Rigid solid Semi- rigid gel	Proteins, peptides, nucleic acids,
Ironexcha nge	Agarose Strong base Weak base Strong acid Weak acid	Sephadex Partisil- SAX Micropak -NH_2 Parisil- SCX Zipak- WAX	Soft gel Porous Porous Porous Pellicular	Polysaccharides, Aminoacids, Peptides, proteins, Nucleotide, Polysaccharides.

Column packing procedure :- The packing must be done uniformly without cracks. A high pressure slurry technique is used. In this the column at one end is plugged and slurry of packing material is pumped into column at high pressure. This is then equilibrated with solvent system for long duration to attain uniformity. The gels used in HPLC are to be swollen and then pumped.

The guard column :- This column is used while working with biological materials like serum, fermentation sample etc. This is a small column of 2 – 10cm placed above working column and functions to retain elements which otherwise would clog working column.

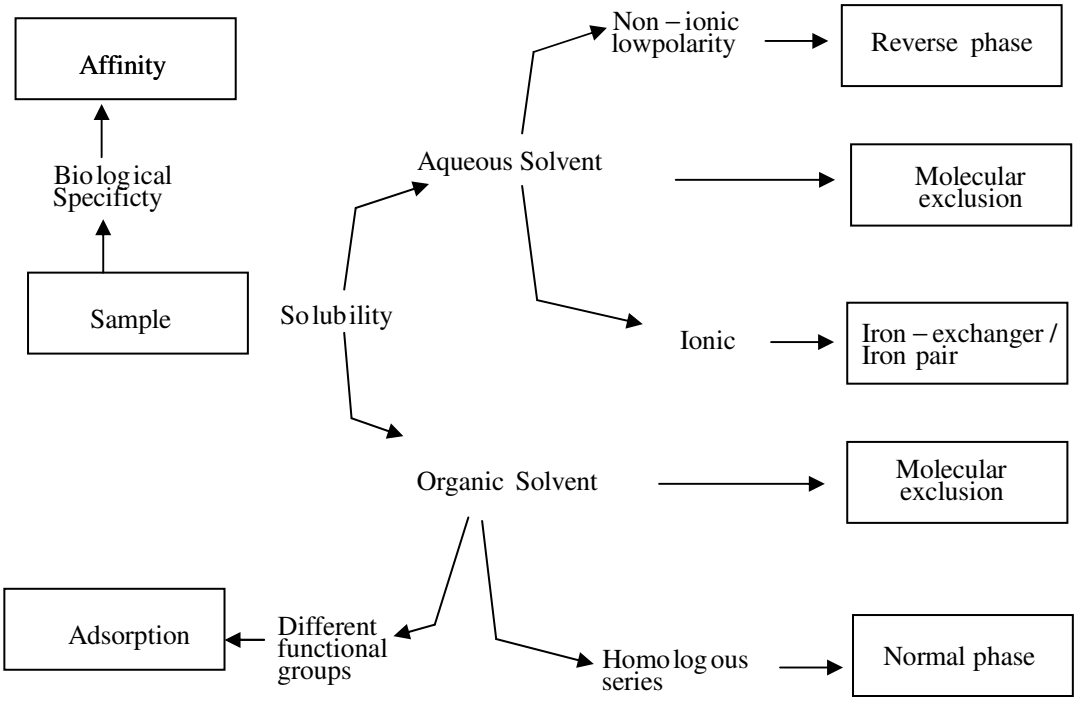
Detectors :- HPLC operates on various principles and the resulting sample is detected generally using UV/Vis photometers, spectrophotometers. Fluorimeters etc.

Selection of separation system :-

HPLC operates in several modes. Based on the sample of interest and its solubility either partition or adsorption chromatography are used.

Weakly ionic substances which are water soluble can be separated by reverse phase chromatography. Strongly ionic ones can be separated by ion-exchange chromatography. The various methods that can be adopted based on solute properties is illustrated in figure 5.18.

Figure 5.18: Guide to HPLC mode of selection



FAST PROTEINLIQUID CHROMATOGRAPHY (FLPC)

Fast protein liquid chromatography (FPLC) is a form of high-performance chromatography that takes advantage of high resolution made possible by small-diameter stationary phases. It was originally developed for proteins and features high loading capacity, biocompatible aqueous buffer systems, fast flow rates, and availability of stationary phases in most common chromatography modes (e.g., ion exchange, gel filtration, reversed phase, and affinity). The system makes reproducible separation possible by incorporating a high level of automation including autosamplers, gradient program control, and peak collection. In addition to proteins, the method is applicable to other kinds of biological samples including oligonucleotides and plasmids. The most common type of FPLC experiment is anion exchange of proteins.

FPLC provides the full range of chromatography various modes based on particles with average diameter sizes in a similar range as those used for HPLC separations. These columns can accommodate much higher protein loadings than conventional HPLC, however, and use a wide range of aqueous, biocompatible buffer systems.

Components of FLPC (Figure 5.19):

A typical laboratory FPLC consists of one or two high-precision pumps, a control unit, a column, a detection system and a fraction collector. Although it is possible to operate the system manually, the components are normally linked to a personal computer or, in older units, a microcontroller.

1. Pumps: FLPC utilizes two two-cylinder piston pumps, one for each buffer, combining the output of both in a mixing chamber. Waters systems use a single peristaltic pump which draws both buffers from separate reservoirs through a proportioning valve and mixing chamber. In either case the system allows the fraction of each buffer entering the column to be continuously varied. The flow rate can go from a few milliliters per minute in bench-top systems to liters per minute for industrial scale purifications. The wide flow range makes it suitable both for analytical and preparative chromatography.

2. Injection loop: A segment of tubing of known volume which is filled with the sample solution before it is injected into the column. Loop volume can range from a few microliters to 50ml or more.

3. Injection valve: A motorized valve which links the mixer and sample loop to the column. Typically the valve has three positions for loading the sample loop, for injecting the sample from the loop into the column, and for connecting the pumps directly to the waste line to wash them or change buffer solutions. The injection valve has a sample loading port through which the sample can be loaded into the injection loop, usually from a hypodermic syringe using a Luer-lock connection.

4. Column: The column is a glass or plastic cylinder packed with beads of resin and filled with buffer solution. It is normally mounted vertically with the buffer flowing downward from top to bottom. A glass frit at the bottom of the column retains the resin beads in the column while allowing the buffer and dissolved proteins to exit.

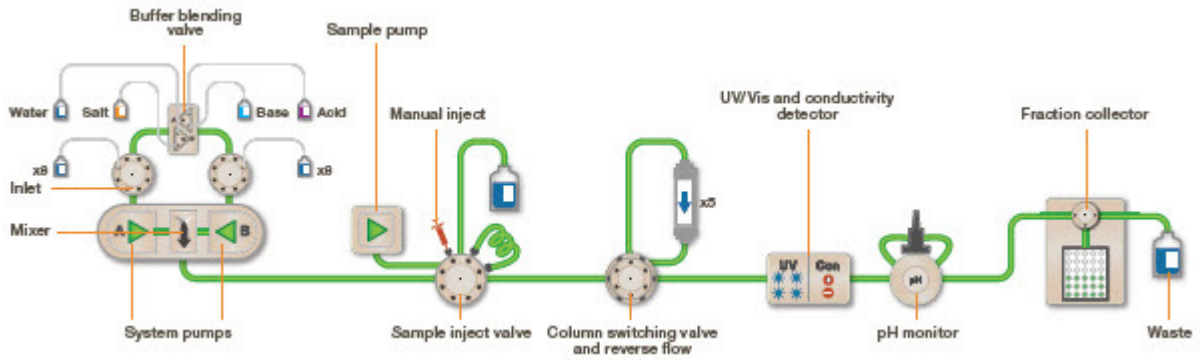
5. Flow Cells: The effluent from the column passes through one or more flow cells to measure the concentration of protein in the effluent (by UV light absorption at 280nm). The conductivity cell measures the buffer conductivity, usually in millisiemens/cm, which indicates the concentration of salt in the buffer. A flow cell which measures pH of the buffer is also commonly included. Usually each flow cell is connected to a separate electronics module which provides power and amplifies the signal.

6. Monitor/Recorder: The flow cells are connected to a display and/or recorder. On older systems this was a simple chart recorder, on modern systems a computer with hardware interface and display is used. This permits the experimenter to identify when peaks in protein concentration occur, indicating that specific components of the mixture are being eluted.

7. Fraction collector: The fraction collector is typically a rotating rack that can be filled with test tubes or similar containers. It allows samples to be collected in fixed volumes, or can be controlled to direct specific fractions detected as peaks of protein concentration, into separate containers.

Many systems include various optional components. A filter may be added between the mixer and column to minimize clogging. In large FPLC columns the sample may be loaded into the column directly using a small peristaltic pump rather than an injection loop. When the buffer contains dissolved gas, bubbles may form as pressure drops where the buffer exits the column; these bubbles create artifacts if they pass through the flow cells. This may be prevented by degassing the buffers, or by adding a flow restrictor downstream of the flow cells to maintain a pressure of 1-5 bar in the effluent line.

Figure 5.19: Components of FLPC



Columns:

The columns are large tubes that contain small particles or gel beads that are known as stationary phase. The chromatographic bed is composed by the gel beads inside the column and the sample is introduced into the injector and carried into the column by the flowing solvent. As a result of different components adhering to or diffusing through the gel, the sample mixture gets separated.

Columns used with an FPLC can separate macromolecules based on size, charge distribution (ion exchange), hydrophobicity, reverse-phase or biorecognition (as with affinity chromatography). For easy use, a wide range of pre-packed columns are available. FPLC differs from HPLC in that the columns used for FPLC can only be used up to maximum pressure of 3-4 MPa (435-580 psi). Thus, if the pressure of HPLC can be limited, each FPLC column may also be used in an HPLC machine.

Optimizing Protein purification:

The purpose of purifying proteins with FPLC is to deliver quantities of the target at sufficient purity in a biologically active state to suit its further use. The quality of the end product varies depending the type and amount of starting material, efficiency of separation, and selectivity of the purification resin. The ultimate goal of a given purification protocol is to deliver the required yield and purity of the target molecule in the quickest, cheapest, and safest way for acceptable results. The range of purity is >99% target molecule. No chromatographic techniques provide 100% yield of active material and overall yields depend on the number of steps in the purification protocol. By optimizing each step for the intended purpose and arranging them that minimizes inter step treatments, the number of steps will be minimized.

A typical multistep purification protocol starts with a preliminary capture step which many times utilizes ion exchange chromatography (IEC). The media (stationary phase) employed range from large bead resins (good for fast flow rates and little to no sample clarification at the expense of resolution) to small bead resins (for best possible resolution with all other factors being equal). Short and wide column geometries are amenable to high flow rates also at the expense of resolution, typically because of lateral diffusion of sample on the column. For techniques such as size exclusion chromatography to be useful, very long, thin columns and minimal sample volumes (maximum 5% of column volume) are required. Hydrophobic interaction chromatography (HIC) can also be used for first and/ or intermediate steps. Selectivity in HIC is independent of running pH

and descending salt gradients are used. For HIC, conditioning involves adding ammonium sulphate to the sample to match the buffer A concentration. If HIC is used before IEC, the ionic strength would have to be lowered to match that of buffer A for IEC step by dilution, dialysis or buffer exchange by gel filtration. This is why IEC is usually performed prior to HIC as the high salt elution conditions for IEC are ideal for binding to HIC resins in the next purification step. Polishing is used to achieve the final level of purification required and is commonly performed on a gel filtration column. An extra intermediate purification step can be added or optimization of the different steps is performed for improving purity. This extra step usually involves another round of IEC under completely different conditions.

Although this is an example of a common purification protocol for proteins, the buffer conditions, flow rates, and resins used to achieve final goals can be chosen to cover a broad range of target proteins. This flexibility is imperative for a functional purification system as all proteins behave differently and often deviate from predictions.[

Advantages of Fast Liquid Protein Chromatography:

FPLC is the fast and simple chromatography system for the separation of various proteins present in body fluids such as plasma, urine and specially cerebrospinal fluid (CSF).

- It is used for the separation of peptides, polynucleotides.
- Used for the purification of synthetic oligonucleotides.
- Used for the purification of plasmid DNA.
- Rapid purification of RNA.
- Used for the identification of protein profiles and variability within single proteins.
- It can separate the proteins from a more complex mixture in the liquid phase.
- Used in the enzymology and biology.
- Lipoprotein can be separated by FPLC.
- For the analysis of nitrogenous constituents of beer.
- Used to measure the levels of Tubular Protenuria: During Tubular Protenuria, patient's kidney secrets more than 150mg of urinary proteins daily. By using FPLC, the specific proteins can be isolated from the extracted proteins.
- Profiling of plasma proteins in cerebrospinal fluid: Proteins in cerebrospinal fluid or CSF can be profiled by using anion exchange column in FPLC. FPLC can easily track changes occurring in patient's body.
- Analysing Pancreatitis: In pancreatic juice, certain type of proteins can be determined by using FPLC, which lead to disease.
- By FPLC peptide sequencing and protein structures/functions can be studied.

Drawbacks:

- Needs glass columns.
-FPLC cannot handle high pressure.
- Instrument does not support HPLC columns.
- A purifying thermo labile (heat sensitive) protein is a hard task.

PRACTICE QUESTIONS

1. Total volume of a column of gel (v_t) is indicated by which among the following equation

   (A) $V_t = V_0 + V_i$, $V_0 =$ void volume

   (B) $V_t = V_O + V_i - V_g$, $V_i =$ inner volume

   (C) $V_t = V_g + V_o$ $V_g =$ volume of gel matrix

   (D) $V_t = V_0 + V_i + V_i$

2. Identify the correct statement

   (A) Column efficiency depends upon its dimensions

   (B) Column efficiency depends upon it gel packing

   (C) Column efficiency is indicated based upon the number of theoretical plats formed in minimum column length.

   (D) More number of theoretical plates in a lengthy column indicates its efficiency

3. If the partition coefficient of a sample which is added for a mixture of two immiscible solvents A & B is 0.02 and the number of molecules added are 1000 what is the count of molecules in solvents A : B

   (A) 1000 : 20 (B) 20 : 980 (C) 20: 1000 (D) 980 : 20

4. Cut of capacity of a gel is

   (A) Indicates all molecules that cannot enter

   (B) Indicates smallest molecules size that cannot enter gel.

   (C) Indicates largest molecule that has entered

   (D) Indicates size of molecules that can enters gel.

5. The number in scphadex G – 10 indicates

   (A) Water gaining capacity x 10

   (B) cut of capacity x 1000

   (C) Water gaining capacity alone

   (D) Water gaining capacity and cut of capacity of gel

6. Selection of resins and pH of buffer helps in resolution of a solute mixture in ion exchange chromatography. Say the mixture has 5 solutes A to E whose pI values are 5.2, 5.8, 7.3, 7.4, 7.5.

   At pH 6.5, what would be their charges

   (A) A^-, B^-, C^+, D^+, E^+ (B) A^-, B^-, C^-, D^+, E^+

(C) A^+, B^+, C^-, D^-, E^- (D) All will be positively charged

7. Selection of resins and pH of buffer helps in resolution of a solute mixture in ion exchange chromatography. Say the mixture has 5 solutes A to E whose pI values are 5.2, 5.8, 7.3, 7.4, 7.5.

If A & B are to eluted first, which type of buffer and ion- exchangers are to be used.

(A) Basic buffer pH-8 and anion exchangers

(B) Acidic buffer pH-4 and cation exchangers

(C) Basic buffer pH 6.5 to 7.2 and cation exchangers

(D) Basic buffer pH 6.5 to 7.2 and anion exchangers

8. Match the following

(A) Avidin	1. Concavalin A
(B) Hormone	2. Epitope
(C) Ab	3. Biocytin
(D) Glycoprotein	4. Receptor

(A) A – 4, B -1, C-3, D-2 (B) A-1, B-2, C-4, D-3

(C) A-3, B-4, C-2m D-1 (D) A-2, B-3, C-1, D-4

9. In gas liquid chromatography, the chromatogram has

(A) Peak Vs concentration

(B) No. of peaks with respect to time

(C) No. of peaks and concentration

(D) No. of peaks and retardation factor

10. ____ detector has a radioactive source which ionizes the carrier gas coming out of the column in GC.

(A) Flame Ionization detector (B) Electron capture detector

(C) Thermionic emission detector (D) Both A & B

ANSWER KEYS

1	D	**2**	C	**3**	B	**4**	B	**5**	A	**6**	A	**7**	C
8	C	**9**	B	**10**	D								

EXPLANATIONS

1. Total volume of a column is the sum of all the volumes.

2. Column efficiency to decided by the number of theoretical plates formed in minimum length of column, indicating better partitioning of substance .

3. $K = X_A / X_B$
   $K = 0.02,\ 20/980 = 0.02$
   $0.02 \times 1000 = 20$, i.e 20 are in solvent A and hance rest of 980 in solvent B.

4. Cut of capacity or exclusion limit of a gel is expressed in terms of molecular weight of the smallest molecule that cannot enter gel pores.

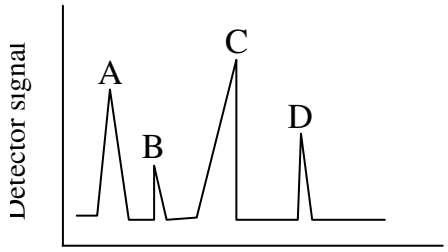
5. Sapheads G – 10, indicates that, 1gram of gel can take up 1.0 gm of water multiplied by 10.

6. At pH 6.5, the pH is basic to A & D so they loose proton and become A^- & B^-, while for the rest it is acidic, hence they gain proton and result in C^+, D^+, E^+ formation.

7. At pH between 6.5 to 7.2, A & B will be negatively charged and elute out of a cation exchanger column without binding to it

8. In affinity chromatography, the molecules of interest and their ligands are as follows Avidin – biocytin, Receptor – hormone, antibody –epitope, glycoprotein- concanavalin A

9. A GLC chromatogram is as below



Indicating 4 peaks, A, B, C, D for components, which are eluted at different time intervals

10. Electron capture detector has radioactive source that ionizes carrier gas in Gas Chromatography.

6. Electrophoresis

Electrophoresis is the migration of charged particles or molecules in a medium under the influence of an applied electric field. The first recorded Electrophoretic phenomenon was performed in 1861 by Quincke. In the year 1937, Tiselius popularized this technique by developing moving Boundary Electrophoresis. In recent days, this is the most routinely used technique by Biochemists & molecular biologists and has wide array of applications.

Principle in brief :- Upon suspension of particles or molecules in aqueous solvent they acquire either positive or negative charge. This acquisition of such charges depends upon the nature of the particle / molecule and the solvent. Say, a protein having amino and carboxyl groups are placed in a solvent, these groups ionize and give the protein a net charge density. This determines the movement of molecules in an electric filed and its velocity (figure 6.1). The charge density which depends upon the chemical groups can be modified by varying the solvent (changing pH of solvent). Even uncharged molecules can be made to attain charge through derivatization, Eg :- Carbohydrates having weak charge are derivatized as borates or phosphates. If two molecules have same charge, yet their separation is ensured due to the variation in charge/mass ratio.

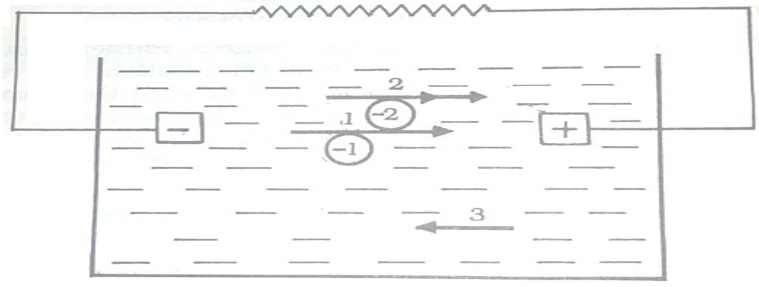


Figure 6.1 A charged particle migrates towards the oppositely charged electrode. Arrow 1 indicates movement of a particle bearing a net charge density of -1. Arrow 2 indicates faster movement of particle bearing a net charge density of -2. Arrow 3 indicates the direction of frictional force

Migration of Ion in an electric field

Consider a situation where a spherical molecule of net charge 'q' is placed in an electric field. The force 'F' which will act upon this partied will depend upon (i) the net charge density of the molecule and (ii) the strength of the field in which it is placed. The above relationship may be represented as,

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

Where, $\Delta E / d$ is the field strength applied, ΔE is the potential difference between the two electrodes, d is the distance between them. Since the particle has been suspended in a solution, friction between the accelerating molecules and solution is to be considered as it effects electrophoretic migration. The extent of friction as described by Stoke's equation, will depend upon, (i) The size and shape of the molecule and (ii) on the viscosity of the medium through which the molecule will migrate. Thus, $F = 6\pi rn\nu$

where, F is the friction exerted on the spherical molecules, r is the radians of the molecule, η is the viscosity of the solution, and ν is the velocity at which the molecule is migrating. The frictional force will oppose the accelerating force generated by the electric field

Equating the force of acceleration with stoke's equation will result in,

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

By rearranging we get

$$\nu = \frac{\Delta E q}{6\pi r\eta d}$$

Hence, Velocity of molecules is proportional to the field strength $(\Delta E / d)$ charge (q), and insanely proportional to particle size (r) and Viscosity of solution (η).

Faction Affecting electrophoretic mobility:

1) **The Sample:** The size and shape of molecule are important. The charge/mass ratio primarily dictates samples electrophoretic mobility. Greater the charge, fast will be he migration of molecules. Molecules electropherotic mobility. Molecules which are larger have low electrophoretic mobility due to the friction experienced by them, while small molecules migration is fast. Molecules which are spherical experience lesser frictional force and electrophoretic retardation as compared to other.

2. **The Electric Field :** The force acting upon an ion of charge q is $\Delta E\ q / d$. The rate of migration under unit potential gradient is referred to as mobility of the ion. An increase in potential gradient increases the rate of migration.

   The current (total charge carried per second to the electrode) in the solution placed between two electrodes is carried mainly by the buffer ions, and only a small proportion is carried by sample ions. An increase in the potential difference therefore increases the current. But as current is increased, temperature of solution increase resulting in increase in viscosity of sample, that can lead to retardation of molecule. Hence, maintaining temperature in important. Ideally the systems are covered with lid and have cooling systems.

3. **The Medium:** The medium must be inert and offer some principle for separation of molecules

(i) Adsorption: It is retention of component on the surface of supporting medium

(ii) Molecular Sieving : Supporting media such as polyacrylamide, agar, starch and sephadex, which are cross – linked structures give rise to porous gel beads. In sephadex the molecules larger than pores are excluded out of gel beads and migrate faster, while in case of polyacrylamide, starch and agarose the larger molecules have to squeeze through the pores, hence the their migration is retarted.

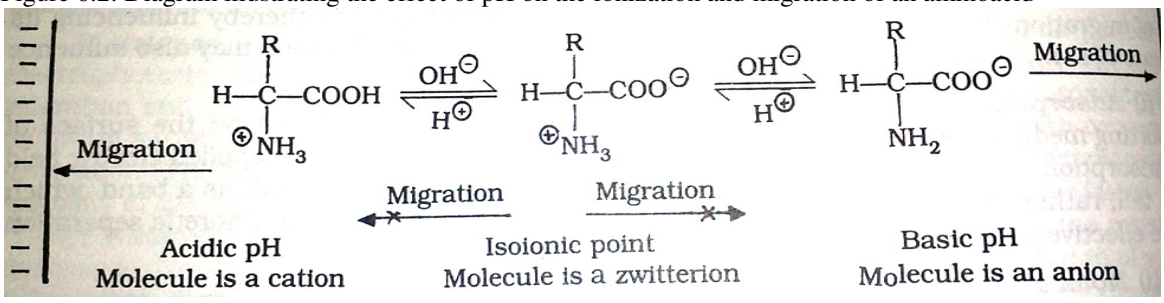
4. Buffer :- Buffer effects electrophoretic mobility of the sample in many ways

(i) Composition :- Commonly used buffers are formate, citrate, phosphate, EDTA, acetate, pyridine, Tris and barbitone etc. The buffer must not effect electrophoretic mobility of sample, hence care must be taken in its selection.

ii) Ionic strength.: Increase in ionic strength of buffer means large share of current as carried by buffer ions and only a small proportion is carried by sample ions. A decrease in ionic strength would mean an large share of current is being carried by sample ions leading to their faster movement. The ionic strength of buffer is usually maintained between 0.05 too 0.1M.

iii) pH : It determines the degree of ionization of organic compounds. Increase in pH increases ionization of organic acids and a decrease in pH increases the ionization of organic bases. For an ampholyte such as an amino acid, which has both acidic and basic properties, both the above effects apply (figure 6.2)

Figure 6.2: Diagram illustrating the effect of pH on the ionization and migration of an aminoacid



Types of Electrophoresis :-

Electrophoresis can be divided into two main types-

Free electrophoresis and zone electrophoresis

Free electrophoresis does not use any stabilizing medium while in zone electrophoresis a stabilizing medium is used.

1. Free electrophoresis : The best example for this is moving boundary electrophoretic technique (figure 6.3). The prototype for this technique was developed by A. Tiselius of Sweden in 1930s. R is a "U" tube having electrodes at the open end. Buffer is filled initially in the tube and the sample having a mixture of components say x, y & z is layered. The entire apparatus is placed in constant temperature bath insulated from external vibration. In the initial stage (figure 6.3A), the three molecular species are seen diffused throughout the 'U' tube. When power is switched on, the molecular species are seen to attain a net negative charge and start moving towards the anode.

Based on PH, the charge attained by molecule varies. As seen in figure 6.3B, the molecular species, X attained more negative charge and hence moved fast towards anode, followed by Y and then Z. The movement of molecules is observed by measuring refractive index along the walls of the glass tube. Rayleigh interference optics yields electrophoretic pattern that shows the direction and relative rates of migration of major molecules in the samples. For many years this techniques was popular and used for the observation of blood plasma components. An electrophoritic pattern as shown in figure 6.4 shown the serum protein composition of plasma. The major drawback with this technique is the heat produced as the convection currents increases. The samples keep moving as electrophoresis is conducted, but as the electric current is stopped, samples tend to diffuse. This does not allow satisfactory separation of constituents of a sample mixture.

Figure6.3: Moving Boundary Electrophoresis, indicating migration of solutes X,Y and Z

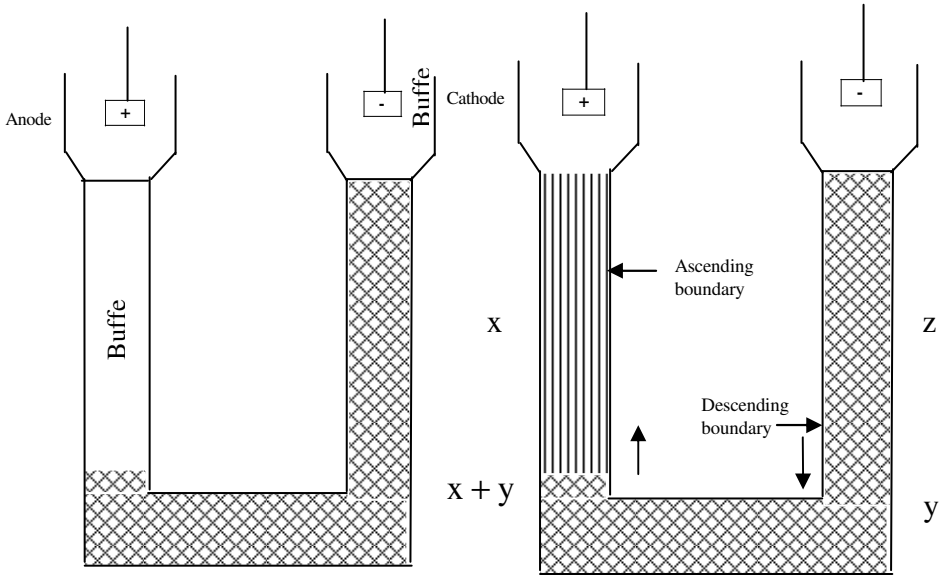
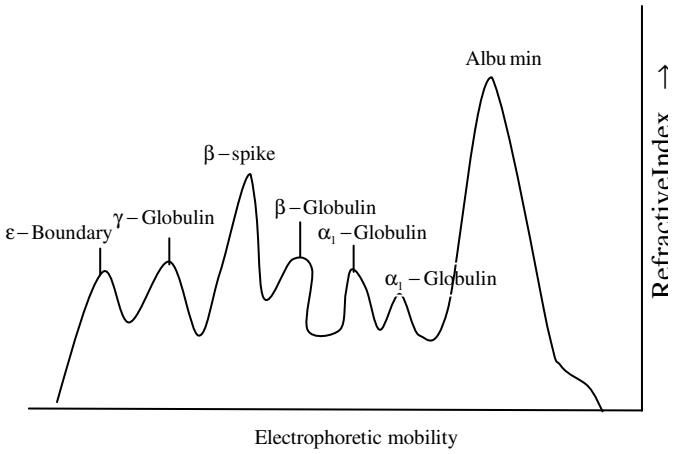


Figure 6.4: Representation of Electrophoretic pattern of serum proteins



2. Zone electrophoresis :- By the year 1937 alternatives methods were under investigation of which filter paper electrophoresis became popular. Research paved the way for other porous stabilizing agents which are gels like agar, starch and acrylamide, which are considered as stabilizing media. The advantage with this techniques is that the sample requirement is very less (Microliters). Upon separation the molecules are immobilized by fixation in different zones which are deterred by staining the supporting media.

TECHNIQUES IN ZONE ELECTROPHORESIS

PAPER ELECTROPHORESIS

In this technique a filter paper is used as the stabilizing medium. It is used for the study of normal and abnormal plasma proteins. A good quality filter paper having 95% cellulose and has very low adsorption capacity is used.

Apparatus: The equipment required for electrophoresis comprises of a power pack and an electrophoresis cell. The power pack provides a stabilized direct current and has control for voltage and power output of 0-500 V and 0-150mA respectively.

The electrophoretic cell (Figure 6.5) contains the electrodes, buffer reservoirs, a support for paper and a transparent insulating cover. Te electrodes are made up of platinum. The buffer reservoirs are partitioned into two sections which are interconnected. The outer section has the electrode, while the inner one is in contact with the supporting medium. The supporting medium is saturated with buffer, prior to the start of electrophoretic procedure. At times paper wick is used to connect the supporting medium to the buffer, while direct immersion of supporting medium, i.e., filer paper can be used to close the circuit. The paper is placed on an insulating material, usually a Perspex sheet. The whole electrophoresis unit is then covered with an insulating material to minimize evaporation during the run and also to ensure electrical insulation. The figure 6.5 shows both vertical and horizontal paper electrophoresis.

Sample application: Sample application is critical in paper electrophoresis. Usually strips of filter paper are used, hence during sample application it is applied as a spot or as a narrow uniform streak.

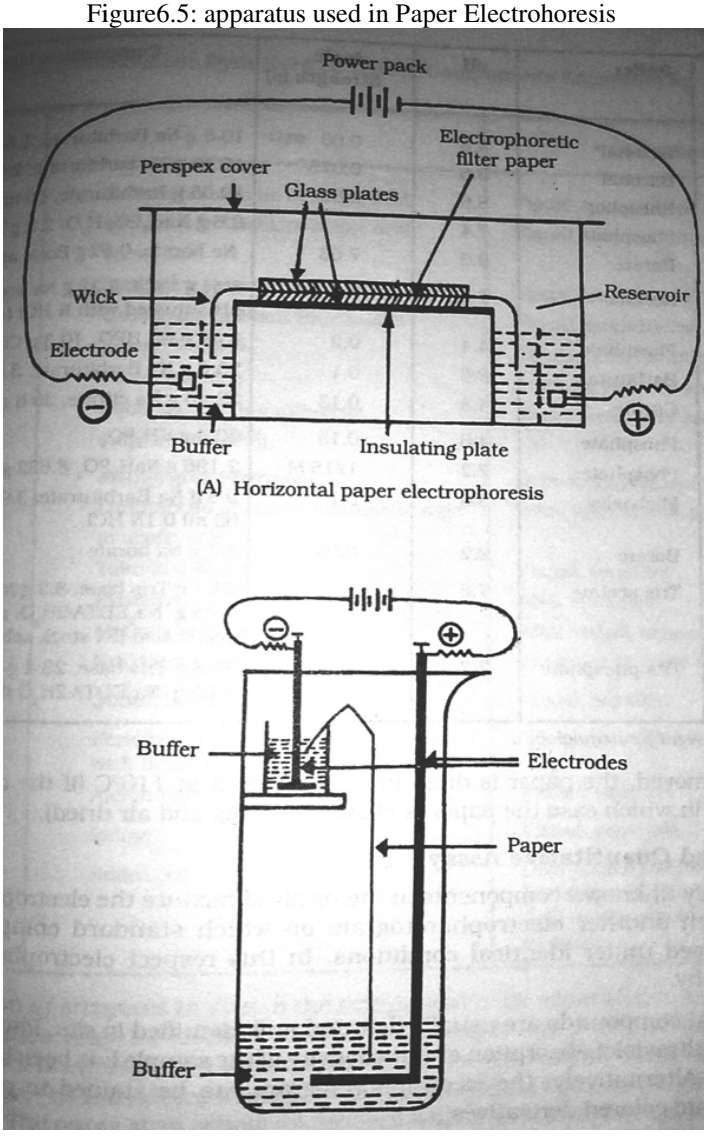
Electrophoretic run: The current is switched on and as the buffer ions move, based on the charge attained by solute molecules, they start separating and more towards anode or cathode. Buffer dictates the charge attained by solute molecules. The process usually does not take longer than two hours. The power is switched off after the run and before the paper is removed. The paper is dried in vacuum oven at 110°C before going for detection and quantitative assay.

Detection and Quantitative assay:

The resolved components in the electrophoretogram has to be visualized as most biomolecules are colorless. Hence the staining techniques were developed to view the components.

i) Fluoresance :- Staining of DNA and RNA samples with ethidium bromide helps their visualization under UV light. Similarity Fluorescamine can be used to stain amino acids, their derivations, peptides and proteins. Dansyl chloride is another stain that and be used to stain proteins.

Figure6.5: apparatus used in Paper Electrohoresis



ii) Direct ultraviolet absorption by samples: Proteins, peptides and nucleic acids absorb in the range 260-280mm which is detected by eluting the sample into buffer.

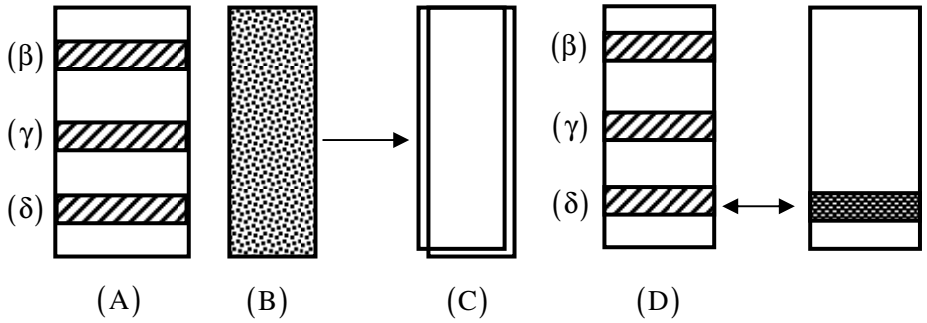
iii) Staining : Different dyes are used for detecting different compounds which are listed in the table below.

iv) Detection of enzymes in situ: If the component is an enzyme, it can be detected in situ then based on its location it can be detected in situ. Then based on its location it can be eluted. Another paper strip of same dimensions having coated with a chromogenic substrates is placed opposed to the migrated samples strip. Upon leaving for some duration when the substrates coated strip is observed, color development will be seen, and that indicates the location of enzyme on the electrophoretogram (figure 6.6).

Table- Representing stains that can be used for Biomolecules in Electrophoresis

Compound	Dye	Visualization
Proteins	Bromophenol blue in acetic acid	Visual quantification
	Nigrosine in Trichloro acetic acid	Visual, very sensitive
	Lissamine green in acetic acid	Visual, Quantitative
	Coomassie Brilliant Blue R-250	Visual, Quantitative
	Dansly Chloride	Fluorescent, quantitative
	Fluorescamine	Fluorescent, very sensitive
Nucleic acids	Methyl Green-Pyronine	DNA-Blue, RNA-Red
	Toluidine Blue	Visual sensitive
	Pyronine	RNA, sensitive
	Methylene Blue	RNA, Visual, sensitive
	Ethidium Bromide	Fluorescent, very sensitive
Lipoproteins	Sudan Black in 60% ethanol	Visual sensitive
Glycoproteins	Periodate oxidation + Treatment with Schiff's reagent	Visual, quantitative
	Alcian Blue	Visual , sensitive
Polysaccharides	Iodine	Visual, sensitive

Figure 6.6: Detection of enzymes in situ



A) A given sample containing enzyme has separated into four bands as determined by UV absorption, and one of these has enzyme
B) Paper strip having same size as electrophoretogram coated with crhomogenic substrate is taken
C) The paper strip is placed juxtaposed over the electrophoretogram
D) Upon removal, color is seen where enzyme is present on the electrophoretogram.

v) Quantitative estimation: This is done by eluting the sample in a suitable solvent. The color obtained is measured to obtain quantity of solute in the mixture.

In some cases, the paper as made transparent by immersion in organic mixtures of known refractive index (paraffin, oil bromonapthalens, anidoletic) the transparent paper is then placed between two glass plates and absorption of the coloured region is measured by using densitometer, which give approximates quantity of solute.

CELLULOSE ACETATE ELECTROPHORESIS

Cellulose acetate as medium was introduced by Kohn in 1958. It is now available as strips with highest purity and of uniform pore size. The disadvantage of adsorption phenomenon, during paper electrophoresis is over come completely by usage of cellulose strips.

Additional advantages of cellulose acetate strips are-

i) It is chemically pure and does not contain lignins, hemicelluloses or nitrogen.
ii) These strips are transparent and this makes them suitable for direct photoelectric scanning for separated bands of components.
iii) Due to low content of glucose, cellulose acetate strips are suitable for electrophoresis of polysaccharides. Upon staining by Schiff's base, back ground staining appearance is negligible (Contrast to cellulose strips).

Cellulose acetate electrophoresis is especially used for clinical investigations such as separation of glycoproteins, lipoproteins and hemoglobin from blood. The strips can also be used for immune diffusion and immuno electrophoresis. The buffer holding capacity is less and much better resolution is obtained in short span of time. The equipment is same as paper electrophoresis, including sample application and buffers usage.

Some solvents that render the strips transparent are, glacial acetic acid, whithmore oil 120, cotton seed oil, decalin, liquid paraffin etc. Transparency allows quantitative estimation of solute molecules.

GEL ELECTROPHORESIS

Usage of porous gels allows separation of molecules not just based on charge but even based on size of the molecule. Larger molecules movement within the gel will be retarted while allowing easy movement of smaller molecules. various types of gels are prepared to study biomolecules.

Types of gels :- Few important gels that are developed to enable study are

i) starchs gels
ii) Agar gels
iii) Polyacrylamide gels
iv) Agarose-Acrylamide gels

1. Starch gels : Introduced in 1955 by Smithies, starch gel was introduced for electrophoretic separation of biomolecules. Potato starch is hydrolyzed in acidified acetone at 37°C, and the suspension thus obtained is neutralized with sodium acetate and then washed with water. It is then treated with acetone and dried to get pure starch. This upon boiling in water or a buffer and cooling results in formation of gels (amuylopetin chain interwine to form a 3d matrix). The concentration of starch dictates porosity of gels. 2% starch gels are highly porous, while a 15% starch gel has very small pores. Both have their own advantages and disadvantages. High porosity does not resolve complex mixture but all own free movement and separation very large macromolecule. Low porosity gives better resolution while working with molecules of less molecular weight. Hence based upon the composition of mixture, gel percentage is decided.

Starch gels are unsatisfactory for separation of basic proteins. These are easily prone to fungal contamination. One major disadvantage with starch gels is that they turn opaque during staining, which prevents detection of samples.

ii) Agar gels: These are first described by Gordon in 1949 and are being increasingly used for biochemical separations. Agar is a polymer of galatose and comprises of two components agarose and agar pectin. The later component is sulphated and this charge can be detrimental during separation process. Hence, sulphate groups are removed during its purification. Agarose can be separation from agaropectin. For this, a solution of agar is prepared, kept at 80°C and to this equal volume of 40% polyethylene glycol is added. This precipitates agarose, which is collected washed with distilled water and dread with acetone.

Agar solubilizes in aqueous buffers at temperatures above 40°C and they set as gels at temperature below 38°C. The quantity of agar taken dictates the pore size of gels, and the gels allow good molecular sieving i.e, separation of molecules based on size. They have low diffusion resistance and are consequently of much use in immune electrophoresis, for detection of antigenic proteins. These are now better used for separation of nucleic acids and proteins.

iii) Polyacrylamide : The components used for the preparation of gel are neurotoxins. The chemicals used for preparation of matrix are,

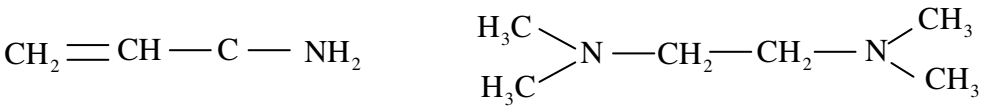
Acrylamide monomers
N,N'- methylene bisacrylamide (bio)
Ammounium persulphate and
Tetra methylene diamine (T E M E D)

The chemical structures of these are shown in figure 6.7.

Ammounium persulphate when dissolved in water generates free radicals. These free radicals activate acrylamide monomer inducing them to form long chains. These chains become cross become cross linked by N, N'-methylene bisacrylamide. T E M E D acts as a catalyst. The entire sequence of events in polyacrylamide gel formation are represented in figure 6.8 The pore size of

gel is determined by the amount of acrylamide used per unit volume of reaction medium and the degree of reaction medium and the degree of cross linking depends upon of these two components hence dictates pore size. Poly acrylamide gels are best used for separation of proteins and nucleic acids. They exhibit very low adsorption capacity, reproducible results are obtained as they lack electroosmosis and are best suited for in situ histochemical and qualitative analysis.

Figure 6.7: Components of Polyacrylamide gel



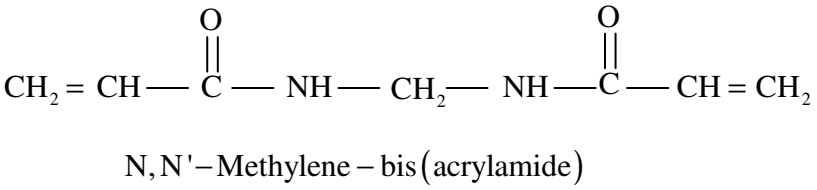
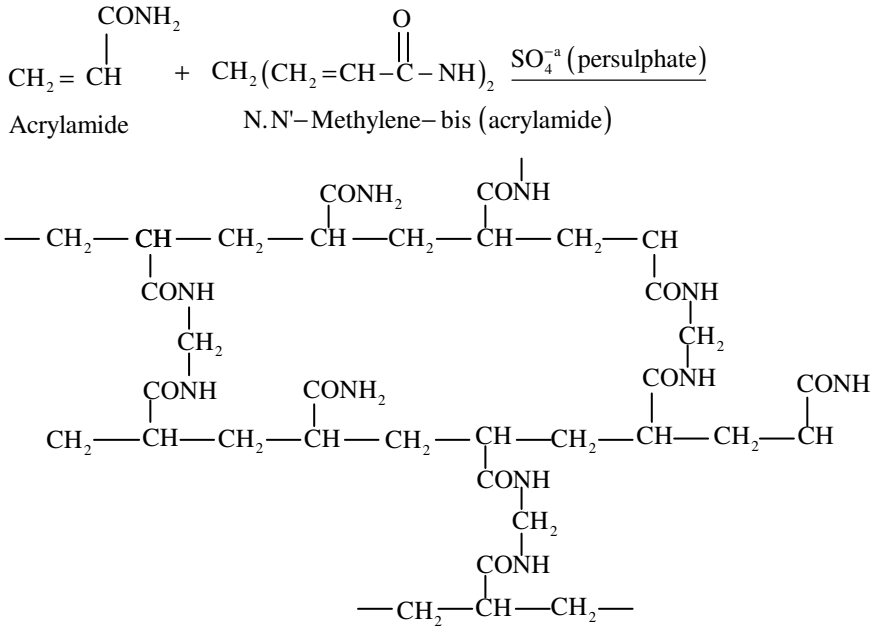


Figure 6.8:Reaction involved in polyacrylmaide gel formation



iv) Agarose acrylamide gels: These are complex gels that allow separation of large molecules (> 200kd). Usage of agarose provides mechanical support to the low percentage of polyacrylamide gels that have best sieving action. These gels enable to overcome the drawbacks of agarose gel i.e, has less resolution capacity and that of polyacrylamide i.e, low concentration of acrylamide yields

large pore, but a liquid matrix. These are overcome by agarose - acrylamide gels. The preparation of gels involved, initial preparation of 0.5% agarose gel and bringing its temperature down to 40°C. At this temperature, reagents required for polyacrylamide gel formation are added. They are mixed thoroughly and then added to the mould for setting.

Solubilizers : Proteins are made up of polypeptide chains whose structure is stabilized by hydrogen bonding, disulphide bridges hydrophobic association. When these proteins migrates in a gel electrophoresis, they are seen as single band. Subunit structure can be obtained only upon destabilization of this quaternary structure. The substances used for this propose are teamed as solubilizers, examples include Urea, Sodium dodecyl sulphate and □-mercapto ethanol.

i) Urea :- It is used to disrupt hydrogen bonds which are common and enable macromolecules to maintain structure. Concentrations of 3 – 12M urea are added in the gel in which electrophoresis is conducted.

Addition of urea enabled to identify that □-globulin has light and heavy chains. It is also used in studies with α and β casein, hepatoglobins, ribosomal proteins, double stranded natural of DNA etc.

ii) Sodium dodecyl sulphate $\left(CH_3(CH_2)_{10}CH_2OSO_3^-Na^+\right)$: It is an anionic detergent that disrupts hydrophobic association. SDS binding also imparts a large negative charge to the denatured polypeptide. This will result in migration of molecules based on size and not or charge.

Cetyltrimethyl ammonium bromide (CTAB) is used as a cationic detergent which can be used in place of SDS.

iii) β mercaptoethanol :- This is used to disrupt disulphide bridges. The bonds found in proteins are broken by heating the protein solution in the presence of mercaptoethanol. An alkylating agent Iodoacetate is added to prevent reformation of broken disulphide bonds.

Electrophoretic procedure :-

The equipment comprises of two components

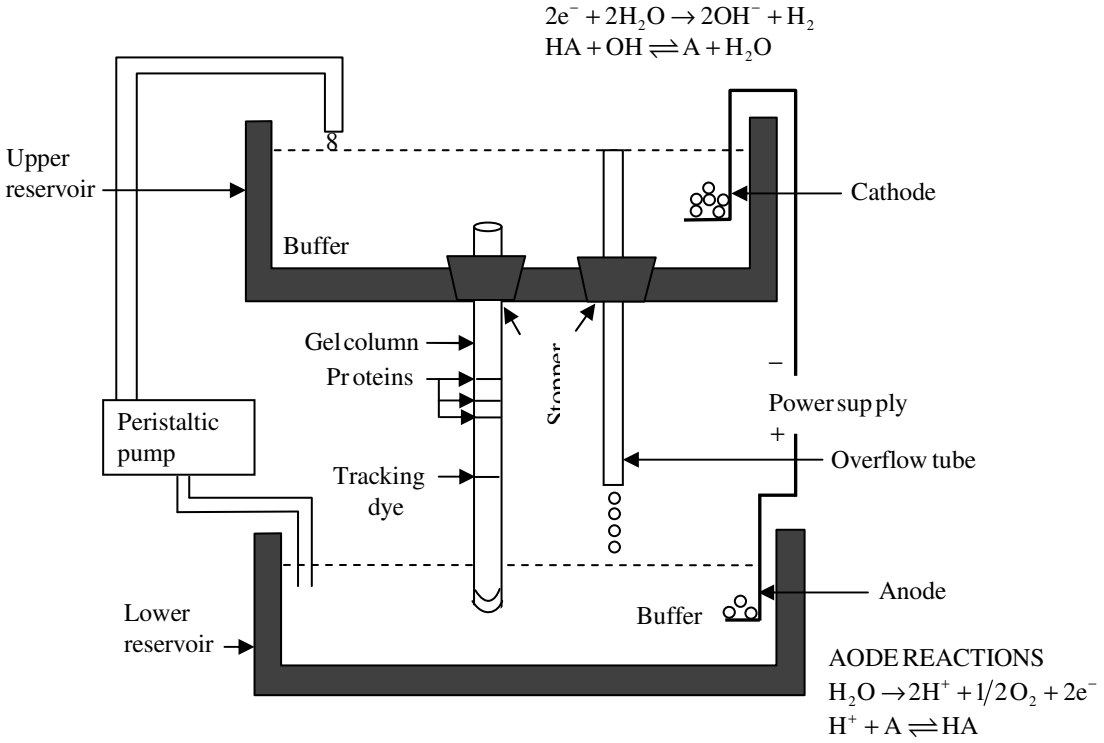
i) A buffer reservoir and
ii) A D.C power supply (figure 6.9)

The buffer reservoir system comprises of an upper reservoir and a lower reservoir each having platinum electrodes. Gel connects the two reservoirs, and if gel in removed there exists no electrical connection. The electrodes are connected to terminals extending from the top of the unit. The whole assembly are covered by Perspex shield.

The sample in this procedure is to be added into wells which are immersed in the buffer. Direct addition of sample causes diffusion of solute into buffer tank which can never contribution to sample separation. To avoid this, the density of sample is increased by the addition of glycerol or Ficoll. A tracking dye like bromophenol blue is mixed with sample which acts as an index of electrophoresis process. The dye migrates faster than macro molecules. The electrophoretic process is stopped when the dye reaches around three fourth of the gel.

The pH of the gel is also kept at 9, which imparts negative charge to macromolecules and hence anode is placed in the lower buffer reservoir.

Figure 6.9: Electrophoretic system with buffer recycling system



When the power is switched on, bubble formation is seen at both the electrodes, while more bubbles are seen at anode (figure 6.9). It is due to electrolysis of water producing hydrogen at cathode and oxygen at the anode. For each mole of hydrogen produced at the cathode only one half mole of oxygen is produced at anode, leading to formation of less number of bubbles. If electrophoresis is run for longer duration, it will lead to decrease in buffer level in the upper reservoir, which is replenished by pumping buffer from the lower reservoir using peristaltic pump. An over flow tube placed in upper reservoir prevents undue increase in its volume, and the over flow goes back to the lower reservoir.

Modes of gel electrophoresis:

Gel electrophoresis is carried out in two modes,

i) Column electrophoresis and

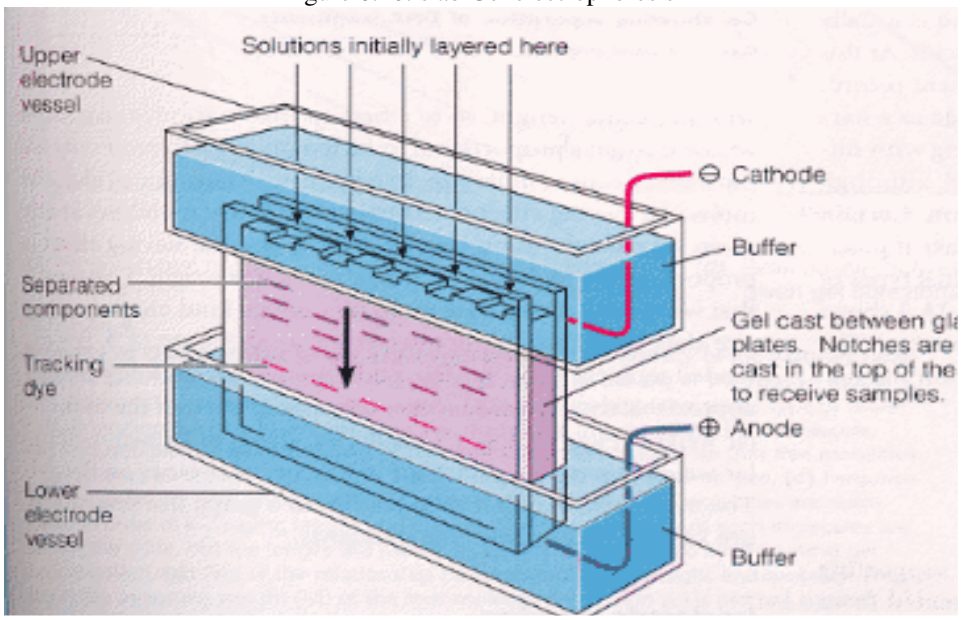
ii) Slab gel electrophoresis.

Column Electrophoresis :-The gel is set or polymerized in a column. It is then fitted between upper and lower reservoirs. At a time 8 to 12 columns can be fitted into buffer reservoirs (Figure 6.9).

Slab gel electrophoresis :- The gel is set or polymerized into a thin slab between two glass plates. The thickness of the slab of gel is adjusted by using appropriate spacers whose thickness is important. Sample wells are made at one end of the gel by placing a comb-shaped jig into the gel

before it sets or polymerizes. After the gel is set, the gel plates are set to the apparatus, buffer reservoirs are filled with buffer and then comb is removed. This leaves wells into which sample can be loaded (figure 6.10). The slab gel electrophoresis became much popular in the field of molecular biology.

Figure 6.10: slab Gel electrophoresis



Detection, Recovery and Estimation:

To detect the samples separated in gels, the gel is to be taken out first. In the case of column, gel is removed by forcing water using hypodermic syringe around the walls of column which extrudes the gel. Slab gels are removed by introducing a thin metal plates between the two gel plates and separating them apart. The gels are then immediately exposed to 7% acetic acid, which is a fixative that prevents diffusion of sample,. These gels are then subjected to appropriate stains. This can make the gel opaque, hence they are treated with glycerol and acetic acid which renders the gel transparent allowing visualization of separated molecules.

To perform quantitative analysis, the gels are subjected to various treatments. Starch gels are cut in the places where sample is present and it then exposed to amylases, that solubilize starch, bringing the solute into solution. Polyacrylamide gels on the other hand are rubbery and thin. These are cut into required size and is squeezed out through microsyringe or by using eppendorfs having small pore. As the gel strip extrudes, it is collected in a buffer, centrifuged and the supernatant is used for quantitative determination of solutes.

At times radio labeled samples may be used. These gels are subjected to autoradiography that enables visualization. However, they are pretreated with H_2O at 60°C to eliminate radioactive quenching by gel polymers.

Applications of gel electrophoresis

Gel electrophoresis has wide variety of analytical applications:

a)Applications in Molecular Biology:

i) DNA sequencing: DNA Sequencing is done by Maxam and Gilbert techniques and dideoxy nucleotide technique of Sanger. Both techniques use polyacrylamide gel electrophoresis which enables preparation of fragments that vary with respect to single nucleotide.

ii) Southern and Northern blotting: Southern blotting is used to identify the presence of a desired DNA sequence, while Northern blotting enables to identify RNA sequences. In both the techniques, the DNA or RNA sample is subjected to denaturation, electrophoresis followed by blotting onto nitrocellulose membrane. Gel electrophoresis is used during separation of sequences.

iii) Restriction mapping of DNA : This techniques is used to study genomic DNA or Cloned DNA. The technique involves, digesting the DNA using restriction enzymes, their separation by agarose or polyacrylamide gel electrophoresis and then visualization of fragments. The fragments are estimated for their size. The map of fragments generated is unique for a DNA. In this separation of fragments is done by gel electrophoresis.

iv) DNA foot printing: This technique is used to identify regions of DNA which interact with proteins. The fragmented DNA before binding to protein and after binding are separated by gel electrophoresis.

v) Restriction fragment length polymorphism (RFLP): It is used to identify mutations in various genes in carcinogenesis and other diseases uses gel electrophoresis.

vi) Used to study various conformations of DNA or all nucleic acids.

b) Applications in Protein study :

i) To determine subunit Structure of proteins by using solubilizers or SDS-PAGE.

ii) To determine molecular weight of proteins by using gel electrophoresis. SDS-PAGE is used as in this gel electrophoresis, the migration of molecules is purely based on molecular weight. A marker lane is added in one well in which proteins of known molecular weight are added. Comparing the unknown with known bands, molecular weight of proteins as well as nucleic acids is determined.

SPECIALIZED ELECTROPHORETIC TECHNIQUES.

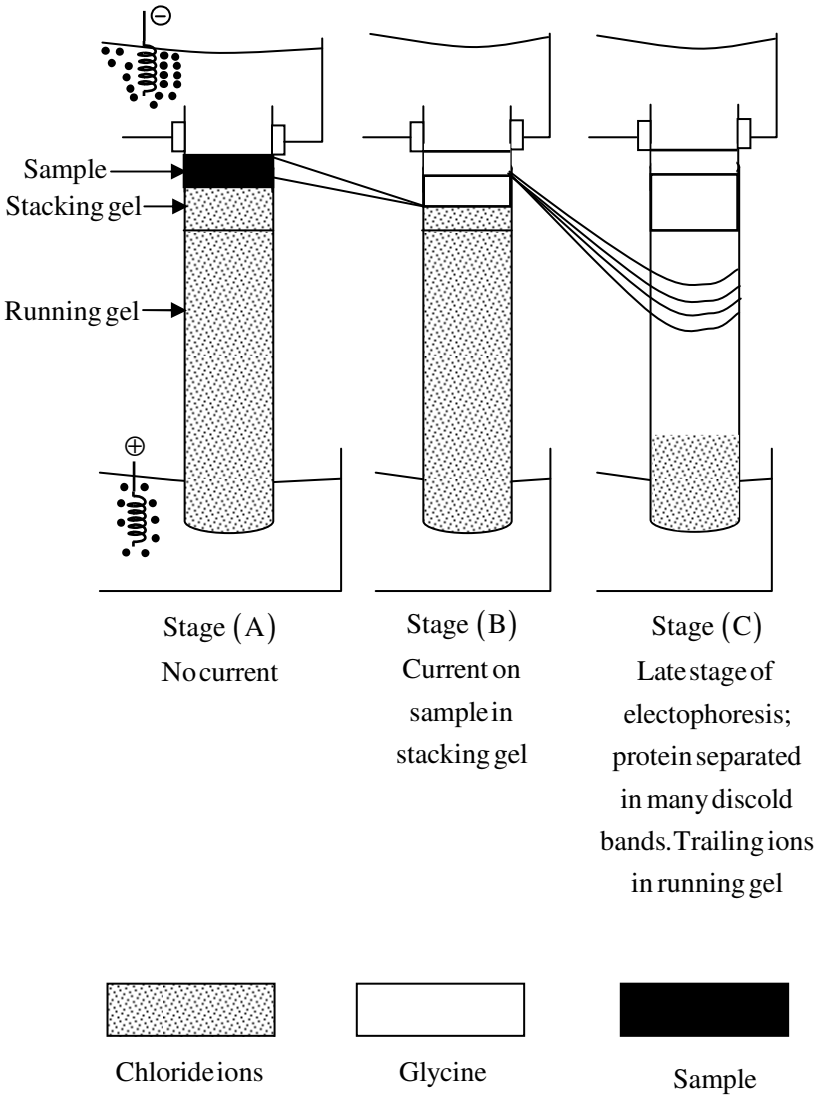
DISCONTINUOUS (DISC) GEL ELECTROPHORESIS

During sample addition onto gel or into gel wells, even if utmost care is taken, sample can ever be loaded as a sharp band and this diffused layer of sample impedes in a sharp resolution of the components. Disc gel electrophoresis is so called because of the discontinuous buffer employed and discoid appearance of the macromolecular zones. It is a modification of conventional zone electrophoresis, which allows the sample to enter the gel as a sharp band, thereby helping further resolution. Here, the macromolecular mixture to be analyzed is subjected to an electric field in a retarding gel support that is separated into two sections differing in porosity and buffered at different pH's. The macromolecular mixture migrates from the more porous into the less porous gel, a process accompanied by a change in pH. As a result, each macromolecular species becomes

concentrated into a very thin, sharp band, producing much higher resolution than that can be achieved in a continuous buffer.

The gel that is preferred for disc gel electrophoresis is polycrylamide. The two different porosity gels used are known as the stacking gel (high porosity) and separating or running gel (low porosity). The two gel system example is illustrated in figure 6.11.

Figure 6.11: Schematic representation of disc gel electrophoresis showing migration of ions at various stages



Separating gel : The lower gel is called as separating gel or running gel which is prepared by using 5 – 10 % acrylamide. Consequently the pores in this gel are numerous and are of smaller diameter, imparting molecular sieving property to the gel. The buffer used in this gel preparation is Tris buffer, whose pH is adjusted to around 8.3, using hydrochloric acid. This gel constitutes 2/3rds of the column length or the gel plates.

Stacking gel : This is the second layer of gel which is polymerized on the separating gel. The concentration of acrylamide is kept very low (2.3%) making it highly porous and is devoid of any sieving action.

Buffer used in its preparation is identical to separating gel while its pH is about two units lower than separating gel (pH-6.7).

The buffer used in the preparation of sample is identical to stacking gel, and the buffer added to both the reservoirs is identical to separating gel. The pH of buffer in reservoirs is adjusted using glycine.

Electrophoretic process :

Glycine in the upper buffer reservoir exists in two forms- as a zwitter ion which does not have a net charge, and as a glycinate anion with a charge of minus one.

$NH_3CH_2COO^- \rightleftharpoons NH_2H_2COO^-H^+$

Zwitterions Negative charge.

When the power is switched on, Chloride, protein and glycinate anions begin to migrate toward the anode. Upon entering the stacking gel, the glycenate ions encounter a condition of low pH which shifts the equilibrium towards formation of zwitterions, which has zero charge and hence immobile. The immobility of glycine zwitterions to migrates into the stacking gel coupled with high mobility of the chloride ions creats a very high localized voltage gradient between the leading chloride and the trailing glycinate ions. Since proteins have their mobility intermediate between the trailing and the leading ions, they carry the current in this region and migrate rapidly in this strong local electric field. The proteins, however cannot over take the chloride ions as the strong local field exists only between the chloride and the glycinate ions. As a result, the proteins migrate quickly until they reach the region rich in chloride ions and then drastically slow down. This two speed movement of the proteins results in piling up of the proteins sample into a tight, sharp disc between the glycinate and the chloride ions. It is in this sharp band form that the macro molecules enter the running gel. The smaller pores of the running gel retard the movement of the sharp band of the macromolecules for duration that is sufficient for glycinate to catch up to form anions. As the pH in running gel is higher, glycine ions become fully charged again and the localized high voltage gradient disappears. From now on, the protein separation takes place as in zone electrophoresis, but sharp bands are seen.

GRADIENT GEL ELECTROPHORESIS

A Pore- gradient gel is generated and the size of pores goes an continuously decreasing in the direction in which the macromolecules migrate in an electric field. The gel used in polyacrylmamide and decrease in pore size in achieved by increments in acrylamide concentration. When electric field is applied initially the macro molecules move according to their electrophoretic mobility and as the pore size decreases, the migration of molecules is based on size.

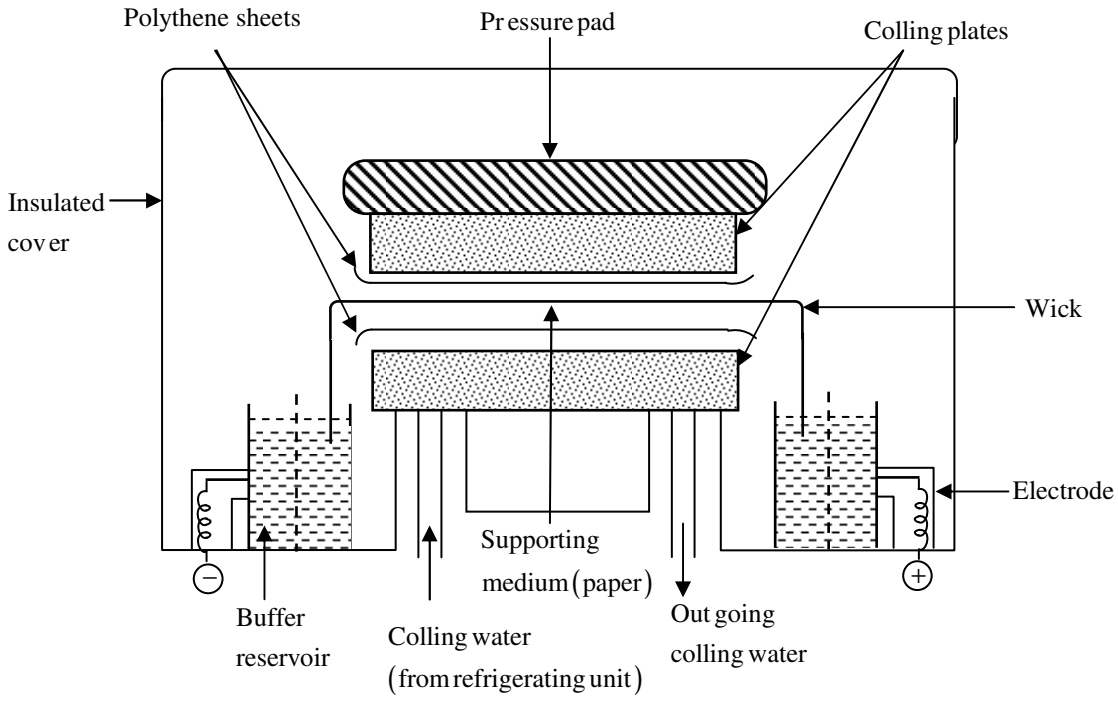
HIGH VOLTAGE ELECTROPHORESIS (H.V.E)

Low molecular weight substances when subjected to conventional electrophoresis, exhibit the tendency to diffuse back, leading to unsatisfactory resolution of sample mixture. To overcome this problem, High voltages of the order of 2500 to 10000V are used. This gives better resolution, better yield and very rapid separation.

The components of HVE are same as conventional electrophoresis except that these are equipped with cooling system, as high temperatures are generated due to usage of high voltage. Coolents like chlorobenzene, toluene are used, but if temperatures of above 10,000V are used water – glyerol mixture or water are used to cool the system. The whole apparatus is covered with an insulating surface to avoid accidents which may be fatal.

The equipment is represented in figure 6.12. The contact between the paper strip and the buffer compartment in made by means of pads of wet filter paper enclosed in cellophane sleeves. The buffer vessels are usually made up of thick sheet of polythene. The communication between the two halves of buffer vessel is afforded by a narrow slit between the two. The slit is usually plugged with glass wool and the electrodes are usually platinum.

Figure 6.12: Diagram of High voltage Electrophoresis unit



H.V.E can be combined with chromategraphy too. This allows best separation of amino acids, peptides and amines. Sample application is done at the centre of the strip. The paper can be equilibrated two times with two different buffers and thus yield superlative resolution of amino acids and peptides.

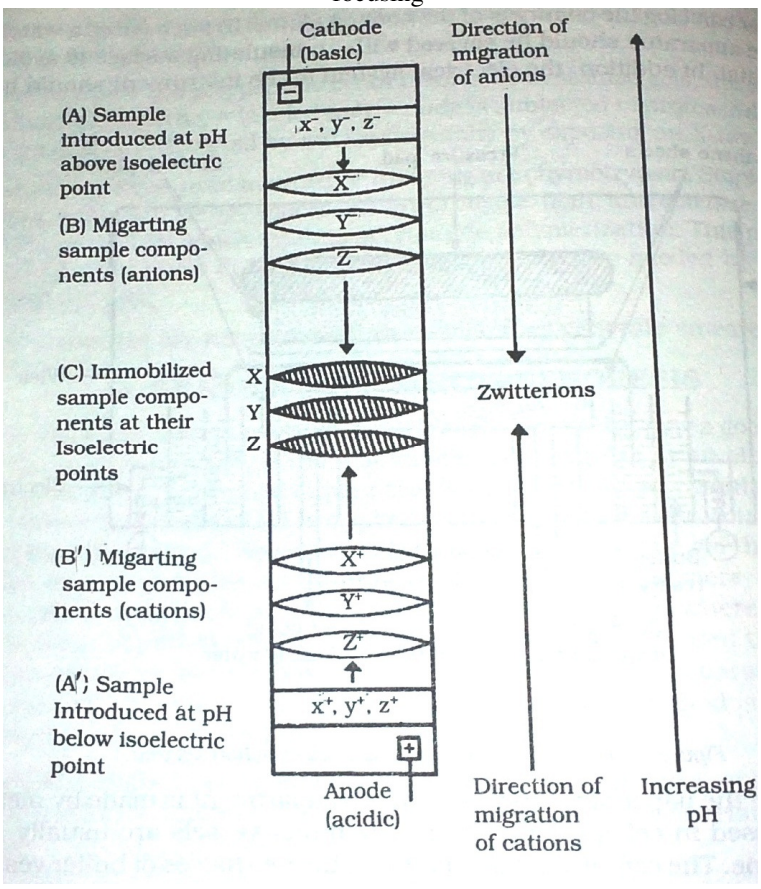
ISOELECTRIC FOCUSSING

This technique is discovered by H.Svenson of Sweden and has very high resolution power. If plasma proteins are subjected to isoelectric focusing, it is resolved into around 40 bands, indicating its resolving power.

Proteins when placed in acidic solution attain positive charge, while in basic solution they attain negative charge. At certain value of pH, called the isoionic point, the net charge on the protein is zero. In conventional electrophoresis, the pH between anode to cathode is constant and the positively charged ions migrate towards the cathode and negative ions migrate to anode. In isoelectric focussing, a stable pH gradient is generated with pH increasing from anode to cathode. A protein introduced into this system at a point where the pH is lower than its isotonic point, it will possess a net positive charge and will migrate to cathode. As the protein moves, it will be influenced by the buffer and its net charge varies. When the protein encounters a pH where its net charge is zero, its movement will stop. This is the isoelectric point of the protein. In this technique point of application of sample is not critical and diffusion is not a problem in this. Thus, once a final, stable focussing is reached, the resolution will be retained even if the experiment is continued for longer duration.

A schematic representation of the principle of isoelectric focusing is represented in figure 6.13.

Figure 6.13: Schematic representation of principle of Isoelectric focusing



Carrier ampholytes are used to generate pH gradient. These are isomers and homolog's of aliphatic polyaminopolycarboxylic acids which are special buffer substances.
Their general formula is.

$$R-N-(CH_2)_n-N-(CH_2)_n-COOH$$

Where R can be

$$-(CH_2)_n-COOH, H, \text{or} -(CH_2)_n-\underset{\underset{R}{|}}{N}-R$$

Applications :-
Used for separation and identification of proteins.
It is being used by the food and agricultural industries, forensic and human genetics laboratories, and for research in enzymology, immunology and membrane biochemistry etc.

CAPILLARY ELECTROPHORESIS (CE)
This is a new generation electrophoretic technique which is developed to overcome the disadvantages of conventional technique. Few disadvantages of conventional electrophoresis include.
- Conventional electrophoresis apparatus offer low level of automation.
- Long analysis times are needed.
- Detection of separated bands is not done online, bands are visualized by staining after separation.
- Since excessive generation of heat causes loss of separation, only low voltages can be applied : this adds to long analysis time.

During late 1980's, Jorgenson and Lukacs popularized capillary electrophoresis which has the following advantages :
-Very high level of automation is possible
- Fast analysis time.
- Detection of separated peaks is done online.
-Heat generated inside capillary is effectively dissipated through the walls of the capillary; therefore, high voltages can be applied.
-High voltages means a rapid separation. This along with online detection enable faster analysis.

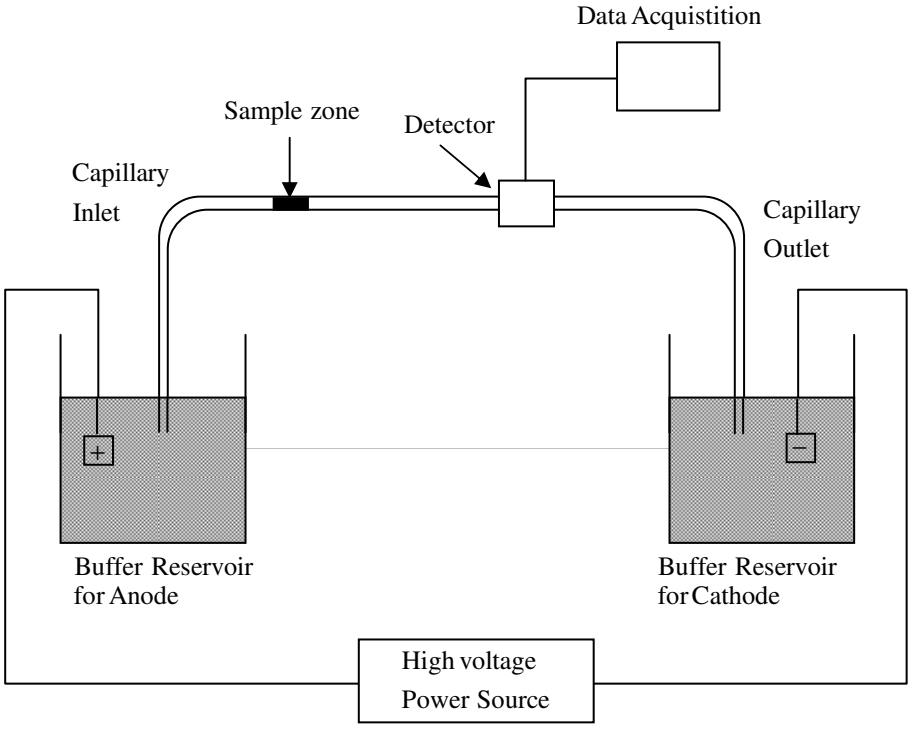
The capillary electrophoresis technique has advanced further, bringing about several variations which include.
-Separation based on size and charge, difference between solutes, as in the techniques - Capillary zone electrophoresis (CZE) or free solution capillary electrophoresis (FSCE).
-Separation of neutral compounds using surfactant micelles; as in Micellar electrokinetic capillary electrophoresis chromatography (MECC).
-Separation by sieving of solutes through a gel network, the technique is termed capillary gel electrophoresis (GCE).

-Separation of zwitter ion solutes within a pH gradient, termed Capillary Isoelectric focusing (CIEF).
- If electrophoresis and chromatography principles are employed, it is termed as Capillary Electrochromatography.

Equipment :-

If comprises of a capillary tube connecting two buffer reservoirs. Electrodes are present dipped in the two reservoirs. The capillary tube passes through a detector which allows online detection of process (figure 6.14).

Figure 6.14: A schematic drawing of capillary electrophoresis



CE system involves application of high voltage across a narrow bore (25-100mm) capillary. The capillary is initially filled with electrolyte and both the ends are dipped in buffer reservoirs which closes the electrical circuit. A small volume of sample is injected at one end of the capillary and high voltage is applied. As the samples migrate, they pass through the detector. The computer attached to detector generates a signal for each sample. The voltage used in this CE are in the range 10-30KV. The capillary tubes used are designed to have enhanced mechanical strength. Hence fused silica capillaries are externally coated with polyimide, which offers mechanical strength and does not interfere with detection procedure. Their length varies from 25-100cm with their inner diameter varies from 50 to 75 microns.

PRACTICE QUESTIONS

1. The velocity of vibration of particle during electrophoresis is inversly proportional to
   (A) Field strength
   (B) Charge, particle size
   (C) Particle size, viscosity
   (D) Field strength & viscosity

2. The advantage of using cellulose acetate paper, rather than pure cellulose paper in paper electrophoresis include.
   (A) Adsorption of solute does not take place
   (B) offer better resolution in short time
   (C) paper holds very little buffer
   (D) All of the above

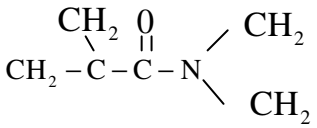
3. Which among the following use voltages of 2500-10,000 V and offer better resolution, vapidly on paper strips?
   (A) Paper electrophoresis
   (B) Capillary electrophoresis
   (C) High voltage electrophoresis
   (D) Cellulose acetate electrophoresis

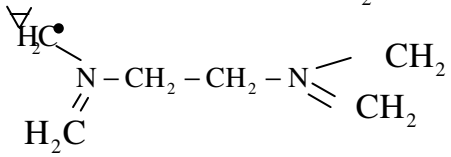
4. Isoelectric focusing exploits the following principle
   (A) Electrophoretic mobility and pI difference in solutes
   (B) Electrophoretic stability and charge
   (C) pK values of solutes
   (D) All of the above

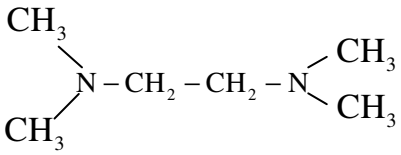
5. The capillaries used in capillary electrophoresis are coated with polyimide which contributes to
   (A) Makes tubes transparent
   (B) Allows UV to penetrate
   (C) Makes tubes hydrophobic
   (D) Increases mechanical strength

6. DNA sequencing and DNA foot printing can be done using
   (A) Ion-exchange chromatography and HVPE
   (B) Polyacrylamide gel electrophoresis
   (C) All electrophoretic methods
   (D) Electrophoresis and IR spectroscopy

7. Polyacrylamide gel electrophoresis involves preparation of polyacrylamide gel. It requires four components to be mixed.
   Which among the following represents TEMED ?

(A) 

(B) 

(C) 

(D) $CH_2=CH-C-NH-CH_2-NH-C-CH=CH_2$

8. Polyacrylamide gel electrophoresis involves preparation of polyacrylamide gel. It requires four components to be mixed.

Match the solubilizers with the types of bonds they break

(a) Urea	1. - S – S – bridges
(b) SDS	2. Hydrogen bonds
(c) β- mercaptoethanol	3. Hydrophobic associations
(A) a-1, b-3, c-2	(B) a-3, b-2, c-1
(C) a-2, b-1, c-3	(D) a-1, b-3, c-1

9. In discontinuous gel electrophoresis two types of gels having different porosities are used.
The top gas having _____ % acrylamide is called _____ gel.
(A) 2.5% , stacking
(B) 7% , separating
(C) 2.5% , separating
(D) 7% , stacking

10. In discontinuous gel electrophoresis two types of gels having different porosities are used.
_____ gel uses _____ % of acrylamide and is the bottom gel is DISC gel electrophoresis.

(A) Stacking gel, 5-10%
(B) Separating gel, 5-10%
(C) Stacking gel, 2-3%
(D) Separating gel, 2-3%

ANSWER KEYS

1	C	**2**	D	**3**	C	**4**	A	**5**	D	**6**	B	**7**	C
8	D	**9**	A	**10**	B								

EXPLANATIONS

1. Form the quotation $V=\frac{\Delta Eq}{6\pi rnd}$

Velocity of molecule is directly proportional to field strength and charge of molecule but inversly proportional to particle size and viscosity

2. The advantage of using cellulose acetate paper, rather than pure cellulose paper in paper electrophoresis include.
(A) Adsorption of solute does not take place
(B) Offer better resolution in short time
(C) Paper holds very little buffer

3. High voltage electrophoresis uses high voltages of 2500-10,000V. This is a paper electrophoresis and is equipped with cooling plates

4. Isoelectric focusing separates charged particles based on their pI value

5. Polyimide coating to capillary tubes increases their mechenical strength and prevents their breakage.

6. Polyacrylauide gels offer better resolution of samples and hence are suitable for DNA sequencing and DNA foot printing

7. TEMED is tetramethylene diamine

8. urea – hydrogen bonds

SDS breaks hydropholic associations

β- mercaptoethanol breaks disulphide bridges.

9. Separating gel is the top gel which has high porosity and is used at 2-3% acrylamide.

10. Separating gel or running gel uses 5-10% acrylamide. It is the gel that separates molecules based on size.

7. Microarrays

Introduction to Microarray

Molecular Biology research evolves through the development of the technologies used for carrying them out. It is not possible to research on a large number of genes using traditional methods. DNA Microarray is one such technology which enables the researchers to investigate and address issues which were once thought to be non traceable. One can analyze the expression of many genes in a single reaction quickly and in an efficient manner. DNA Microarray technology has empowered the scientific community to understand the fundamental aspects underlining the growth and development of life as well as to explore the genetic causes of anomalies occurring in the functioning of the human body.

A typical microarray experiment involves the hybridization of an mRNA molecule to the DNA template from which it is originated. Many DNA samples are used to construct an array. The amount of mRNA bound to each site on the array indicates the expression level of the various genes. This number may run in thousands. All the data is collected and a profile is generated for gene expression in the cell.

Microarray Technique

An array is an orderly arrangement of samples where matching of known and unknown DNA samples is done based on base pairing rules. An array experiment makes use of common assay systems such as microplates or standard blotting membranes. The sample spot sizes are typically less than 200 microns in diameter usually contain thousands of spots.

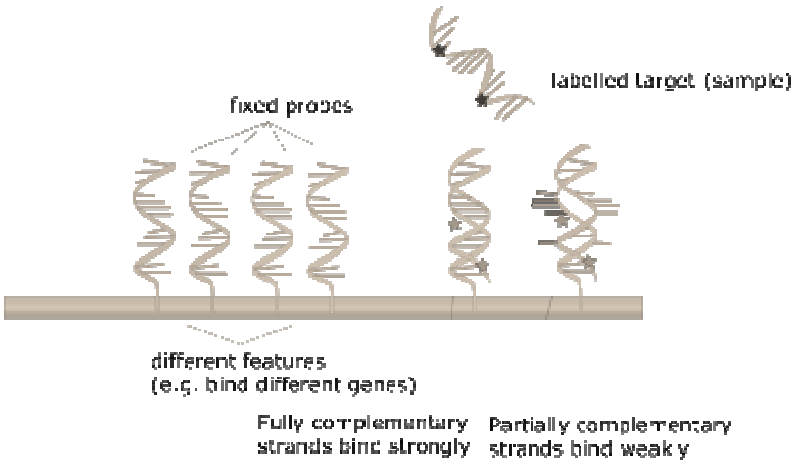
Thousands of spotted samples known as probes (with known identity) are immobilized on a solid support (a microscope glass slides or silicon chips or nylon membrane). The spots can be DNA, cDNA, or oligonucleotides. These are used to determine complementary binding of the unknown sequences thus allowing parallel analysis for gene expression and gene discovery. An experiment with a single DNA chip can provide information on thousands of genes simultaneously. An orderly arrangement of the probes on the support is important as the location of each spot on the array is used for the identification of a gene.

Principle:

The core principle behind microarrays is hybridization between two DNA strands, the property of complementary nucleic acid sequences to specifically pair with each other by forming hydrogen bonds between complementary nucleotide base pairs. A high number of complementary base pairs in a nucleotide sequence means tighter non-covalent bonding between the two strands. After washing off non-specific bonding sequences, only strongly paired strands will remain hybridized. Fluorescently labeled target sequences that bind to a probe sequence generate a signal that depends

on the hybridization conditions (such as temperature), and washing after hybridization. Total strength of the signal, from a spot (feature), depends upon the amount of target sample binding to the probes present on that spot (Figure 7.1). Microarrays use relative quantitation in which the intensity of a feature is compared to the intensity of the same feature under a different condition, and the identity of the feature is known by its position.

Figure 7.1: Principle of microarray



Steps involved in Microarray Technology

The principle of DNA microarray technology is based on the fact that complementary sequences of DNA can be used to hybridize immobilized DNA molecules. This involves three major multi-stage steps;

1- Manufacturing of microarrays: This step involves the availability of a chip or a glass slide with its special surface chemistry, the robotics used for producing microarrays by spotting the DNA (targets) onto the chip or for their *in situ* synthesis.

2- Sample preparation and array hybridization step: This step involves mRNA or DNA isolation followed by fluorescent labelling of cDNA probes and hybridization of the sample to the immobilized target DNA.

3- Image acquisition and data analysis: Finally, this step involves microarray scanning, and image analysis using sophisticated software programs that allows us to quantify and interpret the data.

However, here, we will concentrate on how microarray experiments are performed in the laboratory, rather than the technological developments involving array construction or manufacturing using precision robotic devices.

Typically, a microarray experiment involves the comparison of a query or experimental sample representing the expression pattern of genes in a specific set of conditions, with a control sample representing all the genes that are expressed in the cells/tissue to be analyzed.

An example of this is comparisons made between expression profiles of bacteria within infected cells (query) and the same bacteria cultured under standardized *in vitro* conditions of growth (control). Or similarly a comparison made between an isogenic mutant and the wild type strain.

There are four major steps in performing a typical microarray experiment (figure 7.2)

1. Sample preparation and labeling
2. Hybridization
3. Washing
4. Image acquisition and Data analysis
5.

Sample preparation and labelling

There are a number of different ways in which a DNA microarray sample is prepared and labelled. All of these different approaches however have their own advantages and disadvantages with respect to many factors such as the starting amount of RNA or DNA required, through to cost, time and data acquisition and transformation. However the choice of which one to use depends on these factors as well as the type of microarray technology used, for example the slide type and the detection equipment. Here, we used the term "sample" to refer to the free, fluorescently labelled cDNA, not to confuse with the immobilized DNA known as reporter element (s)

Initially, the sample preparation starts by isolating a total RNA containing messenger RNA that ideally represents a quantitative copy of genes expressed at the time of sample collection (experimental sample & reference sample). This step is crucial, simply because the overall success of any microarray experiment depends on the quality of the RNA.

For example purity in the sense of homogeneity or uniformity of the mRNA is a critical factor in the downstream hybridisation performance, particularly when fluorescence is used, as cellular proteins, lipids, and carbohydrates can mediate significant nonspecific binding of labeled cDNAs to matrix surfaces. The sample mRNA extracted from the biological sample of interest and the reference are then separately converted into complementary DNA (cDNA) using a reverse-transcriptase enzyme. This step also requires a short primer to initiate cDNA synthesis. Next, each cDNA (Sample and Control) are labelled with a different tracking molecule, often fluorescent cyanine dyes (i.e. Cy3 and Cy5)

Array hybridization

Hybridization is the process of joining two complementary strands of DNA to form a double-stranded molecule. Here, the labelled cDNA (Sample and Control) are mixed together, and then purified to remove contaminants such as primers, unincorporated nucleotides, cellular proteins, lipids, and carbohydrates. Purification is usually carried out using filter spin columns such as Qiaquick from Qiagen. After purification, the mixed labelled cDNA is competitively hybridized against denatured PCR product or cDNA molecules spotted on a glass slide. Ideally, each molecule in the labelled cDNA will only bind to its appropriate complementary target sequence on the immobilized array.

Before hybridization however, the microarray slides are incubated at high temperature with solutions of saline-sodium buffer (SSC), Sodium Dodecyl Sulfate (SDS) and bovine serum albumin (BSA) to reduce background due to nonspecific binding.

The slides are washed after hybridization, first to remove any labelled cDNA that did not hybridize on the array, and secondly to increase stringency of the experiment to reduce cross hybridization.

The later is achieved by either increasing the temperature or lowering the ionic strength of the buffers.

Image acquisition and data analysis is the final step of microarray experiments. The aim is to produce an image of the surface of the hybridized array. Here the slide is dried and placed into a laser scanner to determine how much labelled cDNA (probe) is bound to each target spot. Laser excitation of the incorporated targets yield an emission with characteristic spectra, which is measured using a confocal laser microscope. Classically, microarray software often uses green spots on the microarray to represent genes upregulated compared to control, red to represent those genes that are downregulated in the experimental sample, and yellow to represent those genes of equal abundance in both experimental and control samples.

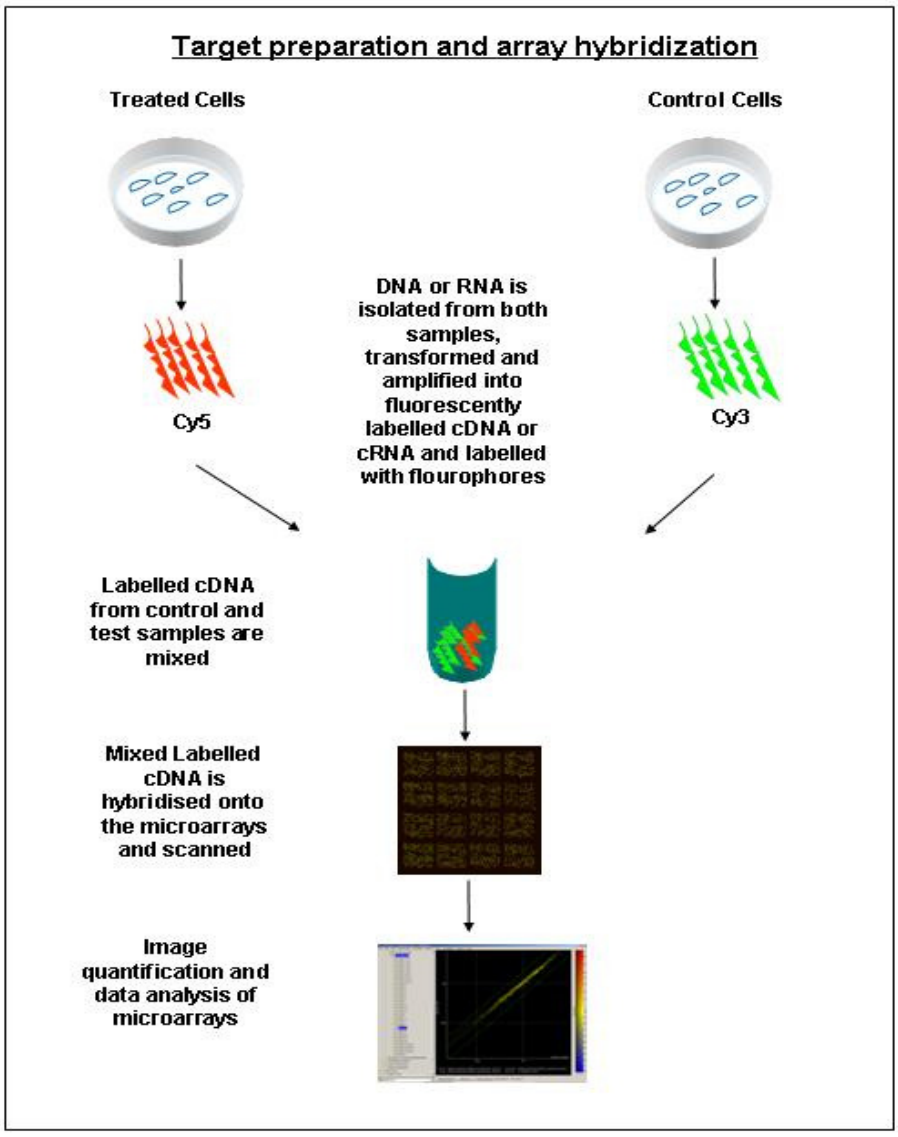


Figure 7.2. Microarray experimental principles.

Types of Microarrays

Depending upon the kind of immobilized sample used construct arrays and the information fetched, the Microarray experiments can be categorized in three ways:

1. **Microarray Expression Analysis:** In this experimental setup, the cDNA derived from the mRNA of known genes is immobilized. The sample has genes from both the normal as well as the diseased tissues. Spots with more intensity are obtained for diseased tissue gene if the gene is over expressed in the diseased condition. This expression pattern is then compared to the expression pattern of a gene responsible for a disease.
2. **Microarray for Mutation Analysis:** For this analysis, the researchers use gDNA. The genes might differ from each other by as less as a single nucleotide base. A single base difference between two sequences is known as Single Nucleotide Polymorphism (SNP) and detecting them is known as SNP detection.
3. **Comparative Genomic Hybridization:** It is used for the identification in the increase or decrease of the important chromosomal fragments harboring genes involved in a disease.

Application of microarryas

DNA microarrays can be used to detect DNA (as in comparative genomic hybridization), or detect RNA (most commonly as cDNA after reverse transcription) that may or may not be translated into proteins. The process of measuring gene expression via cDNA is called expression analysis or expression profiling.

Applications include:

Application or technology	**Synopsis**
Gene expression profiling	In an mRNA or gene expression profiling experiment the expression levels of thousands of genes are simultaneously monitored to study the effects of certain treatments, diseases, and developmental stages on gene expression. For example, microarray-based gene expression profiling can be used to identify genes whose expression is changed in response to pathogens or other organisms by comparing gene expression in infected to that in uninfected cells or tissues
Comparative genomic hybridization	Assessing genome content in different cells or closely related organisms
GeneID	Small microarrays to check IDs of organisms in food and feed (like GMO), mycoplasms in cell culture, or pathogens for disease detection, mostly combining PCR and microarray technology.
Chromatin immunoprecipitation on Chip	DNA sequences bound to a particular protein can be isolated by immunoprecipitating that protein, these fragments can be then hybridized to a microarray (such as a tiling array) allowing the

	determination of protein binding site occupancy throughout the genome. Example protein to immunoprecipitate are histone modifications (H3K27me3, H3K4me2, H3K9me3, etc.), Polycomb-group protein (PRC2:Suz12, PRC1:YY1) and trithorax-group protein (Ash1) to study the epigenetic landscape or RNA Polymerase II to study the transcription landscape.
SNP detection	Identifying single nucleotide polymorphism among alleles within or between populations. Several applications of microarrays make use of SNP detection, including genotyping, forensic analysis, measuring predisposition to disease, identifying drug-candidates, evaluating germline mutations in individuals or somatic mutations in cancers, assessing loss of heterozygosity, or genetic linkage analysis.
Alternative splicing detection	An *exon junction array* design uses probes specific to the expected or potential splice sites of predicted exons for a gene. It is of intermediate density, or coverage, to a typical gene expression array (with 1-3 probes per gene) and a genomic tiling array (with hundreds or thousands of probes per gene). It is used to assay the expression of alternative splice forms of a gene. Exon arrays have a different design, employing probes designed to detect each individual exon for known or predicted genes, and can be used for detecting different splicing isoforms.
Fusion genes microarray	A Fusion gene microarray can detect fusion transcripts, e.g. from cancer specimens. The principle behind this is building on the alternative splicing microarrays. The oligo design strategy enables combined measurements of chimeric transcript junctions with exon-wise measurements of individual fusion partners.
Tiling array	Genome tiling arrays consist of overlapping probes designed to densely represent a genomic region of interest, sometimes as large as an entire human chromosome. The purpose is to empirically detect expression of transcripts or alternatively spliced forms which may not have been previously known or predicted.
Double-stranded B-DNA microarrays	Right-handed double-stranded B-DNA microarrays can be used to characterize novel drugs and biologicals that can be employed to bind specific regions of immobilized, intact, double-stranded DNA. This approach can be used to inhibit gene expression. They also allow for characterization of their structure under different environmental conditions.
Double-stranded Z-DNA microarrays	Left-handed double-stranded Z-DNA microarrays can be used to identify short sequences of the alternative Z-DNA structure located

	within longer stretches of right-handed B-DNA genes (e.g., transcriptional enhancement, recombination, RNA editing). The microarrays also allow for characterization of their structure under different environmental conditions.
Multi-stranded DNA microarrays (triplex-DNA microarrays and quadruplex-DNA microarrays)	Multi-stranded DNA and RNA microarrays can be used to identify novel drugs that bind to these multi-stranded nucleic acid sequences. This approach can be used to discover new drugs and biologicals that have the ability to inhibit gene expression. These microarrays also allow for characterization of their structure under different environmental conditions.

PRACTICE QUESTIONS

1. Probes used in microarrays are
   (A) DNA (B) cDNA
   (C) Oligonucleotides (D) All of the above

2. The core principle in microarrays is

   (A) Spotting the DNA
   (B) Hybridization between two DNA strands
   (C) DNA chips
   (D) Fluorescence

3. Which among the following are applications of microarrays?

   (A) SNP detection & Fusion gene microarray
   (B) SNP detection & GFP detection
   (C) Protein profiling
   (D) All of the above

4. Arrange the steps involved in microarray experiment.

   (A) Hybridization
   (B) Sample proportion & labelling
   (C) Washing
   (D) Image acquisition and data analysis.

   (A) B, C, A, D (B) B, A, C, D
   (C) A, B, C, D (D) A, C, B, D

5. Which among the following is used to measure the functionality and expression of genes?

   (A) DNA variation screening
   (B) Gene expression profiling
   (C) Microarray comparision genetic hybridization
   (D) All of the above

ANSWER KEYS

1	D	**2**	B	**3**	A	**4**	B	**5**	B				

EXPLANATIONS

1. Thousands of spotted samples known as probes are immobilized on solid support. The spots can be DNA, CDNA or Oligonucleotides.

2. The core principle behind microarrays is hybridization between DNA strands.

3. Microarrays have several applications which include-
   Gene expression profiling
   Comparative genomic hybridization
   SNP detection
   Alternative splice detection
   Fusion gene microarray, etc

4. Steps involved include:
   - Sample preparation and labelling
   - Hybridization
   - Washing
   - Image acquisition and data analysis.

5. Gene expression profiling is the measurement of the activity of thousands of genes at once to create a picture of cellular function.

Reference Books:

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4. Electron Microscopy: The Basics by Bettina Voutou and Eleni-Chrysanthi Stefanaki