MICROBIOLOGICAL METHODS

(DMB02)

(MSC MICROBIOLOGY)

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1. M I C R O S C O P Y

1.0 OBJECTIVE

In this chapter resolving Power, Principles and light pathways of various light microscopes and electron microscope have been discussed.

1.1 INTRODUCTION

Although the use of lenses for magnification has been known for centuries, modern microscopy began when a Holland's eyeglass maker, Z. Jannsen, lined up two lenses to effectively multiply their individual magnifications. Subsequently, Robert Hook (1665) and Leeuwenhoek (1674) published the initial microscopic examinations of biological tissues.

A microscope can be compared with a human eye, since both have lens systems and in both cases images of the objects are formed. The most important principle involved in microscopy is to get a magnified image, in which structures may be resolved which otherwise cannot be resolved with the unaided eye.

1.1.1 Resolving Power

The ability of lens system to distinguish two adjacent points as distinct and separate or, in other words, the ability of microscope to produce separate images of closely placed objects is known as resolving power. This depends upon the wavelength of light and numerical aperture (NA).

The Swiss physicist E. Abbe developed the theory of microscope resolution and expressed the Resolution Limit (RL) in the following formula:

$$RL = \frac{0.61\,\lambda}{NA}$$

Where λ = Wavelength of light [400-750 nm for visible light used in compound

microscope; blue light (400 nm) is better for resolution than red light (700 nm)].

NA = n sin ϕ (Numerical aperture)

n = Refractive index of the medium present between the specimen and lens. Generally immersion oil (*n* = 1.5) is better than air (*n* = 1.0) (Table 1).

ϕ = Half of the angular width of the cone by the objective lens from the typical point of the specimen (Fig. 1).

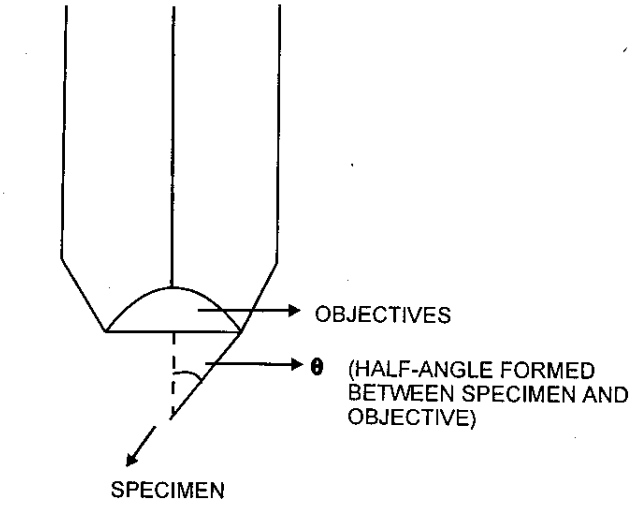

Fig. 1 Determination of Angle θ

Table 1 Refractive Indices of Some Mounting Media

Medium	**Refractive index (*n*)**
Distilled water	1.33
Glycerol	1.47
Cedar wood (immersion oil)	1.51
Eucalyptus oil	1.46
Carbon tetrachloride	1.46
Olive oil	1.47
* Euparal	1.48
Sandalwood oil	1.51
* Canada balsam	1.54
* Polystyrene	1.59

* Permanent mounting media

So numerical aperture is nothing but the light collecting ability of lens system. The higher the numerical aperture, the higher will be the degree of resolution whereas the lower the wavelength of the light, the higher will be the resolution.

Clearly, lowering RL can be achieved in three ways: (1) lowering the wavelength (λ), (2) raising the refractive index, and (3) raising sin θ as lenses of shorter focal length are used, the distance between the object and the lens decreases (Table 2).

*Table 2. Optical Properties of Objective Lenses

Magnification	Focal length (mm)	Lens-specimen distance (mm)	NA
10	16	5.5	0.25
40	4	0.6	0.65
95	2	0.1	1.32

*Adopted from Alberts *et al*. (1994).

If λ = 400 nm, n = 1.5, sin θ = 0.99 and an optical lens of maximal NA, it gives a theoretical maximum resolution of the objective lens of about 0.2 μm which is 1000 times greater than that of unaided human eye.

A wide variety of microscopes are currently available for studying plant structure. These are mainly categorized into two: (i) Light Microscope, and (ii) Electron Microscope. In the former one, magnification is achieved with the help of optical lens system. Brightfield, darkfield, phase-contrast, polarization and fluorescence microscopes come under this category. Whereas in the latter category a beam of electrons is used in place of light to obtain higher magnification; transmission and scanning electron microscopes belong to this category.

The resolution of various biological components is given as follows (adopted from Lewis and John, 1963):

Structure	Atoms, molecules	Macro-mole-cules	Genes, viruses	Bacteria, cytoplasmic organelles	Chromo-somes, cells	Tissues
Scale	1 A^o	10 A^o	100 A^o 1000 A^o	1 μ	10 μ	100 μ
					←Light microscope→	
Means of Resolution	←Electron microscope→ ←x-ray diffraction→					

1.2 LIGHT MICROSCOPY

1.2.1 Bright-Field Microscope

Bright-field microscope is absolutely indispensable to the biologists. It provides magnification of the objects which are not visible to naked eye. In this commonly used compound microscope, dark image of the object appears against bright background. This

is accomplished by the condenser, a series of lenses and diaphragms below the stage on which the object is located.

Lens systems

Compound microscope consists of three lens systems: (1) objective, (2) eyepiece or ocular, and (3) condenser (Figs. 2, 3).

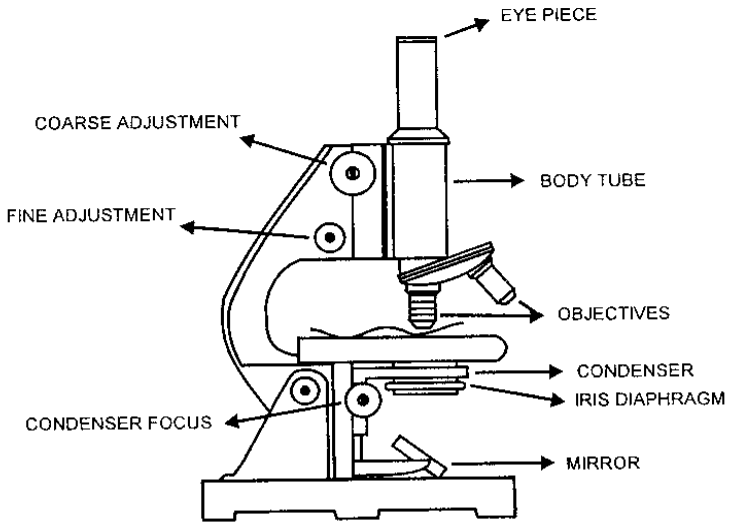

Fig. 2 The Compound Microscope

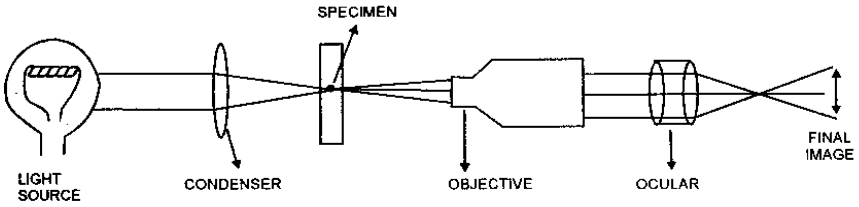

Fig. 3 The Path of Light through Bright-field Microscope

(1) **Objective:** Objective is considered to be the most important component of the light microscope since it affects the quality of image formation. On the basis of degree of correction for chromatic aberration, objectives are of two types: (i) achromatic objectives that are corrected for two regions of the spectrum, and (ii) apochromatic objectives that are corrected for three regions of the spectrum (for details see Desai and Desai, 1980; O'Brien and McCully, 1981).

Light focused on the specimen is either differentially transmitted (absorbed) or, for the background, fully transmitted to the objective. This lens resolves the specimen to produce a magnified image and focus in the microscope tube. Generally, compound microscopes are fitted with three objectives with different magnifying powers. They are low power, high power and oil-immersion objectives. These are easily recognised by

their length and NA value engraved on the barrel. Low power objective is the shortest and oil-immersion is the longest.

In bright-field microscope, both direct (rays not hitting the specimen) and indirect rays (rays diffracted or scattered from specimen) enter the objective, hence dark image is seen against bright background.

(2) Eyepiece (ocular): The main purpose of ocular is to magnify the intermediate image and to correct certain aberrations produced by the objective. Ocular is composed of mainly two lenses; the upper component or eye lens is the magnifier, whereas the lower component is known as field lens.

(3) Condenser : The quality and method of use of condenser are important factors for a good microscopy. The primary function of condenser is to supply sufficient cone of light to the objective to gain the maximum resolution. So, it should be properly positioned during the microscopy. The Abbe condenser, aplanatic condenser and achromatic condensers are available for general purposes. Condensers are also fitted with iris diaphragm and filter holder to control the light intensity.

All the four optical components (lamp, condenser, objectives and eyepiece) are installed in the common axis in the microscope. The following steps should be carefully taken for the effective use of research microscope (see also Desai and Desai, 1980; O'Brien and McCully, 1981):

1. Turn on the lamp, adjust it to a considerable intensity.

2. Adjust the mirror in such a way that the light travels up the body tube of microscope.

3. Take out the eyepiece and examine the back focal plane of the objective. Close the condenser iris until about ¾ of the field of vision is left clear. In some binocular microscopes, it is difficult to see the image of the condenser iris in the back focal plane of the objective. In this case, close the iris, then open it slowly and watch the ‘Circle of light’ as it increases. It comes to a certain position after which further increase in iris diameter does not increase the ‘Circle of light’. Close down the iris a little from that position.

4. Place the specimen on the stage.

5. Now keep the low power objective in position. Lower the body tube with the help of coarse adjustment knob until the distance between the specimen and low power objective becomes just about 5-7 mm.

6. Bring the specimen in focus with the help of coarse adjustment knob by looking through the eyepiece.

7. Sharpen the image with fine adjustment knob.

8. When higher magnification is needed, change the objective by shifting the nosepiece in the appropriate direction till it clicks into place. Care should be taken not to touch the objective lens with our fingers.

9. Focus the specimen again with the help of coarse and final adjustments. Adjustment of iris is necessary again since different power objectives have different field areas.

10. To set up oil-immersion objective, especially x90 or x100, first rack the body tube up and place a drop of immersion oil (free of bubbles).

11. By observing from the sides of the instrument, rack the objective down to touch the oil and almost near to the specimen. Care should be taken to see that the objective does not touch the specimen.

12. By looking through the eyepiece, slowly focus upwards with the fine adjustment until clear image appears. It comes quickly, since the working distance of oil-immersion objective is about 0.1-0.15 mm.

13. Remove and clean the oil from both objective lens and slide with lens paper immediately after the use of instrument

1.2.2 Dark-Field Microscope

In dark-field microscope, bright image of specimen is seen against the dark background. A specially devised condenser with star diaphragm is used in this microscope. It blocks the light rays (direct rays) that would normally enter the objective lens. Instead, it supplies a hollow cone of light and illuminates the specimen. Only indirect rays scattered from specimen would enter the objective. As a result bright image against the dark background is seen (Fig. 4). In this microscope, special condensers of very high NA, such as cardoid and the paraboloid, are employed with oil-immersion objective.

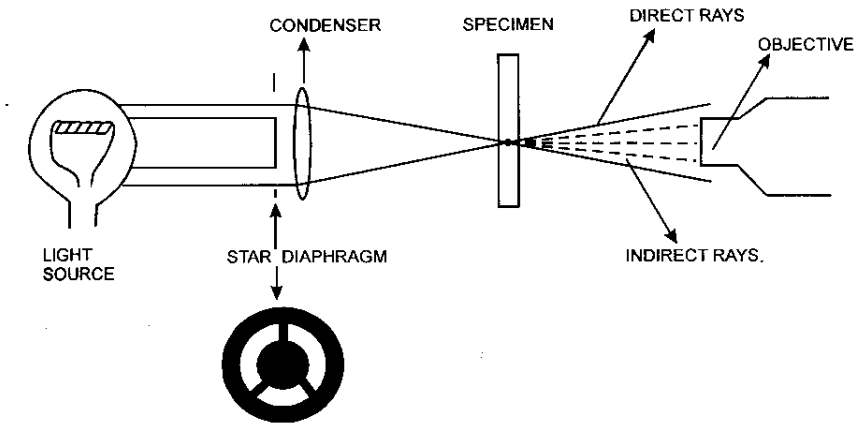

Fig. 4 Dark-field Microscope

Since the contrast is quite vivid (just like stars against a dark sky) organelles such as mitochondria and lysosomes are easily detected by this microscope. It is also useful for the observation of plant structures and microbes in an unstained condition. It has a diagnostic significance especially in the case of syphilis (Desai and Desai, 1980).

1.2.3 Phase-Contrast Microscope

Phase-contrast microscope is based on phase-contrast principles propounded by Fritz Zernike. He got the Nobel Prize in Physics in 1953 for his phase-contrast principles. According to him, light waves have variable character for frequency and amplitude. Human eye cannot notice a phenomenon when two light rays have similar amplitude and frequency, but different phase (Fig. 5). This can be achieved by phase-

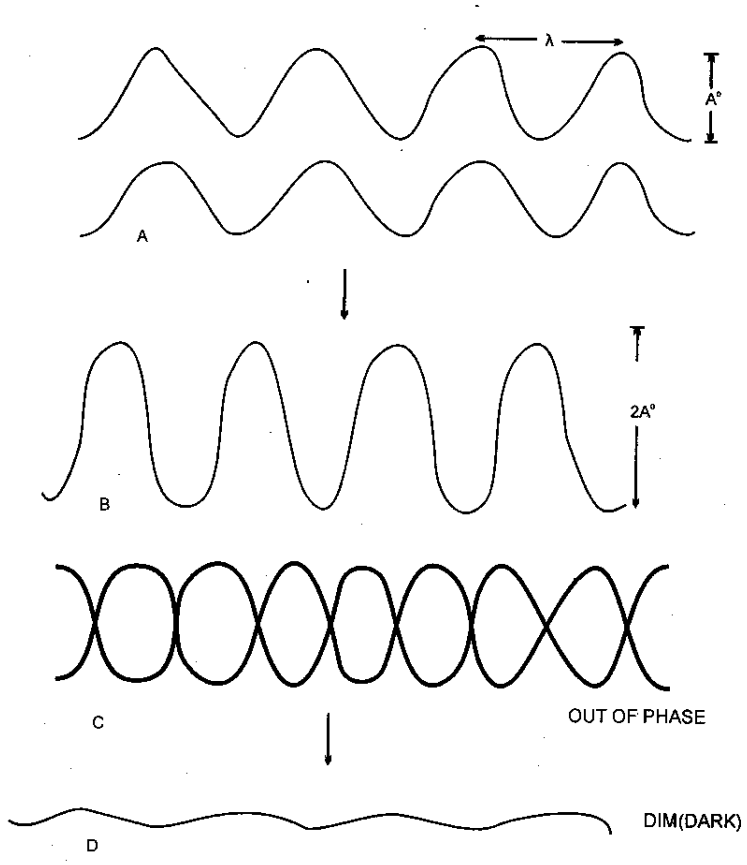

Fig. 5. Properties of Light

(a) Both rays are in phase (rays go up at the same time and down at the same time), (b) Constructive interference in which amplitude becomes doubled ($2A^o$), brightness increased, (c) Rays are in out of phase, and (d) Destructive interference, rays that are out of phase partially cancelled, brightness decreased and finally becomes dark.

contrast microscope. When the light rays are passing through the vacuum, they travel in high speed. When they pass through the transparent cells, light rays become slow due to change in the velocity. When a beam of light changes speed, it is refracted at an angle depending on the magnitude of the velocity change. So the light rays are refracted and alter their phase. This phase change may not be noticed by the human eye. In the phase-contrast microscope, both refracted rays (indirect rays) and direct rays are undergoing interference. As a result clear image of the specimen is seen.

Phase-contrast microscope is fitted with two additional optical lenses, namely annular diaphragm and phase-shifting plate (Fig. 6). Annular diaphragm is attached in place of iris diaphragm to the condenser. This allows only a ring of light to pass through the condenser and then to the specimen. Each objective requires different size of annular diaphragm according to its numerical aperture. Phase-shifting plate is attached at the back of the objective. It involves phase-shifting of direct rays with respect to indirect rays.' Phase-shifting plate is composed of a disk of glass having circular trough etched in it and when light passes through it, it creates a phase difference of a quarter of a wavelength as compared with the rest of the plate. It enhances the difference between indirect and direct rays so that interference takes place and clear image of the object is seen.

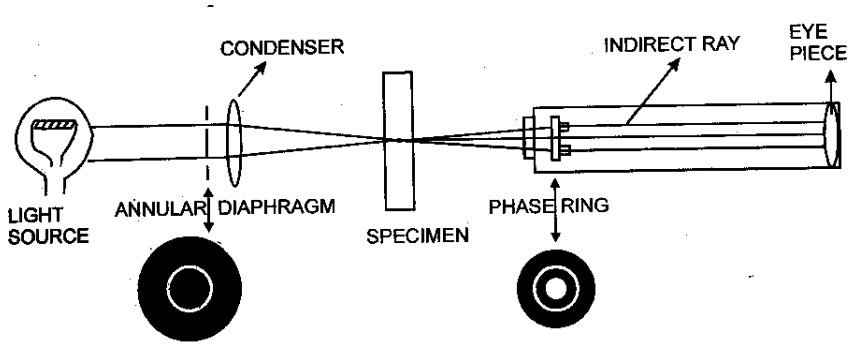

Fig. 6 Phase Contrast Microscope

Depending on the type of phase-shifting element employed, the specimen appears darker against light background (positive contrast) or lighter against dark background (negative contrast) (see also O'Brien and McCully, 1981). Phase-contrast microscope is widely used to observe living cells and organelles.

1.2.4 Polarization Microscope

Polarization microscope is used to visualize birefringents. Various biological structures like microtubules, micro fibrils and crystals show birefringence property because they refract polarized light in different ways depending on the direction from which the light strikes the specimen. Anisotropic materials are briefringents, because they show double refractions. The principle of the polarizing microscope is to deduce and measure the structural anisotropy of the specimen from the optical anisotropy- that it displays (see also Bennett, 1961; Bartels, 1966; James, 1976; O'Brien and McCully, 1981).

When a beam of polarised light passes through the birefringent object, the ray splits into two rays polarised in mutually perpendicular lines; the one which follows the ordinary laws of refraction is known as the 'ordinary' ray and the other, whose velocity through the object is different, is known as the 'extraordinary' ray. The difference in refractive indices $(n_e - n_o)$ is the value of birefringence (B). The two polarised rays, after emerging from the object, recombine but, because of different velocities through the object, one shows retardation when compared to the other. The value of 'retardation' (T), which is based on the birefringent property, is measured in the following manner:

$T = B \times t$

where T = Value of retardation

B = birefringence, i.e., $(n_e - n_o)$ and

t = thickness of the object

Finally $T = (n_e - n_o)\ t$; it is expressed in terms of wavelength in Angström units (Sharma and Sharma, 1972).

Polarization microscope is fitted with two additional optical lenses: (1) a vertical polarizer placed between the light source and substage condenser to produce polarized light, and (2) an analyzer horizontally placed above the objective lens (Fig. 7). In this, a bright image of specimen against dark background appears. Polarizing microscope is also useful for studying the optical properties of cell wall and starch grains.

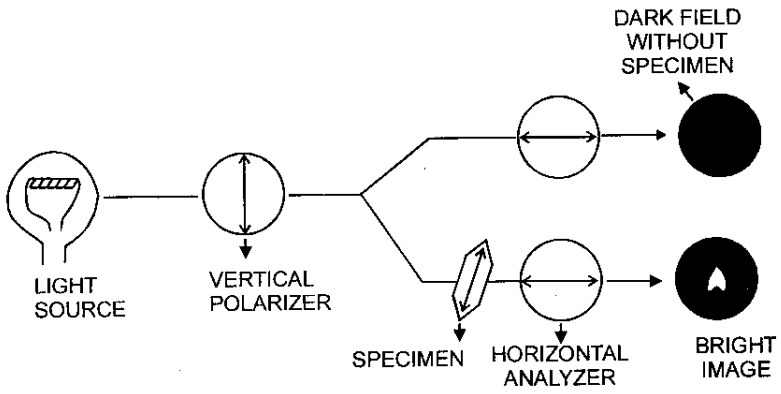

Fig. 7 Polarization Microscope

1.2.5 Fluorescence Microscope

Fluorescence microscope is based on the principle 'Fluorescence'. In this microscope, dyes are used that absorb light energy of one wavelength and emit light energy of longer wavelength; for example, the dye fluorescein absorbs at 490 nm and emits at 520 nm appearing yellow green and rhodamine absorbs at 546 nm and emits at 580 nm appearing red. So each compound has a characteristic absorption and emission spectrum (see also Pringsheim, 1963; Udenfriend, 1964, 1969).

A fluorescence microscope is fitted with two filters, these are (1) a *primary* or *exciter filter* placed between the light source and specimen, and it allows only the excitation wavelength, and (2) a *secondary* or *barrier filter* placed between the specimen and viewer, and it ideally transmits only the wavelengths emitted by the fluorescing compounds (Fig. 8).

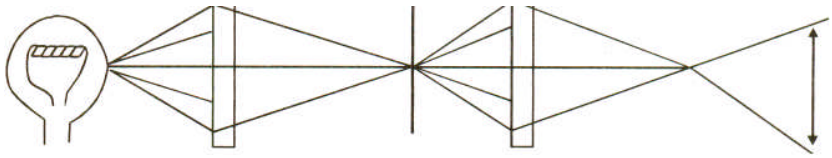

Fig. 8 Fluorescence Microscope

Fluorescence in histological specimens is induced either in compounds inherent in the tissue known as autofluorescence or in compounds (fluorochromes) that have been added to the tissue. Freehand sections, peels and whole mounts are enough to study the autofluorescence of lignins and chlorophylls present in higher plants. Plant cuticles (van Gijzel, 1975) and suberized walls of cork (Mader, 1954) are also auto fluorescent. The compounds of biological origin like berberin sulphate (from *Berberis)* and Primulin *(Primula)* and other compounds are still widely used as fluorochromes. Peirson and Dumbroff (1969) used an extract from *Chelidonium* roots to show effectively the casparian strips in roots. The other fluorochromes are acridine orange for DNA (Bertalanffy, 1963; Pearse 1972; Kasten, 1967), ethidium bromide for DNA and lignified walls, and aniline blue for callose of sieve elements (Currier and Strugger, 1956; Eschrich and Currier, 1964; Smith and McCully, 1978b; O'Brien and McCully, 1981).

The major use of fluorescence microscope in microbiology is in immuno-fluorescence studies. The antibody is made fluorescent by conjugating it with a fluorescent dye. With the help of fluorescence microscope, it is possible to detect specific types of antigens using an antibody tagged with a fluorescent dye (see also Nairn, 1969; Walker *et al*., 1971; Desai and Desai, 1980).

1.3 ELECTRON MICROSCOPY

In electron microscope instead of light a high speed electron beam is used (Table 3). Visualization of minute structures like ribosomes, mitochondria and membranes is possible with this microscope. In 1924, F. deBrogile (quoted in Prescot *et al*., 1999) opined that electrons could behave as waves with the wavelength inversely proportional to the square root of the voltage used to generate the electrons.

$$\lambda = 12.3 / \left[\left(\sqrt{V}\right)(0.1)\right] \text{ nm}$$

Table 3. Characteristic differences between electron and light microscopes

Sl. No.		Electron microscope	Light microscope
1	Best resolution	0.0005 μm (0.5 nm)	0.2 μm
2	High vacuum	Necessary	Not necessary
3	Specimen	Dead; ultrathin sections	Living and non-living material; sections of 2-10 μm thickness
4	Radiation source	Electron beam	Visible light
5	Lenses	Electromagnetic lenses	Glass lenses
6	Source of contrast	Scattering of electrons	Differential light absorption
7	Staining	Heavy metals	Dyes
8	Focussing mechanism	Adjacent current to the magnetic lens	Adjacent lens position
9	Specimen mount	Metal grid (copper)	Glass slide

1.3.1 Transmission Electron Microscope (TEM)

In TEM, the commonly used voltage is 100,000, so the wavelength of the electrons is 0.004 nm. Aberrations inherent in the lenses of the instrument require its operation at very low numerical apertures (0.001). So the resolution limit, according to Abbe's formula, is approximately 0.2 nm. Compared to the unaided human eye (200 μm or 200,000 nm), this represents the magnification upto 1,00,000 X (see also Alberts *et al.*, 1994).

Although the source of illumination is electron beam and not light, in general, the TEM is quite similar to the bright-field light microscope (Fig. 9). There are some differences between these two (Table 3). In TEM, high speed electron beam generated from electron gun is used. Electrons are focussed by electromagnetic lenses. Since air molecules interfere with the movement of electrons, high vacuum is created within the microscope. Also due to very poor penetrating capacity of electrons, sections should be ultrathin. These conditions do not allow the observation of living material through electron microscope.

There are three electromagnetic lenses in electron microscope. The condenser lens, placed between the source of illumination and specimen, collimates the electron beam on the specimen and an enlarged image is produced by two other lenses similar to the objective and ocular of light microscope. Since the electrons are not visible to the human eye, the final image is projected on the fluorescent screen.

Since most of the constituent elements in biological matter are of low mass, the contrast of these materials is very poor. This can be enhanced by staining with salts of heavy metals like uranyl acetate, lead citrate etc. These metals may be either fixed on the specimen (positive staining) or used to increase the opacity of the surrounding area (negative staining). The latter is useful 'for observation of virus particles and bacteria.

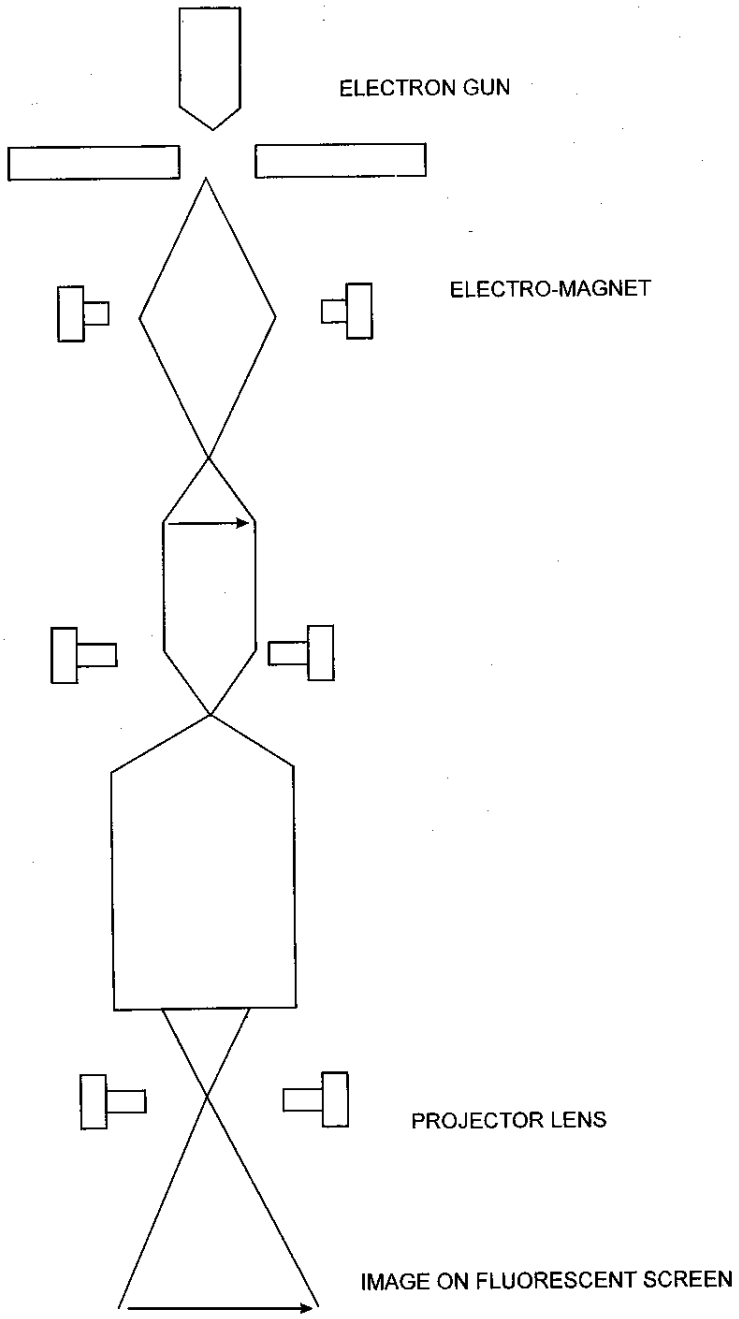

Fig. 9 Transmission Electron Microscope

1.3.2 Scanning Electron Microscope (SEM)

The electron microscope has been modified to provide three dimensional image of the specimen. The specimen is surface coated with a thin layer of heavy metal and is then scanned by a narrow electron beam. The intensity of various radiations released from a portion of the specimen depends on the shape and chemical composition of the irradiated object. Electrons scattered or emitted from the specimen's surface will form the image. The final image can be viewed from the fluorescent screen. So the topology of the object can be studied with SEM.

SEM lacks high resolution capacity when compared to TEM but it has the advantage of revealing three-dimensional impression. With this microscope, the surface features of the specimen can be studied with a high contrast.

Some of the important features of the microscopes are given in Table 4.

Table 4. Characteristic Features of Some Important Microscopes

Type of microscope	Image formation	Magnification	Applications
Bright-field microscope	Dark image of the object against bright background	1000-2000 x	To study the anatomy, embryology of plants and gross morphology of yeast, moulds, algae, protozoa, bacteria etc.
Dark-field microscope	Bright image of the object against dark background	1000-2000 x	To study the gross morphology of the living specimen
Phase contrast microscope	Based on phase contrast principles; Darker against the light background (positive contrast) or lighter against the dark background (negative contrast)	-do-	To study the gross morphology of living specimen and cell organelles.
Polarization microscope	Bright image of the object against dark background	-do-	To study the birefringents, viz., microtubules, microfibrils, crystals and also to study the optical properties of cell wall.
Fluorescent microscope	Based on fluorescent principle	-do-	To study the plant cuticles, suberized walls, lignins, chlorophylls, DNA, callose of sieve elements; immuno-fluorescence studies in microbiology
Electron microscope	Viewed on fluorescent screen	Upto 10,00,000 x	To study the ultrastructure of cell organells, microbial cells, viruses etc.

1.4 SUMMARY

Resolving Power

The ability of microscope to produce separate images of closely placed objects is known as resolving power. This depends upon the wavelength of light and numerical aperture (NA). This can be expressed in the form of *Resolution Limit* (RL) in the following formula:

$$RL = \frac{0.61\,\lambda}{NA}$$

Light Microscopy

Bright-field microscope is commonly used compound microscope, in which dark image of the object appears against bright background. In general, compound microscope consists of three lens systems: (1) Objective, (2) Eyepiece or ocular, and (3) Condenser. The objective lens resolves the specimen to produce a magnified image and focus in the microscope tube. The ocular is to magnify the intermediate image and to correct certain aberrations produced by the objective. The primary function of condenser is to supply sufficient cone of light to the objective to gain the maximum resolution. In *Dark-fied microscope*, bright image of specimen is seen against the dark background. *Phase-contrast microscope* is based on phase-contrast principles propounded by Fritz Zernike. In this microscope, both refracted rays (indirect rays) and direct rays are undergoing interference. As a result, clear image of the specimen is seen. *Polarization microscope* is based on the birefringence property of various biological structures like microtubules, microfibrils and crystals. The principle of the polarizing microscope is to deduce and measure the structural anisotropy of the specimen from the optical anisotropy that it displays. *Fluorescence microscope* is based on the principle 'Fluorescence'. In this microscope, dyes are used that absorb light energy of one wavelength and emit light energy of larger wavelength. Biological specimens are stained with dyes and observed under this microscope.

Electron Microscopy

In *Transmission electron microscope*, high speed electron beam is used. Visualization of minute cell organelles like ribosomes, mitochondria and biological membranes is possible with this microscope. Electrons are focussed onto the specimen by the electromagnetic lenses. Since most of the constituent elements in biological matter are of low mass, the contrast of these materials is very poor. This can be enhanced by staining with salts of heavy metals like uranyl acetate, lead citrate etc., *Scanning electron microscope* provides three dimensional image of the specimen. With this microscope, the surface features of the specimen can be studied.

MODEL QUESTIONS

Essay Questions

1. Define resolving power. Discuss the principles and light pathways of various light microscopes with special reference to phase-contrast and polarization microscopes.

2. Write an essay on Transmission Electron Microscope and discuss the characteristic differences between electron and light microscopy.

Short Notes

1. Resolving power of the microscope
2. Compound microscope
3. Phase-contrast microscope
4. Polarization microscope
5. Fluorescent microscope
6. Scanning electron microscope

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-- S.M. KHASIM

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LESSON – 2

STAINING METHODS AND CULTURE MEDIA

2.0. OBJECTIVE

After studying this lesson you will become familiar with staining of microorganisms. Simple and Negative staining help in comparing morphological shapes and arrangements of bacterial cells.

Differential staining is useful to differentiate between the two principle groups of bacteria Importance of culture medium to grow microorganisms in laboratory. Different types of media to isolate microorganisms and their composition. Preparation procedures for broth/liquid and solid media.

2.1. INTRODUCTION

Microscopes are of little use unless the specimens for viewing are prepared properly. Though resolution and magnification are important in microscopy, the degree of contrast between structures to be observed and their backgrounds is equally important. Nothing can be seen without contrast, so special techniques have been developed to enhance contrast. Staining (dyeing) is used to increase the contrast between the specimen and the background. Preparation of specimens for the microscope Wet Mount: Wet mounts, in which a drop of medium containing the organisms is placed on a microscope slide. This can be used to view living microorganisms. Fast moving microorganisms can be studied by making them slow moving organisms. Addition of a 2% solution of carboxymethyl cellulose, a thick slurry solution helps to make fast moving organisms to slow moving.

Smears: Smears, in which microorganisms from a loopful of medium are spread onto the surface of a glass slide. This can be used to view killed microorganisms. Though they are living when placed on the slide, the organisms are killed by the techniques used to fix or attach them to the slide.

Beginners who make smear preparation should make smear carefully, like:

* if the smear is too thick, individual cells will not be seen.
* if the smear is too thin, no organism may be found.
* if the drop of medium is stirred too much, cell arrangements will be disrupted.

After a smear is made, it is allowed to air dry completely. Then it is quickly passed three or four times through an open flame. This process is called heat-fixation. Heat fixation helps in three ways:

* It kills the organisms
* It causes the organisms to adhere to the slide.
* It alters the organisms so that they accept stains readily.

If the slide is exposed to high flame, the organisms will be boiled and destroyed. If heat fixing is too little, the organisms may not stick. They will wash off

the slide in next steps. Any cells remain alive will stain poorly. Certain structures like the capsules found on some microbes are destroyed by heat fixing. So this step is omitted and these microbes are fixed to the slide just by air-drying.

Some of the microorganisms can not be studied properly because they are transparent, colourless and difficult to see when suspended in an aqueous medium. Specimens are stained to increase visibility, which help to identify microbes. Today several stains and staining procedures which employ dyes of different colours are available to study the properties of various microorganisms. Most staining procedures for light microscopic observation of microorganisms begin with the transfer of a suspension of microorganisms to a glass microscopic slide (Fig.2.1).

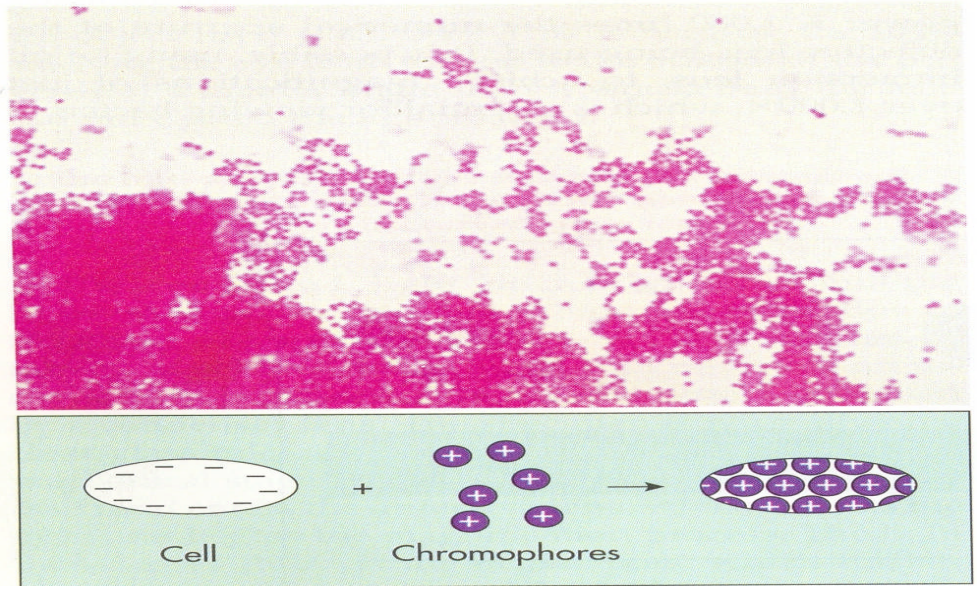


Fig.2.1. In a simple staining procedure, microorganisms are affixed to a glass slide and stained with an appropriate dye.

Staining techniques help investigator to group major categories of microorganisms, examine structural and chemical differences in cellular structures and

look at the parts of the cell. The chemical substances commonly used to stain bacteria are known as dyes or stains. It is a molecule that can bind to cellular structure and give colour to it. Dyes are classified as natural or synthetic. Synthetic dyes are used for bacterial stain preparations. Chemically a dye is defined as an organic compound containing a benzene ring plus a chromatophore and auxochrome group. Chromatophores give colour to the dye and they bind the cells by ionic, covalent or hydrophobic bonding. Based on the ionic charge, dyes can be divided into acidic, basic and neutral.

Basic dyes: These dyes have positive charge (cationic). They bind to negatively charge like nucleic acids and many proteins and bacterial cell surfaces.
Ex: Methylene blue, Basic fuchsin, Crystal violet, Safranin and Malachite green.

Acidic dyes: These have negative charge (anionic). They bind to positively charged structures.
Ex: Eosin, Rose bengal and Acid fuchsin.

Neutral stains are formed by mixing together aqueous solutions of certain acidic and basic dyes. The colouring matter in neutral stains is present in both negatively and positively charged components.

There are two kinds of staining procedures, simple staining and differential staining.

2.2. Simple staining

In simple staining, the smear (cells) is stained by the application of a single staining reagent and all cells and structures generally stain the same colour, regardless

of type. The staining procedure may be positive, in which the stain is attracted to the cells and take on the colour, or negative, in which the stain is repelled by the cells and the background takes on the colour.

The simple stain that stains the bacteria is a direct stain. This technique is used to determine cell morphology, size and arrangement of bacterial cells. In positive staining procedures for light microscopy, basic stains with positively charged chromophore (from Greek ***Chroma*** meaning colour; coloured portion of the stain molecule) that is attracted to the negatively charged structures like nucleic acids and cell wall components that carry a negative charge. A stain such as methylene blue has a blue chromophore, resulting in positive blue staining of the microorganisms. Exposure time varies among bacterial species for example crystal violet, 2-60 seconds, carbol fuchsin 15-30 seconds and methylene blue, 15-120 seconds (Fig.2.2).

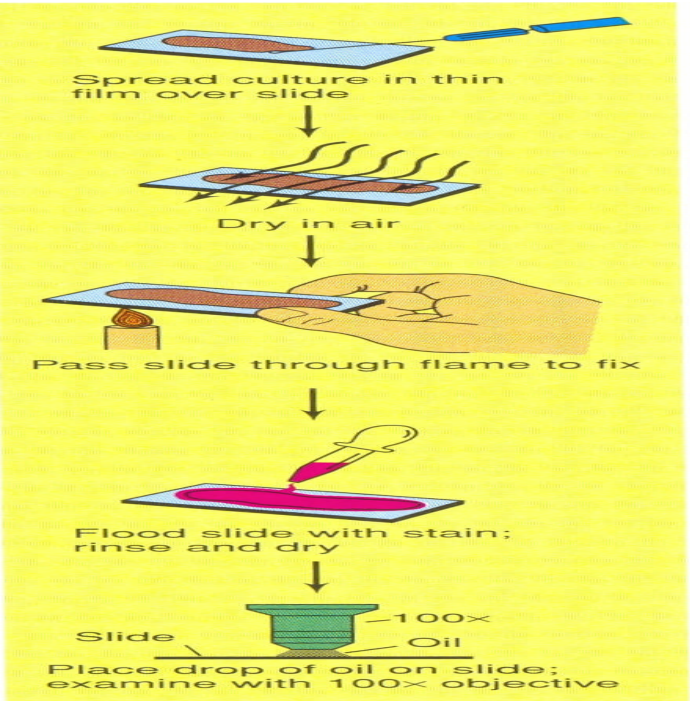


Fig.2.2. Simple staining of cells for microscopic observation.

Procedure

* Take clean glass slide, wash and dry it.
* Prepare bacterial suspension and place a loopful of suspension on the slide.
* Heat fix the smear and apply about 5 drops of a stain for the designated period.
* Pour off the stain and wash the smear gently with slow running tap water.
* Blot dry the slide with blotting paper.
* Examine the slide under oil-immersion objective.

2.3. Negative staining

In negative staining technique, a simple stain is used that does not stain the bacteria but stains the background. This staining requires the use of an acidic stain such as Indian ink

or Nigrosin. In negative staining procedures for light microscopy, the stain which is acidic, with its negatively charged chromogen repelled by the negatively charged microorganisms, which also carry a negative charge on their surfaces. The unstained bacterial cells appear transparent upon examination. Negative staining is advantageous for two reasons. Cells appear less shriveled or distorted because heat fixing is not done, their natural size and shape can be seen, capsulated bacteria and some spirilli can be observed by this technique. For electron microscopy, negative stains include heavy metal salts such as uranyl acetate is used (Fig.2.3).

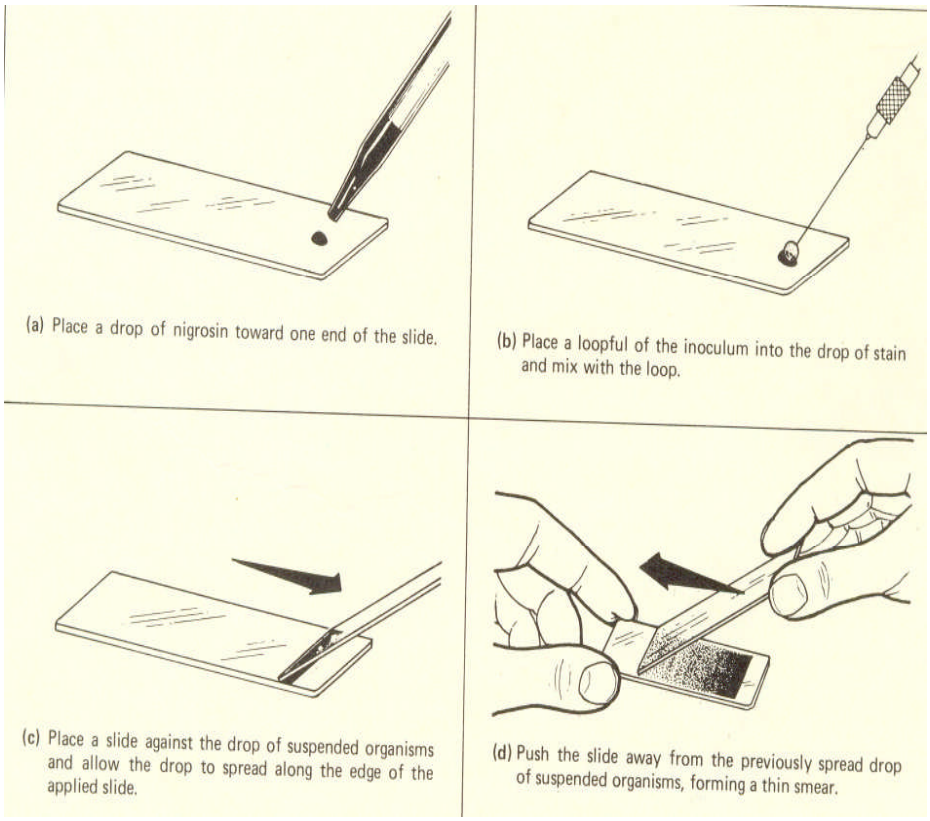


Fig. 2.3. Negative staining procedure

Procedure

* Place one drop of nigrosine at one end of a clean glass slide.
* With the help of a sterile inoculating loop, transfer a loopful of inoculum from the broth culture in the stain and mix with the loop. Never heat fix the slide.
* Take another clean slide, place it against the drop of suspended organism at an angle of 30^{o}C and allow the droplet to spread across the edge of the top slide. The thickness of the film should be uniform.
* Spread the mixture (suspension + culture) into a thin wide smear by pushing the

top slide to the left along the entire surface of the bottom slide.

2.4. Differential staining

In differential staining procedures, multiple staining reactions are employed. Differential stains take advantage of the fact that specific types of microorganisms and for particular structures of a microorganism exhibit different staining reactions that can be readily distinguished by their different colours. They distinguish between structures within a cell and types of cells by staining them different colours. This stain separates microorganisms into Gram positive and Gram negative, capsulated and non-capsulated, spore formers and non-spore formers and acid fast and non-acid fast. It is also useful to visualize the structures like capsule, spore, flagella etc. Stains used for visualizing morphology of fungi include cotton blue in lactophenol, indian ink, nigrosin and orange G. Geisma stain is used for staining fungal nuclei.

The semirigid cell wall lies outside the cell membrane in nearly all bacteria. Peptidoglycan or murin is the most important component of the bacterial cell wall. It forms a supporting net around bacterium that resembles multiple layers in chain-like fence (Fig. 2.4).

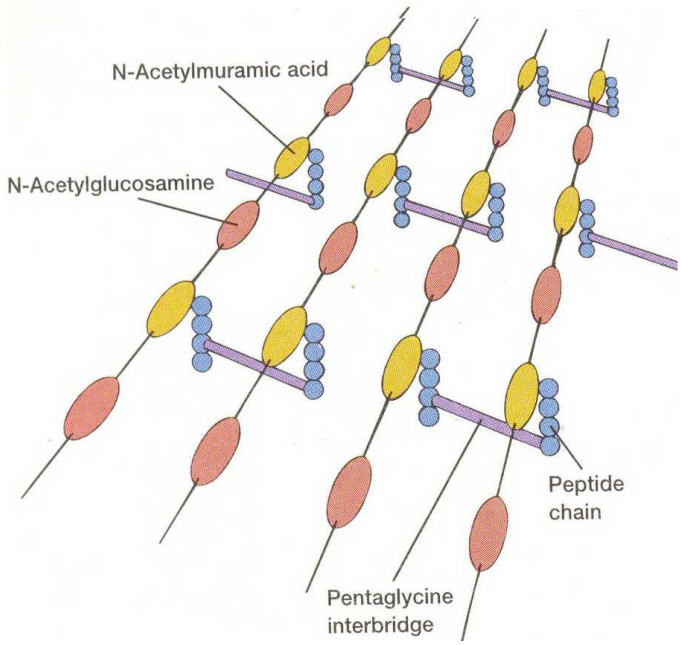
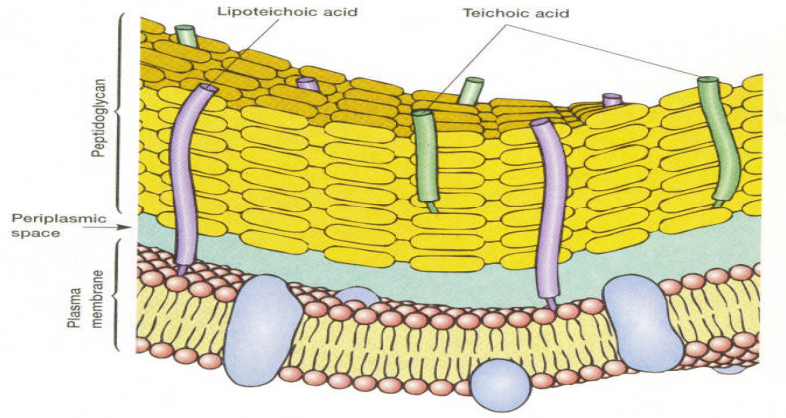
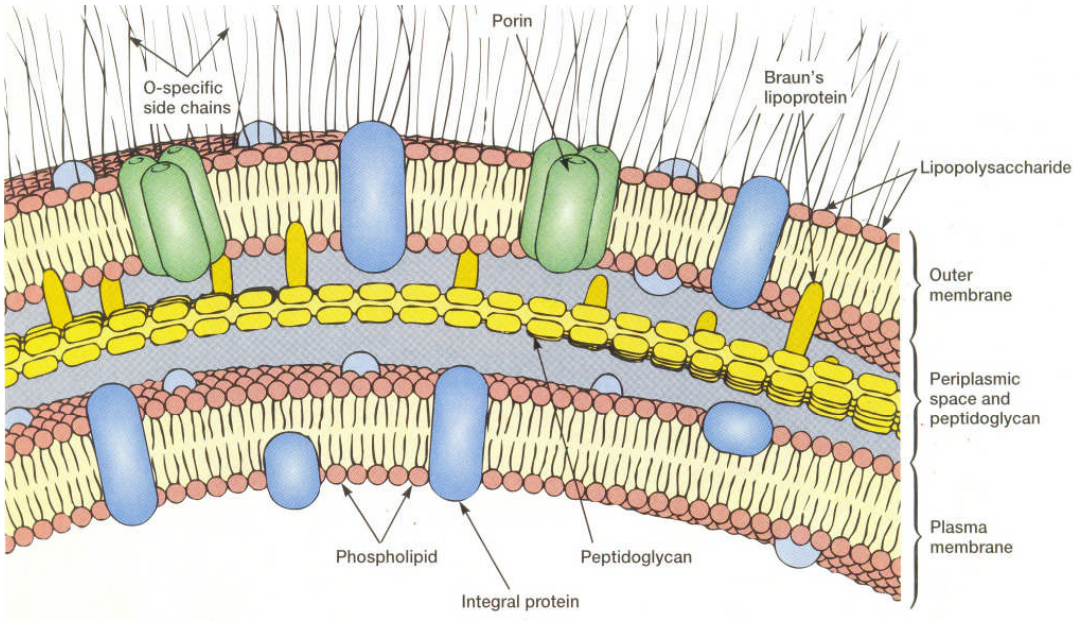


Fig. 2.4. Peptidoglycan structure

Gram positive cells may have as many as 40 such layers. In the peptidoglycan polymers, N-acetylglucosamine, N-acetylmuramic acid and four amino acids are present. In most Gram-positive organisms, the third amino acid is lysine and in Gram negative organisms, it is diaminopimelic acid. Gram positive cell walls have an additional molecule teichoic acid. It consists of glycerol, phosphates and sugar alcohol ribitol. Outer membrane in Gram negative bacteria is a bilayer membrane. It forms the outermost layer of the cell wall. It attaches to the peptidoglycan by small lipoprotein molecules. Proteins called porins form channels through the outer membrane (Fig. 2.5 A, B).



(A)



(B)

Fig.2.5. A) Gram-positive and B) Gram-negative envelope

Lipopolysaccharide (LPS) or endotoxin is an important part of outer membrane. It is used to identify Gram negative bacteria. LPS consists of polysaccharides and lipid A. The space or gap present between cell wall and cell membrane is periplasmic space. It is commonly seen in Gram negative organisms and rarely observed in Gram positive bacteria. Certain properties of cell walls produce different staining reactions.The thick cell walls of Gram positive bacteria retain such stains like crystal violet-iodine dye in the cytoplasm. Retention of Gram stain seems to be directly related to wall thickness and not to peptidoglycan.

The cell wall of Gram negative bacteria is thinner but more complex than Gram positive bacteria. Only 10 to 20 percent of the cell wall are peptidoglycan, the remainder consists of polysaccharides, proteins and lipids. The cell wall contains an outer membrane separated by a narrow periplasmic space. Gram negative bacteria fail to retain the crystal violet-iodine dye during the decolourizing procedure partly because of their thin cell walls and partly because of the relatively large quantities of lipoproteins and lipopolysaccharides in the walls.

2.4.1. Gram staining

The most important differential stain used in bacteriology is the gram stain, named after Dr. Christian Gram. It divides bacterial cells into two major groups, gram positive and gram negative. The gram stain uses four different reagents that are applied to a heat fixed smear. The first reagent is called the primary stain (Crystal Violet). Its function is to impart its colour to all cells. To establish a colour contrast, the second reagent used is the decolourizing agent (Alcohol). The final reagent, the counter stain (Safranin) has a contrasting colour to that of the primary stain. Following decolourization, the primary stain (violet/dark blue) may be retained or lost by the bacteria.

The bacteria, which retain the primary stain, are called Gram-positive and those that lose the crystal violet and counter stained by safranin (appear red) are referred to as Gram-negative. If the primary stain is removed, the decolourized cellular components will accept the contrasting colour of the counterstain.

Primary stain

The first stain used in Gram staining procedure is Crystal Violet that stains all cells purple. Gram's iodine reagent serves as mordant, a substance that forms an insoluble complex by binding to the primary stain. The resultant crystal violet-iodine (CV-I) complex serves to intensify the colour of the stain and all the cells will appear purple-black.

In Gram positive cells only, this CV-I complex binds to the magnesium-ribonucleic acid component of the cell wall. The resultant magnesium-ribonucleic acid-crystal violet-iodine (Mg-RNA-CV-I) complex is more difficult to remove than the smaller CV-I complex. Ethyl alcohol (95%) is a decolourizing agent in Gram staining procedure. The difference in staining responses to Gram stain can be related to chemical and physical differences in their cell walls. This reagent serves a dual function as a lipid solvent and as a protein-dehydrating agent. Its action is determined by the lipid concentration of the microbial cell walls. The Gram negative bacterial cell wall is thin, complex multilayered structure with high lipid contents in addition to protein and mucopeptides.

The high lipid concentration found in outer layers of the cell wall is dissolved by alcohol, creating large pores in the cell wall that do not close on dehydration of cell wall proteins. This facilitates release of the unbound CV-I complex, leaving these cells colourless or unstained. In Gram positive cells, the low lipid concentration is important for the retention of the Mg-RNA-CV-I complex. The small amount of lipid content is readily dissolved by the action of alcohol. This causes formation of minute pores in cell wall. These are closed by the dehydrating action of alcohol. Because of this, the tightly bound primary stain is difficult to remove and the cells remain purple.

The final reagent and counter stain is Safranin. This stain gives red colour to the cells that are previously decolourized. As the Gram-negative cells undergo decolourization, they

absorb the counterstain. Gram-positive cells retain the purple colour of the primary stain (Fig.2.6).

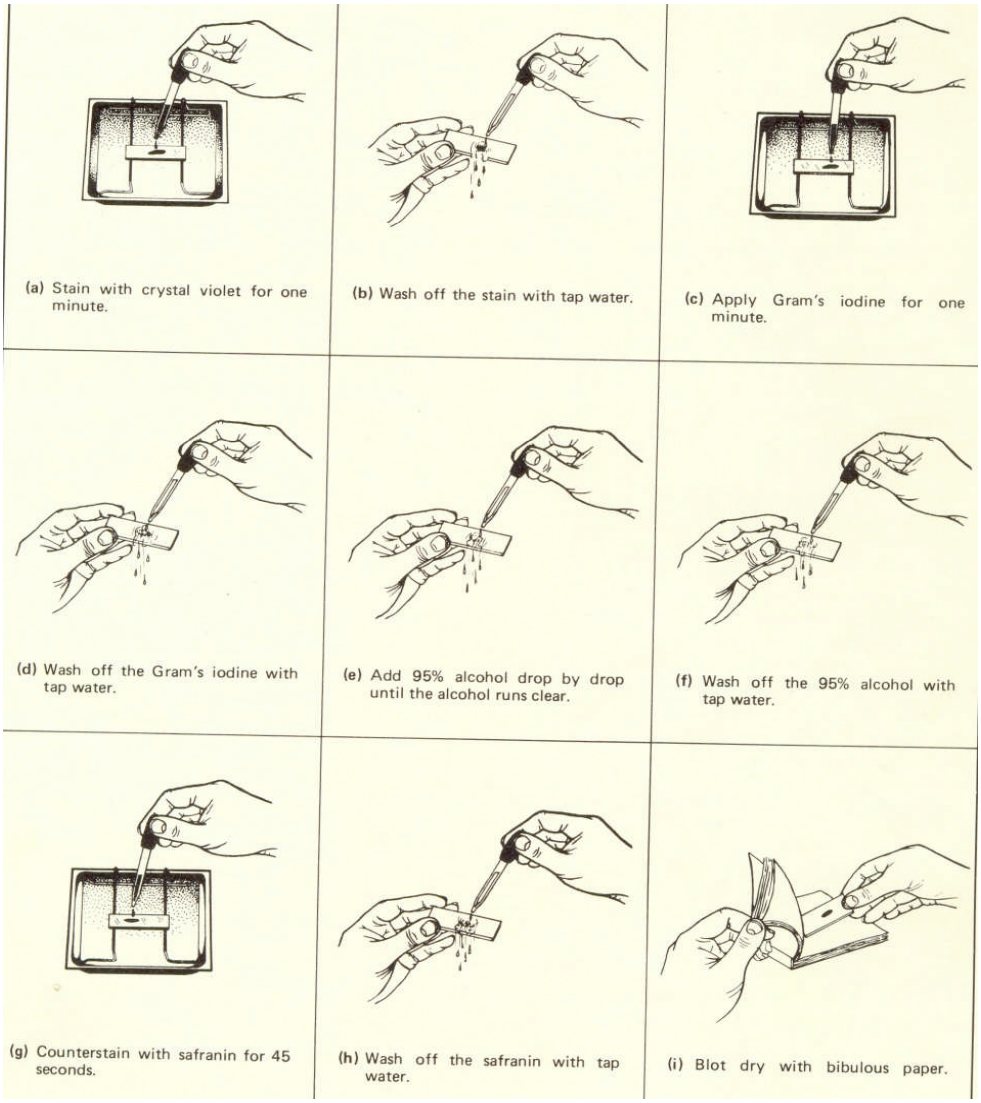


Fig. 2.6 Gram's staining procedure

Procedure

* Take clean glass slide
* Place a drop of water on the slide and transfer the organism to the water drop with a sterile cooled loop. Mix and spread the organism
* Allow the smear to air dry and heat fix.
* Flood smear with crystal violet and let stand for 1 minute.
* Wash with tap water.

* Flood smear with mordant, that is Gram's iodine, and let stand for 1 minute.
* Wash with tap water.
* Decolourize with 95% ethyl alcohol. Do not over-decolourize. Add reagent drop by drop until crystal violet fails to wash from smear.
* Wash with tap water.
* Counterstain with safranin for 45 seconds.
* Wash with tap water.
* Blot dry with blotting paper and examine under oil immersion.
* Bacteria that appear blue are Gram-positive and those appear pink are Gram negative

After completion of the Gram stain procedure, the Gram positive bacteria appear blue or purple and the remaining Gram negative bacteria appear red or pink. This occurs because Gram-positive bacterial cells retain the primary stain when the acetone-alcohol is applied. Gram-negative cells are decolourized by acetone-alcohol. The Gram stain procedure has great diagnostic value because of its ability to easily differentiate among bacterial species. It is a key feature employed in many bacterial classification and identification systems.

2.4.2. Endopore staining

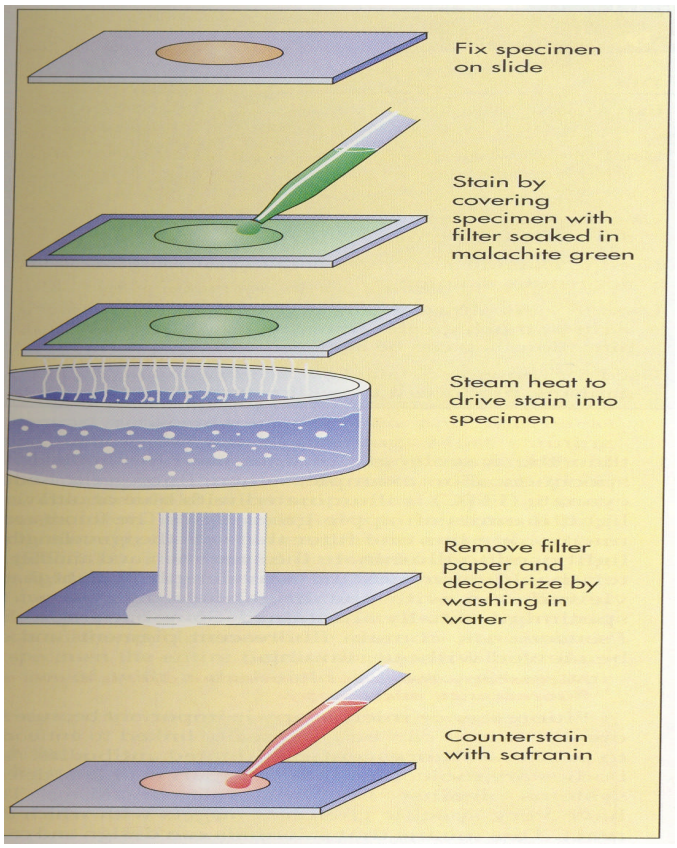
Another key differential staining procedure reveals the presence or absence of bacterial endospores. Members of the anaerobic genera ***Clostridium*** and ***Desulfotomaculum*** and the aerobic genus ***Bacillus*** are examples of organisms that have metabolically active vegetative cells or metabolically inactive and highly resistant cell types called spores. Spores that are produced inside a parent cell is called endospore. Bacterial endospore is a heat resistant structure even to boiling water. For example, ***C. botulinum*** sometimes survives the heat treatment of canning and causes a food poisoning disease known as botulism, when contaminated food is eaten.

Impervious layers surround spore called spore coats. When conditions are not favourable to parent cell, the endospore is released from the degenerating vegetative cell and

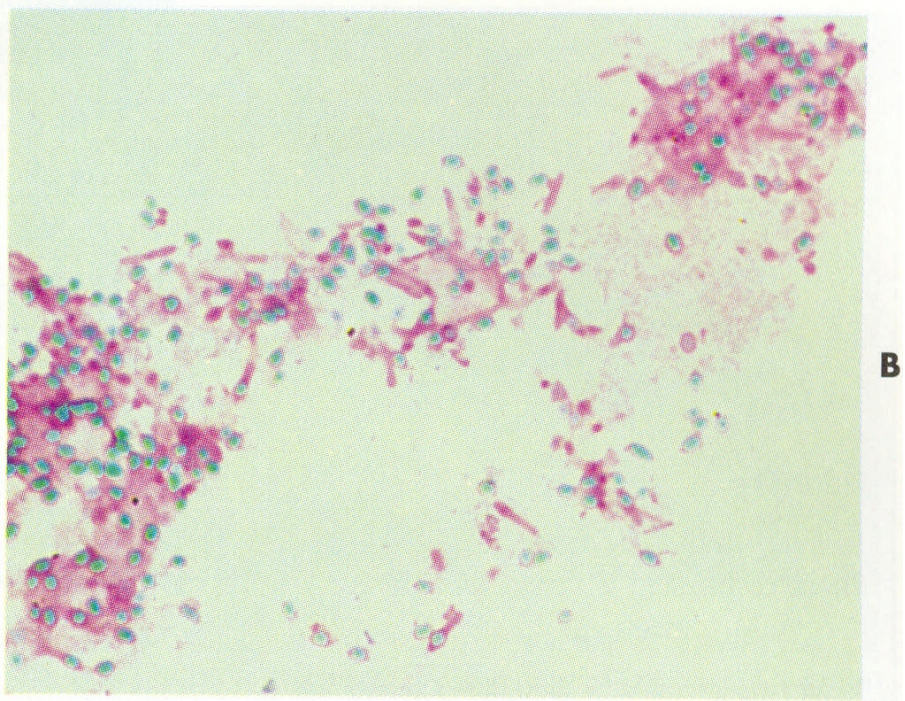
becomes independent. This is called a spore. The spores are differentially stained by using special procedures that help dyes penetrate the spore wall. Endospores are not easily stained and in normal simple staining procedures the endospore remains colourless while the rest of the cell is stained. Primary stain in spore stain is Malachite Green. Endospores can be stained using malachite green and steam to drive the stain into the endospore. Spore will not accept the primary stain easily. To further penetration, application of heat is required. After the primary stain is applied, heat the smear. Both the vegetative and the spore will appear green.

Once the spore accepts the malachite green, it cannot be decolourized by tap water. In a typical endospore staining procedure, water is used as decolorizing agent. This removes only excess staining. The spores remain green and vegetative cells become colourless as water washes the primary stain out of vegetative cells but not the endospores.

The second stain in spore stain is Safranin, which is used as counter stain. This gives colour to the decolourizing vegetative cells. At the end of endospore staining procedure, the spores retain green and the vegetative cells absorb counterstain and appear red, permitting differentiation of the endospore from the vegetative cell (Fig.2.7).


Fix specimen on slide
Stain by covering specimen with filter soaked in malachite green
Steam heat to drive stain into specimen
Remove filter paper and decolorize by washing in water
Counterstain with safranin

A



B

Fig. 2.7 A) The endospore staining procedure B) ***Clostridium tetani*** endospores after staining

Procedure

* Make smear of bacteria on clean slide
* Air dry and heat fix the smear
* Flood the smear with malachite green
* Heat the slide to steaming and steam for 5 minutes adding more stain to the smear from time to time.
* Wash the slide with slow running tap water.
* Counter stain with safranin for 30 seconds.
* Wash the slides with distilled water and blot dry the slide with blotting paper.
* Examine the slide under oil-immersion objective.
* Endospores stain green and the vegetative cells stain red.

2.4.3. Capsule staining

Some bacterial cells are surrounded by a mucilagenous protective structure. This forms a viscous coat around the cell wall and adheres to the cell wall. This structure is called capsule. It is not common to all organisms. The ability to form capsule is genetically determined. Cells that have a heavy capsule are generally virulent and produce disease. Capsule gives protection against phagocytic activities of host cells. Chemically, the capsular material is a polysaccharide, a glycoprotein or a polypetide and the composition vary among species of bacteria. Capsules occur in pneumonia causing strains of ***Streptococcus pneumoniae, Haemophilus influenzae*** **and** ***Klebsiella pneumoniae.*** Some ***Bacillus*** species, in contrast, produce capsules composed of glutamic acid, largely in the D form, rather than polysaccharide capsules.

Capsule staining is more difficult than other types of differential staining procedures because the capsular materials are water-soluble and may be removed with vigorous washing. Smear should be heated, because the resultant cell shrinks and creates a clear zone around the organism that is an artifact that is mistaken for the capsule. The capsule stain uses two reagents, Crystal Violet and Copper Sulfate (20%). Crystal Violet is applied to a non-heat-fixed smear. The cell and the capsule will take on the dark colour. Copper Sulfate (20%) is the decolourizing agent and counterstain. The capsule is non-ionic; the primary stain adheres to the capsule without binding to it. Since the capsule is water-soluble, copper sulfate is used to wash the purple primary stain out of the capsular material without removing the stain that is

bound to the cell wall. At the same time, it acts as a counterstain as it is absorbed into the decolorized capsular material. The capsule will now appear light blue in contrast to the deep purple colour of the cell. The presence of a capsule in an ***Acinetobacter*** species can also be demonstrated by negative staining with indian ink observed by Phase contrast microscopy. The indian ink does not penetrate the capsule and so it is revealed in outline as a light structure on a dark background (Fig.2.8)

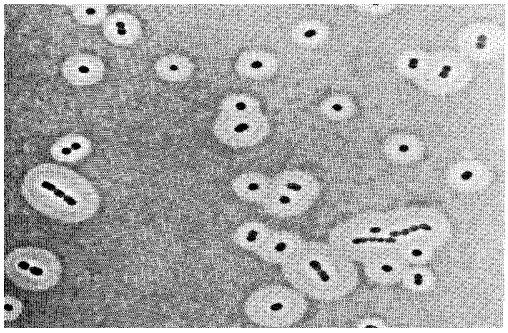


Fig. 2.8. Bacterial capsules

Procedure

For capsule staining, the culture must be grown on medium supplemented with starch.

* Take clean glass slide
* Using sterile technique, prepare a heavy smear of the organism
* Allow smear to air dry. Do not heat fix.
* Flood smear with crystal violet and let stand for 5 to 7 minutes
* Wash smear with 20% copper sulfate solution
* Gently blot dry and examine under oil immersion.

2.4.4. Acid fast staining or Ziehl-Neelsen stain

Another differential staining procedure frequently used in bacteriology is Acid fast staining. The characteristic difference between ***Mycobacterium*** and other microorganisms is the presence of a thick waxy wall that makes the penetration of stains extremely difficult. In this procedure once the stain, carbol fuchsin has penetrated, it can not be readily removed by vigorous use of acid-alcohol as a decolourizing agent. Acid - fast bacteria retain the red colour

of the carbol fuchsin and are not decolourized. Because of this property, these organisms are called acid-fast, all other microorganisms, which are easily decolourized by acid-alcohol, are non-acid-fast. Non-acid-fast bacteria are decolourized and when counterstained with methylene blue, they appear blue.The acid-fast stain uses three different reagents.

Carbol fuchsin: Most bacteria are not stained with common dyes like methylene blue and crystal violet. Major portion of mycobacterial cell wall contains lipoidal material. Carbol fuchsin, a red phenolic dye, is soluble in this lipoidal material. The stain penetrates and is retained. Penetration is enhanced by heating. This drives the carbol fuchsin into the cytoplasm through the lipoidal wall. All the cells will appear red.

Acid-alcohol : 3% HCl + 95% ethanol is the acid-alcohol which is a decolourizing agent in acid-fast staining procedure. The smear is cooled prior to decolourization. This allows the waxy cell substances to harden. On application of acid-alcohol, acid-fast cells will be resistant to decolourization, since the primary stain is more soluble in the cellular waxes than in the decolourizing agent. The primary stain is retained and the mycobacteria will stay red. In non-acid-fast organisms, the primary stain is more easily removed during decolourization, leaving these cells colourless or unstained.

Counter stain: This is used as the final reagent to stain previously decolourized cells. Non-acid-fast cells absorb the counterstain and take on blue colour. The acid-fast cells retain the red of the primary stain (Fig.2.9).

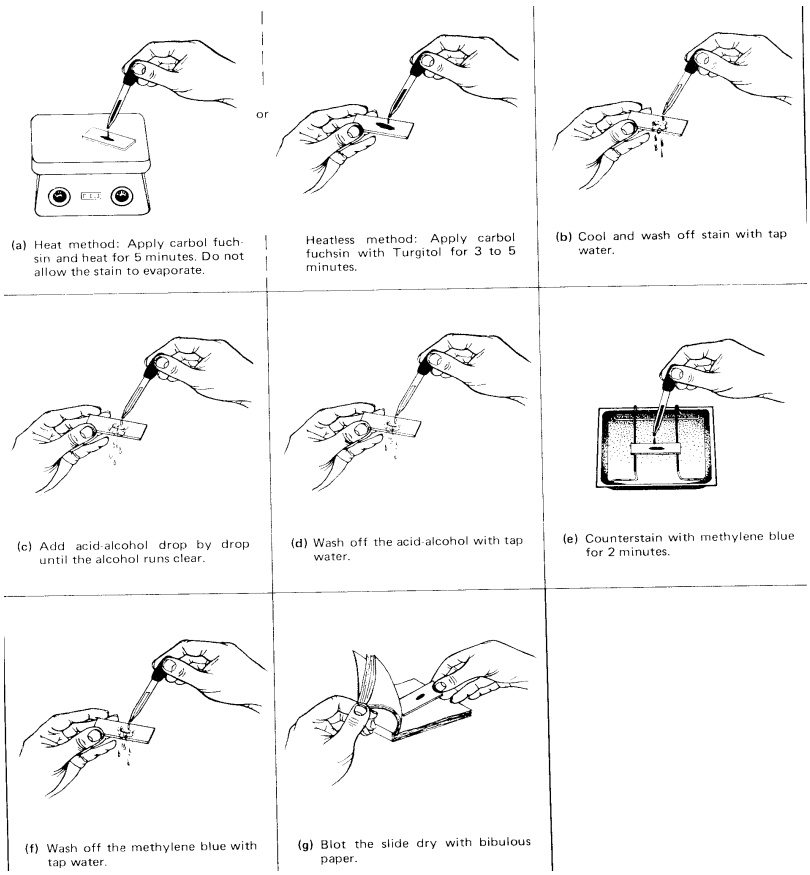


Fig. 2.9 Acid-fast staining procedure

Procedure

* Take clean glass slide
* Using sterile technique, prepare bacterial smear
* Allow smear to air dry and heat fix.
* Flood smear with carbol fuchsin and place on a warm hot plate, allowing the preparation to steam for 5 minutes. Do not allow stain to evaporate. Prevent stain from boiling by adjusting the hot plate temperature.
* Cool the slide and wash with tap water.
* Decolourize with acid-alcohol. Add the reagent drop by drop till carbol fuchsin fails to wash from smear.
* Wash with tap water.
* Counter stain with methylene blue for 2 minutes

* Wash smear with tap water.
* Dry with blotting paper and examine under oil immersion.

Staining with the fluorescent dye, ammonia – rhodamine also has become important in the clinical microbiology laboratory for the detection of acid-fast mycobacteria. This stain binds to the mycolic acids of the cell walls of mycobacteria and resists decolourization with acid alcohol. When viewed with a fluorescence microscope, acid-fast bacteria fluoresce orange-yellow against a black background.

2.4.5. Flagella stain

Many bacteria are motile as they have one or more very fine thread like, filamentous appendages called flagella (Singl. Flagellum). Bacterial flagella are long, thin appendages free at one end attached to the cell at the other end. These are proteinaceous structures. They originate in cytoplasm and project out from the cell wall. The presence, location and the number of flagella are useful in identification and classification of bacteria. As they are very thin (about 20 nm) a single flagellum can never be seen directly with the light microscope but only after staining with special flagella stains that increase their diameter. So they are made thick first by using some chemicals called mordents. After mordent's treatment, stain the bacteria with dye. Flagella are also readily seen with the Electron Microscope (Fig.2.10).

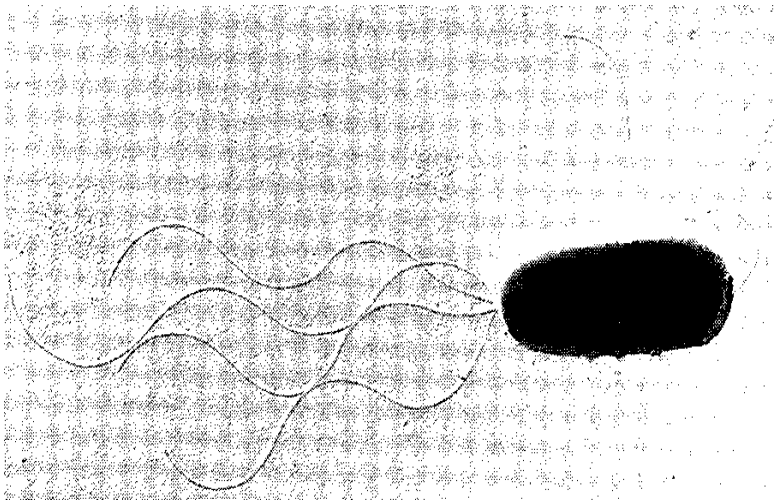


Fig. 2.10. A bacterial cell showing flagella

Procedure 1

* Take grease free slide
* Prepare bacterial suspension with distilled water
* Place a loopful of the suspension on one end of the slide
* Tilt the slide and allow the drop to spread to form a thin film on it. Do not heat fix the smear.
* Allow the film to air dry at room temperature.
* Cover the slide with flagella mordant for 10 minutes.
* Wash the slide with distilled water
*Flood the slide with carbol fuchsin for 15 minutes
* Wash the slide with distilled water. Do not blot dry the smear
* Air-dry the slide.
* Examine the slide under oil immersion.

Procedure 2

There is another procedure for flagella staining, which is known as the Leifson flagella stain. The Leifson flagella stain involves the following steps:

- The suspension of bacteria is fixed chemically, with formalin and spread on a glass slide.

- Allow the slide air dry without heating.

- Prepare freshly a mixture of tannic acid and rosaniline dye and then add to the slide. The tannic acid thickens the flagella. The rosaniline stains them.

- Wash off the excess stain by flooding the slide with water.

- Allow the slide to air dry before examining under the microscope.

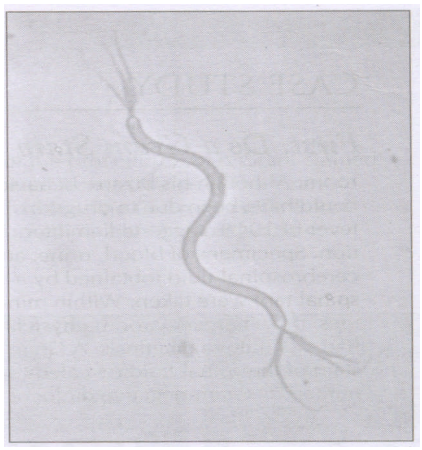


Fig. 11. The Leifson flagella stain of ***Spirillum volutans***

Flagella staining reveals the number and arrangement of flagella on bacteria, which is a vital information for identifying many species. Successful flagella staining is an art that develops only with practice.

2.5 General bacteriological media, composition and preparation

To culture microorganisms, microbiologists must establish the conditions necessary for microbial growth in the laboratory. This is possible only if suitable culture media are available. They must provide suitable environmental conditions and the necessary nutrients, so that the microorganisms can carry out their metabolism and reproduction. Under optimal growth conditions, most microorganisms reproduce very rapidly. Visible colonies of many bacteria, for example, develop in less than 24 hours if the culture is incubated under optimal conditions. The ability to obtain pure cultures within a matter of hours is especially important in the clinical microbiology labs, where speed of identification is essential. Many microbial identifications can be completed in less than a day. This permits the physician to begin appropriate treatment quickly.

To culture microorganisms, microbiologists must ensure that the culture medium contains a variety of organic and inorganic nutrients that are required for microbial metabolism. All organisms require carbon, nitrogen, oxygen, hydrogen, phosphorous, sulfur and various other substances for growth. These substances must be available in a usable chemical form to meet the nutritional requirements of a particular microorganism and to permit that organism to grow. Not all microorganisms have the same nutritional requirements. In fact, the specific nutritional requirements vary greatly for different microorganisms.

Commonly used culture media contain protein that have been digested with enzymes or acids and/or carbohydrates as growth substances that a microorganism can utilize. Because of the difficulty in defining the specific nutritional requirements of individual

microbial species, microbiologists use complex media, that contain various substances whose precise chemical compositions are unknown. Such complex culture media will support the growth of many different types of microorganisms that require organic compounds as their sources of energy. Many complex media contain beef extract, peptones and yeast extract. In some cases, scientists must add specific compounds to get a microorganism to grow in the laboratory. Clinical microbiologists incorporate blood into media that are designed for the culture of disease-causing microorganisms to provide necessary but unspecified nutrients of the growth of these microorganisms.

Scientist sometimes design media to prevent the growth of unwanted microorganisms or to permit the differentiation of specific types of microorganisms. Media are called selective media, if they favor the growth of specific microorganisms and differential media, if they permit recognition of specific types of microorganisms. In some cases a medium is both differential and selective. Selective media are designed to allow some microorganisms to grow while preventing the growth of others.

A medium composed entirely of chemically defined nutrients is termed as synthetic medium. One that contains ingredients of unknown chemical composition is termed as complex medium.

Synthetic or defined media

Some microorganisms, particularly photolithotrophic autotrophs like cyanobacteria and eukaryotic algae, can be grown on relatively simple media containing CO_2 as a carbon source, added as sodium carbonate or bicarbonate; nitrate or ammonia as a nitrogen source; sulfate, phosphate and many minerals (Table2.1).

Such a medium with all components is known as defined medium or synthetic medium. Many chemoorganotrophic heterotrophs can be grown in defined media with glucose as a carbon source and ammonium salt as a nitrogen source. Defined media are used

widely in research, as it is often desirable to know what the experimental microorganism is metabolizing.

Table.2.1 Examples of Defined media.

BG–11 Medium for Cyanobacteria	**Amount (g/liter)**
$NaNO_3$	1.5
$K_2HPO_4 \cdot 3H_2O$	0.04
$MgSO_4 \cdot 7H_2O$	0.075
$CaCl_2 \cdot 2H_2O$	0.036
Citric acid	0.006
Ferric ammonium citrate	0.006
EDTA (Na_2Mg salt)	0.001
Na_2CO_3	0.02
Trace metal solution[a]	1.0 ml/liter
Final pH 7.4	
Medium for *Escherichia coli*	**Amount (g/liter)**
Glucose	1.0
Na_2HPO_4	16.4
KH_2PO_4	1.5
$(NH_4)_2SO_4$	2.0
$MgSO_4 \cdot 7H_2O$	200.0 mg
$CaCl_2$	10.0 mg
$FeSO_4 \cdot 7H_2O$	0.5 mg
Final pH 6.8–7.0	

Complex media :

Media that contain some ingredients of unknown chemical compositions are complex media. Such media are very useful, as a single complex medium, may be sufficiently rich and complete to meet the nutritional requirements of many different microorganisms. Complex media are needed because the nutritional requirements of a particular microorganism are known, and so defined medium can not be constructed.

Complex media contain undefined components like peptones, meat extract and yeast extract. Peptone is a complex mixture of partially digested proteins. Its constituents are proteoses, polypeptides and amino acids, a variety of inorganic salts including phosphates, potassium and magnesium and certain accessory growth factors like riboflavin. They serve as sources of carbon, energy and nitrogen. Beef extract and yeast extract are aqueous extracts of lean beef and brewer's yeast. Beef extract contains amino acids, peptides, nucleotides, organic acids, vitamins and minerals. Yeast extract is an excellent source of B vitamin, nitrogen and carbon compounds. The commonly used complex media are (1) Nutrient broth (2) Tryptic soy broth and (3) MacConkey agar (Table.2.2).

Table. 2.2. Some common complex media.

Nutrient Broth	**Amount (g/liter)**
Peptone (gelatin hydrolysate)	5
Beef extract	3
Tryptic Soy Broth	
Tryptone (pancreatic digest of casein)	17
Peptone (soybean digest)	3
Glucose	2.5
Sodium chloride	5
Dipotassium phosphate	2.5
MacConkey Agar	**Amount (g/liter)**
Pancreatic digest of gelatin	17.0
Pancreatic digest of casein	1.5
Peptic digest of animal tissue	1.5
Lactose	10.0
Bile salts	1.5
Sodium chloride	5.0
Neutral red	0.03
Crystal violet	0.001
Agar	13.5

Simple media (basal media) :

An example for simple medium is nutrient broth. It consists of peptone, meat extract, sodium chloride and water. Nutrient agar, made by adding 2% agar to nutrient broth is the simplest and commonest medium used in the laboratories. If the concentration of agar is reduced to 0.2 to 0.5%, semisolid or sloppy agar is obtained which enables motile organisms to spread. Increasing the concentration of agar to 6% prevents spreading or swarming by organisms such as ***Proteus.***

Agar - agar is universally used for preparing solid media. It is obtained from some types of sea weeds. It is a sulfated polymer composed of D-galactose, 3,6-anhydro-L-galactose and D-glucuronic acid. Its chief constituent is a long chain polysaccharide. It also contains varying amounts of inorganic salts and small quantities of a protein-like substance. It has virtually no nutritive value and is not affected by the growth of bacteria. Agar is hydrolysed at high temperatures, at high acid and alkaline pH. Its unique property is that it melts at 98^{o}C and sets at 42^{o}C depending on agar concentration.

Enriched media

In these media, substances such as blood, serum, egg are added to basal medium. These media are used to grow bacteria which are more exacting in their nutritional needs. Examples are blood agar, chocolate agar and egg media.

Enrichment media

In mixed cultures or in materials containing more than one bacterium, the bacterium to be isolated is often overgrown by the unwanted bacteria. Usually the nonpathogenic or commensal bacterium tend to overgrown by ***Escherichia coli*** in cultures from feces. In such situations, substances which have a stimulating effect on the bacteria to be grown or an

inhibitory effect on those to be suppressed are incorporated in the medium, the result is an absolute increase in the numbers of the wanted bacterium relative to the other bacteria. Such media are called enrichment media, for example, tetrathionate broth, where the tetrathionate inhibits coliforms while allowing typhoid – parathyphoid bacilli to grow freely.

Selective media

Selective media favor the growth of particular microorganisms. Bile salts or dyes lke basic fuchsin and crystal violet favor the growth of Gram-negative bacteria by inhibiting the growth of Gram-positive bacteria. The media such as Endo agar, Eosin methylene blue agar and MacConkey agar are (Table 2.3) widely used for the detection of ***E.coli*** and related bacteria in water supplies. These media contain dyes that suppress Gram-positive bacterial growth. MacConkey agar also contains bile salts. A medium containing only cellulose as a carbon and energy source is quite effective in the isolation of cellulose digesting bacteria.

Indicator media

These media contain an indicator which changes colour when a bacterium grows in them, for example, incorporation of sulfite in Wilson and Blair medium. ***Salmonella typhi*** reduces sulfite to sulfide in the presence of glucose and the colonies of ***S.typhi*** have a black metallic sheen. Potassium tellurite in McLeod's medium gets reduced to metallic tellurium by the diphtheria bacillus to produce black colonies.

Differential media

These media distinguish different groups of bacteria and even permit tentative identification of microorganisms based on a differential medium and an enriched one. It distinguishes hemolytic from nonhemolytic bacteria. Hemolytic bacteria produce clear zones around their colonies because of red blood cell destruction for example, streptococci and staphylococci. MacConkey agar is both differential and selective medium. Since it contains lactose and neutral red dye, lactose-fermenting colonies appear pink to red in colour and are easily distinguished from colonies of nonfermenters.

Sugar media

The term ‘sugar’ in microbiology denotes any fermentable substance. The usual sugar media consists of 1% of the sugar in peptone water along with an appropriate indicator. A Durham’s tube is kept inverted in the sugar tube to detect gas production. For organisms which are exacting in their growth requirements Hiss’ serum sugar is used. They contain 3% serum.

Transport media

In case of delicate organisms (gonococci) which may not survive the time taken for transporting the specimen to the laboratory or may be overgrown by non pathogens, special media are devised and these are termed as ‘transport media’, for example, Stuart’s medium, a non nutrient soft agar gel containing a reducing agent to prevent oxidation and charcoal to neutralize certain bacterial inhibitors for gonococci and buffered glycerol saline for enteric bacilli.

Anaerobic media

These media are used to grow anaerobic organisms for example, Robertson’s cooked meat medium.

Preparation of broth

Bacteria are often cultured in a liquid broth. The most common constituents of basic media used in routine bacteriological laboratory are beef extract and peptones. These may be modified in a variety of ways by supplimenting with some specific chemicals or materials. This provides a medium suitable for the cultivation or demonstration of a reaction for specific types or groups of bacteria. Nutrient broth and glucose broth have been considered as basic liquid media for cultivation of bacteria.

Procedure

- Put the weighed amount of peptone (5g) beef extract (3g) and NaCl (5g) in 500 ml of distilled water.
- Heat with agitation to dissolve the constituents
- Make up the volume to 1000 ml by adding distilled water.
- Adjust pH of the medium to 7.0 by adding either acid or alkali.

- Pour 10 ml per tube or in a conical flask and close them with cotton plugs.
- Autoclave at 121^{o}C, 15lbs pressure for 15 minutes.
- Allow the tubes to cool.
- Store the tubes at room temperature for future use.

Preparation of solid medium

Liquid-growth media containing nutrients are solidified by adding agar. In the solidified state, solid medium may be kept in petri plates or in test tubes. Before cooling the test tubes are kept either in a slant position so that agar slants will be prepared or in an upright position, so that agar deeps will be prepared. Nutrient agar medium is the basic solid medium for cultivation of bacteria.

Procedure

- Put the weighed amount of peptone (5g), beef extract (3g) and NaCl (5g) in 500ml of distilled water.
- Dissolve the constituents and adjust pH to 7.0 either by adding acid or alkali.
- Add weithed amount of agar (15g).
- Make up the volume to 1000ml with distilled water.
- Autoclave at 121^{o}C, 15lbs pressure for 15 minutes.
- Remove the nutrient agar medium and pour either into sterilized petri plates or in test tubes. Test tubes can be used either as slants or deeps. Store at room temperature for future use.

2.5. Summary

- Staining increases the contrast between a specimen and its background so it can be seen under the microscope.
- Stains are dyes used to increase contrast. Most stains are effective only after microorganisms are fixed / killed and attached to a microscope slide. Basic dyes are composed of positively charged ions, and acidic dyes are composed of negatively charged ions.

- A mordant is a compound that increases a specimen’s affinity for a dye or that coasts a structure to make it larger.
- A simple stain used only one dye. A differential stain involves two stains, a primary stain and a counter stain.
- The Gram stain, a type of differential stain, distinguishes between Gram-positive and Gram-negative bacteria, reflecting differences in their outer surfaces.
- The acid-fast stain or Ziehl-Neelsen stain is a differential stain that colours mycobacteria and actinomycetes red and all other bacteria blue.
- The flagella or Leiflson flagella stain uses stains and mordents to thicken flagella, thread like appendages used for motility.
- Negative staining is used to reveal the protective capsule some bacteria have.
- Spore formers can be differentiated from non-spore formers by spore staining procedure. At the end of endospore staining, vegetative cells appear red or pink and spores appear green.
- In nature microorganisms grow on natural medium or the nutrients available in water, soil and living or dead organic material.
- To study microorganisms one has to grow and maintain organisms in the laboratory. This is possible only if suitable culture media are available.

- Culture media can be constructed completely from chemically defined components (defined media or synthetic media) or may contain constituents like peptone and yeast extract whose precise composition is unknown (complex media).
- Culture media can be solidified by the addition of agar, a complex polysaccharide from red algae
- Most routine laboratory cultures make use of peptones or digested meat. Other substances such as yeast extract, casein hydrolysate, serum, whole blood or heated whole blood are sometimes added.
- Diagnostic media are 1. Selective media if they encourage growth of some organisms and inhibit growth of others 2. Differential media , if they allow different kinds of colonies on the same plate to be distinguished from one another or 3. Enriched media , if they provide a nutrient that fosters growth of a particular organism.

- Culture media are classified based on function and composition as, general purpose media, enriched media, selected media, differential media, anaerobic media, transport media, sugar media, indicator media and synthetic media.

2.6. Model questions

1. Give a detailed explanation about the staining that is used to study morphology of bacteria

2. What is Gram staining? Explain the procedure?

3. What do these terms mean: basic dye, acidic dye and mordant?

4. What are the differences between these types of stains?
   A) simple stain B) differential stain, C) Gram stain D) acid-fast stain.

5. What is a negative staining used for?

6. Why is staining usually done?

7. What are the differences between a simple staining procedure and a differential one?

8. Describe the steps in the Gram stain?

9. Describe the steps in the flagella stain and capsule stain.

10. How do clinical microbiologists culture bacteria? Why does it often takeover a day to positively diagnose a disease.

11. What is a selective medium? How is such a medium used to help identify microorganisms in case of gastrointestinal tract infection?

12. What is a differential medium? How is such medium used to help identify microorganisms in case of respiratory tract infection?

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LESSON 3

STERILIZATION TECHNIQUES

3.0. Objective: To study about various physical and chemical sterilization techniques that are used for complete elimination of microorganisms.

3.1 Introduction

According to sterilization aspect the term control is used to refer the reduction in numbers and activity of micro flora. The importances involved in controlling of micro organisms are to prevent transmission of disease and infection, to prevent contamination, to inhibit growth of undesirable microorganisms and to prevent deterioration and spoilage of materials by micro organisms. In the process of sterilization all living cells, spores, viruses and viroids are killed, inactivated or removed from specific object or environment. Objects that are sterile are completely free from these microbial forms. Sterilization can be accomplished by various physical and chemical procedures.

Sterilization Methods

Sterilization is a process by which articles or materials are freed of all microorganisms both in vegetative or spore state. Sterilization is defined as a process of complete elimination or killing of all microorganisms including their spores and making the material free from them. For obtaining pure cultures of microorganism's sterilization of culture media and other instruments is essential. Sterilization can be divided into two broad categories, namely

1) Physical methods 2) Chemical methods

3.2 Physical Methods

Several physical agents can be used to control microbial populations, such as high temperature and ionizing radiations, which kill microorganisms by damaging essential cell components. These physical agents often disrupt enzymes, DNA and Cytoplasmic membranes. Other physical treatments such as filtration remove microorganisms without killing them. The physical treatments are widely used in microbiology laboratories for sterilization of culture

media and lab ware. They are also used in medicine and dentistry for sterilization of instruments and materials contaminated with microorganisms.

The sterilization by physical methods which involves the exposure of the material to physical agents those are lethal to microorganisms.

Sunlight

Sterilization by sunlight is cheap, easy and is a natural method. The use of the sunlight for controlling microorganisms has been followed from older days. Sunlight has an active germicidal effect due to combined effect of UV rays and also the presence of ozone in the outer region of the atmosphere. Suspension of bacteria is readily destroyed after exposure to sunlight. This is one of the natural methods of sterilization in case of water tanks, rivers and lakes.

Drying

Moisture is essential for the growth of bacteria. Half the weight of bacterial cell consists of water. Drying involves the removal of water and so it has deleterious effect on many bacteria. Spores are unaffected by drying. Susceptibility to drying varies with different bacteria and also on the conditions, under which they are exposed to drying i.e., on cloth, furniture, surrounded by water etc., so this method is unreliable.

Sunlight and drying methods mainly destruct bacteria due to coagulation of proteins.

Heat

Heat is most widely used lethal agent for the purpose of sterilization

Dry Heat

Dry heat sterilization is the general application in laboratories mainly for the sterilization of glass materials, which are used in laboratory. The penetrating power of dry heat is less and hence it requires more time and high temperature when compared to moist heat. The principle involved in the dry heat is denaturation of proteins.
Dry heat can be applied in different ways

1) Flaming: The most rapid sterilization method is “Flaming method”. The flame of the Bunsen burner is employed to sterilize the bacterial loop before removing the sample from the culture tube and after preparing the smear. Small articles like inoculating loops, points of forceps, scalpels, spatulas and glass rods are sterilized by holding them in a burner flame, till they become red hot. The mouth of the test tube, flasks and other containers are also routinely passed through the flame of a Bunsen burner to destroy the microorganisms. This method however prevents some bacteria but it is unsatisfactory.

2) Incineration: It is a method of destruction of microorganisms by simple burning into ashes. This is an excellent method used for the destruction of materials like dressings carcasses; infected laboratory animals, beddings and clothing of patients having contagious infection.

3) Hot air oven: This is the most widely used method of sterilization by dry heat. The hot air oven is mainly used for sterilizing glassware like test tubes, petridishes, conical flaks, pipettes and instruments such as forceps, scissors and scalpels. The glass ware should be perfectly dry before sterilization in hot air oven. Because wet glass ware is liable for crack. The materials such as rubber washes, corks are not suitable for sterilization by dry heat.

The dry heat or hot air sterilization is carried out in an electrical appliance called as "hot air oven". The oven is a double walled chamber and is usually heated by electricity and has a "thermostat" that maintains the chamber air constantly at the chosen temperature. The normal duration requested for dry heat sterilization is 2 hrs at 160^0C and 60 minutes at 170^0C temperature. Dry heat is believed to kill microorganisms by promoting a destructive oxidation of essential cell constituents.

Spores of Bacillus subtilis are more resistant to dry heat process. This bacterium is used as a biological indicator for evaluating the efficiency of hot air oven for sterility.

Moist heat

The bactericidal action of moist heat was first demonstrated and used by "Robert Koch". Moist heat is more effective than dry heat. The penetrating power of moist heat is more than dry heat. The time required for sterilization by using moist heat is less when compared with dry heat. The temperatures required for sterilization by using moist heat are also less than dry heat. The moist heat kills microorganisms by coagulating or denaturing their proteins and it is much more effective than dry heat.

Sterilization using moist heat can be achieved at different temperatures

a) Temperature below 100^0C
b) Temperature at 100^0C
c) Temperature above 100^0C

a) Temperature below 100^0C

1) Pasteurization

It is a mild heating process employing temperatures below 100^0C to destroy spoilage causing organisms and other types of pathogens. This process was discovered by "Louis Pasteur"; who discovered the technique of heating food without altering its composition. Milk cream and certain alcoholic beverages (wine and beer) are subjected to pasteurization. For pasteurization of milk two methods are available.

a) Low temperature long time method
b) High temperature short time method

a) Low temperature long time method: In this method the temperature employed for sterilization is 63^0C for 30 minutes.

b) **High temperature short time method:** In this method the temperature employed for sterilization is 71.2^0C for 15-20 seconds.

By using this process all non-sporing pathogens are destroyed.

Temperature at 100^0C

a) Boiling: Boiling in water is another simple method of sterilization. Contaminated materials (or) objects exposed to boiling water cannot be sterilized with certainty. All vegetative cells will be destroyed within minutes by exposure to boiling water. But some bacterial spores can with stand this condition for many hours. The practice of exposing instruments for short periods of time in boiling water is more likely to bring about disinfection i.e. destruction of vegetative cells of disease producing microorganism rather than sterilization. Sterilization by boiling can be enhanced by the addition of 2% sodium bicarbonate to the water. Boiling water cannot be used in laboratory as a method of sterilization.

b) Tyndallization (or) Fractional sterilization

Some microbiological media, solutions of chemicals and biological materials cannot be heated above 100^0C without being damaged. Then the alternative method applied to sterilize the materials is "tyndallization". This method involves heating the material at 100^0C on three successive days with incubation periods in between. Resistant spores germinate during the incubation periods; on subsequent exposure to heat, the vegetative cells will be destroyed. An apparatus known as "Steam Arnold" is used for this technique. However it is also possible to operate an autoclave with free flowing steam for this purpose.

Temperature above 100^0C (or) Autoclave

This is also called as stream heat sterilization. Heat in the form of saturated steam under pressure is the most practical and dependable agent for sterilization than hot air. Steam under pressure provides temperatures above these obtainable by boiling. Moist heat kills microorganisms by coagulating and denaturing the enzymes and structural proteins, a process in which water participates. The laboratory apparatus designed to use steam under regulated pressure is called an autoclave. The process of sterilization of materials in autoclave is called an autoclaving.

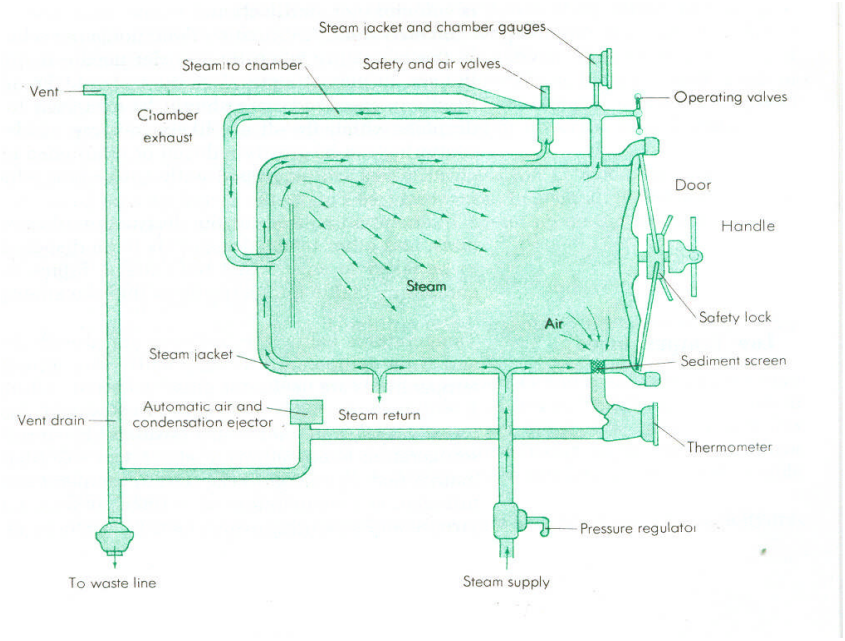

Fig. 3.1: AUTOCLAVE
(Courtesy from Pelczar)

Autoclave is essentially a double-jacketed steam chamber. It is usually equipped with devices for the complete discharge of air from the chamber and filling the chamber with saturated steam. It also consists devices to maintain a designated temperature and pressure for any period of time. When the chamber is completely filled with steam without any air it attains 121^0C temperature and 15 lbs pressure.

If air is present in the chamber it reduces the temperature even at 15lbs pressure. Temperature of the steam is that kills the organisms but not the pressure. This steam sterilization is especially suitable for culture media and aqueous solutions, since the atmosphere of the steam prevents the loss of water by evaporation during heating. In autoclave small volumes of liquids need 20 minutes sterilization and larger volumes require more time. Even the more resistant spores can be killed within 15-30 minutes by autoclaving process.

Sterilization by Filtration

Fluids and gases that cannot be sterilized by heat (or) by chemicals may be sterilized by allowing them to pass through specially constructed filters; without being altered in their physical and chemical properties. This process is generally used for the sterilization of serum, bacterial toxins, cell extracts, enzymatic solutions, antibiotics and carbohydrate solutions etc.

This procedure involves a simple passage of liquid to be sterilized through filters having small pores. These small pores retain the bacterial cells back in the filters. The nature of the fluid, pore size of the filter, change of filter can influence the efficiency of filtration. There are various types of filters made up of different materials. But the common filters that are used in laboratory are:

(i) Candle filters: These are widely used for purification of water for industrial and drinking purposes. They are of two types

a) Unglazed ceramic filters: Ex. Pasteur chamber land filter made up of by "Porcelain".
b) Diatomaceous earth filters: Ex. Berkefeld filter and Mandler filters.

(ii) Asbestos filters: These are disposable single used discs.
Ex. Seitz filters made up of asbestos.

(iii) Sintered glass filters: Made up of by ground glass.

Mainly there are two categories of filters.

I) Depth filters

These filters are fibrous sheets (or) mats made from paper, asbestos (or) glass fibers constructed at a random array of overlapping fibers. During filtration particles get trapped in the paths created throughout the depth.

II) Membrane filters

These are the new type of filters generally composed of cellulose acetate, cellulose nitrate with a large number of tiny holes. Here the particles are trapped on the surface of filters. They are available with different porosity. They are extensively used in laboratories and in industries to sterilize fluid materials.

Example: HEPA (High Efficiency Particulate Air filters): The development of "HEPA" filters has made it possible to deliver clean air to an enclosure such as a cubicle (or) a room. This HEPA filter consists an efficiency of 99.97% for removal of 0.3 μm diameter particles. The best example is "laminar airflow", which is a biological safety cabinet, used to produce dust and bacteria free air.

Sterilization by Radiation

Materials like gasses and liquids, which are heat labile, also can be sterilized by employing different types of radiation. Sometimes the sterilization by radiation is also referred to as cold sterilization". There are two main types of radiation.

(i) Sterilization by using non-ionizing radiation

(ii) Sterilization by using ionizing radiation,

Sterilization by using non-ionizing radiation

Ultraviolet light is the best example for non-ionizing radiation. UV light rays with 290-220nm wavelengths are lethal to various microorganisms. But the rays with wavelength of 253.7nm are the most effective radiation. Even though UV rays are lethal to microorganisms but it exhibits very little ability of penetration through the matter, so only the microbes on the surfaces of object, which are directly exposed to UV rays, are susceptible to destruction. UV

light is mostly absorbed by nucleic acid, in which it forms pyrimidine dimers and ultimately causes damage to the cell.

Sterilization by using ionizing radiation

X-rays, γ-rays and cathode rays are included in the category of ionizing radiation. Ionizing radiation is a good sterilization agent with greater penetration but the technique is very expensive. Bacterial spores are more resistant and gram-negative bacteria are more sensitive to ionizing radiations. X-rays are lethal to microorganisms as well as higher forms of life. Gamma rays are also the best examples of ionizing radiation. This radiation causes damage to cells by producing hyper reactive ions. However at higher does of sterilization with radiation there is a change in color and flavour of the material. Hence this method is commonly used for sterilization of disposable materials.

3.3 Chemical Sterilization

Disinfection is the process of killing, inhibition (or) removal of microorganisms that may cause disease. The chemical agents that are used for this disinfection are called as chemical disinfectants. Disinfectants are potent and toxic in destroying pathogenic microorganisms but not necessarily in killing the resistant spores. Disinfectants are suitable for applications on inanimate objects. There are several chemicals which are often employed in disinfection process. The major disinfectants that are commonly used are

(1) Phenol and phenolic compounds

(2) Alcohols

(3) Heavy metals

(4) Halogens

(5) Dyes

(6) Detergents

(7) Aldehydes

(8) Gaseous agents

Alcohols: Alcohols are one of the most widely used disinfectants. They are bactericidal, fungicidal but not sporicidal. The most widely used disinfectants in alcohols are ethyl alcohol and isopropyl alcohol. Ethyl alcohol in concentration between 50-90% is effective against vegetative and non-spore forming cells. For practical application generally 70% ethyl alcohol is used. The higher alcohols like propylalcohol, butyl alcohol and amyl alcohol and others are more germicidal than ethyl alcohol. Alcohols are effective in reducing the microbial flora of skin and for the disinfection of clinical oral thermometers. In microbial laboratories it is used as common disinfectant. Alcohols act as disinfectants by denaturing proteins, inactivating the enzymes and dissolving the membrane lipids.

Phenol And Phenolic Compounds

Phenol is the first disinfectant used by "Joseph Lister" in 1880's. phenol and phenolic compounds are very effective disinfectants. A 5% aqueous solutions of phenol rapidly kills the vegetative cells of microorganisms. Phenol and phenolic compounds such as bisphenols, cresols, xylenols, orthophenyl phenols are used as disinfectants in most of the laboratories and hospitals.

Lysol is a commercial disinfectant prepared by a mixture of phenols depending on the concentration in which they are used. Phenolic substances are either bactericidal or bacteriostatic. Some phenolics are highly fungicidal. Bacterial spores and viruses are more resistant than vegetative cells. Phenols and Phenolic compounds by their actions, inactivate and denature the enzymes, proteins and ultimately disrupts the cell membrane to irreversible state.

Halogens: Chlorine and iodine are generally used as disinfectants than other halogens. Chlorine is an important and excellent disinfectant. It is generally used in municipal waters, swimming pools, dairy and food industries. Using the halogen tablets can successfully disinfect small quantities of water, which slowly releases chlorine when added to water. Iodine is one of the oldest and most effective disinfectants. At higher concentrations it may even kill some spores. Iodine is normally applied in the form of tincture of iodine and iodophores. Iodophores are the preparatives of iodine, complexed with some organic carriers. They are used in hospitals for pre-operative skin damaging. The antimicrobial action of chlorine and its derivatives is due to the formation of hypochlorous acid. It brings the oxidation of proteins and enzymes and thereby destructs the vegetative cells. The mode of action of iodine and its derivatives is due to the inactivation of essential metabolic compounds such as proteins with sulfhydryl groups as they are highly oxidizing agents.

Heavy Metals: The ions of heavy metals like mercury, zinc, arsenic, silver and copper are used as disinfectants. They are used either alone or in certain compounds. Heavy metals and their compounds act by combining with cellular proteins and inactivating them. Higher concentrations of mercury, copper and silver coagulate cytoplasmic proteins and result in the damage or death of the cells.

Silver nitrate solutions of 1% concentration are often used to prevent ophthalmic gonorrhea in infants. Silver sulfadiazine is used on burns. Copper sulfate is an effective algicide in lakes and swimming pools.

Dyes: Two classes of dye compounds, which have antimicrobial properties, are of special interest to microbiologists. They are

(i) Triphenyl methane dyes (ii) Acridine dyes

Triphenyl Methane Dyes

Mainly malachite green, brilliant green and crystal violet comes under this category. Gram positive organisms are more susceptible to lower concentration of these compounds than gram negative ones. Crystal violet can be used as bactericide as well as fungicide. The modes of

actions of triphenyl methane dyes are uncertain. But these dyes in general interfere with cellular oxidation processes and inhibit the growth of organisms.

Acridine dyes: Acriflavin and tryptoflavin are comes under the category of acridine dyes. These compounds exhibit selective inhibition against bacteria particularly staphylococci and gonococci. They are used to some extent for the treatment of burns and wounds and for ophthalmic application.

Detergents: The wetting agents employed primarily for cleansing surfaces are called detergents. Ex. Soap.

The synthetic detergents called as "surfactants" are the more efficient cleaning agents. They are extensively used in laundry and dish washing powders, shampoos etc.

Detergents are mainly divided into three types.

1) Anionic detergents
2) Cationic detergents
3) Non-ionic detergents,

Anionic Detergents: Those, which ionize with the detergent property resident, in the anion are referred to as anionic detergents.
Ex. "Sodium lauryl sulfate"

Cationic Detergents: Those that ionize with the detergent property resident in the cations are referred to as cationic detergent.
Ex. Cetyl pyridinium chloride; Benzalkonium chloride.

Non-Ionic Detergents: A third category of detergents is non-ionic i.e. they do not ionize. However these substances do not posses significant antimicrobial activity.

Cationic detergents are more effective than anionic detergents. Most of the cationic detergents, which are germicidal, belongs to a class of quartenary ammonium slats. Their mode of action includes denaturation of proteins, interference with glycolsis and membrane damage. The cationic detergents are widely used as skin disinfectants as a preservative in ophthalmic solutions and in preparation of cosmetics. These are also used in hospitals, nursing homes, hotels, and at other public places to sanitize the food, utensils and places etc.

Aldehydes

Several aldehydes having low molecular weight are used as disinfectants. The two important and widely used aldehydes are formaladehyde and glutaraldehyde. Aldehydes are both microbicidal and sporicidal. They combine with proteins and inactivate them. Formaldehyde is the simplest compound of aldehydes. It is usually dissolved in water or alcohol before used. The fumes of formaldehyde are irritating to tissues and eyes. Formalin is one of the aqueous solutions available commercially as a gaseous form. Formaldehyde is used to disinfect the enclosed areas

Glutaraldehyde is used as an effective disinfectant. It causes less irritation than formaldehyde. It exhibits wide spectrum of antimicrobial activity against bacteria, fungi, spores. Glutaraldehydes disinfect the objects within ten minutes of time. It is used for sterilizing biological instruments, lensed instruments, and respiratory therapy equipment in medical fields.

Gaseous Agents

Many heat sensitive surgical instruments and disposable Petri dishes are disinfected by using certain gases

Ex. Ethylene oxide and β-propiolactone are 2 best sterilizing gases.

Ethylene oxide is both microbicidal and sporicidal. It is so effective as it penetrates rapidly through packing materials and through plastic wrappers also. Ethylene oxide combines with organic compounds such as enzymes and proteins and inactivates them by alkylation reactions. Removal of residual ethylene oxide is necessary as it is so toxic. Extensive aeration of sterilized materials removes the residual of ethylene oxide.

β - propiolactone is occasionally employed as sterilizing gas. In liquid form, it is used to sterilize vaccines and sera. It destroys microorganisms more rapidly than ethylene oxide. But the penetration power is less than ethylene oxide. β - propiolactone decomposes to an inactive form after several hours unlike the ethylene oxide.

3.4. Summary

Sterilization is the complete killing of all microorganisms. Sterilization can be accomplished by both physical and chemical methods. In physical methods of sterilization the most widely used methods is the application of heat. The temperature for heat sterilization is selected to eliminate the most heat resistant organisms in the material usually bacterial endospores. Heat can be applied in two forms for sterilization purpose. They are dry heat and moist heat. The most widely used method of sterilization by dry heat is hot air oven. This is mainly used for sterilization of glassware. But the heat sterilization by moist heat is more advantageous than dry heat. For routine sterilization an autoclave is used. This permits application of steam heat under pressure at temperatures above the boiling point of water. Filter sterilization involves the removal of living microorganisms from liquids. Membrane filters are widely used for sterilization of heat sensitive liquids in laboratory. Materials which are heat labile also can be sterilized by employing different types of “Radiation”.

Chemicals are often used to control microbial growth. Chemicals that kill microorganisms are called Cidal Agents; those that inhibit growth are called Static Agents. The value of a chemical agent is assessed by determining the minimum concentration necessary to kill (or) inhibit growth and by determining whether it exhibits selective toxicity. Disinfectants are chemical compounds used to decontaminate (or) sterilize inanimate objects. Antiseptics can be used to decontaminate living tissues. These compounds are used in many commercial, health care & industrial applications.

3.5. Model Questions

Essay Type Questions

1) Define the term sterilization and explain different physical methods of sterilization?
2) What is sterilization? Write the different methods of sterilization.
3) Define disinfection and write a detailed account about the chemical disinfectants?
4) For cultivation of a typical microorganism enumerate sterilization and disinfection methods?

Short Answer Questions

1) Describe sterilization of materials through heat?
2) Hot Air Oven
3) Moist heat sterilization.
4) Describe sterilization of materials through filtration.
5) Describe sterilization of materials through radiation.
6) Disinfectants
7) Pasteurization

3.6. Reference Books

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2) Brock T.D., Madigan, M.T., Prentice Hall Englewood Cliffs Publications, Edition No. 5 – Biology of Microorganisms
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Lesson – 4

ISOLATION OF BACTERIA

4.0. Objective: The main objective of this lesson is to explain in detail the different physical and chemical methods applied for the isolation of the desired microorganisms from different sources.

4.1. Introduction

In natural environments microorganisms usually grow in a complex mixed population containing several species. To study the characteristics of one species, that species must be separated from all other species or in other words it must be isolated into a pure culture form. How ever before attempting for isolation it is often helpful to use a selective method first. Such method can increase the relative proportion of the desired species in the population so that it can be more easily isolated. Once obtained, a pure culture can be maintained or preserved in a culture collection. The isolation of microorganisms in pure form depends on two operations. Isolation and separation of particular microorganisms from a mixed population that exist in nature and culturing these isolated organisms in artificial environment (culture medium under lab conditions).

Bacteria are usually cultured and studied under laboratory conditions. Numerous media (singular medium) have been developed for bacterial cultivation. Different species of bacteria growing on the same kind of medium may appear quite different, thus the knowledge of the appearance or the culture characterization of the species is useful for the recognition of certain kinds of bacteria and may also serve as an aid to the identification of species. The nutritional requirements of bacteria vary widely. There are great differences in the chemical compositions of the media used in laboratory. In addition bacteria also exhibit wide difference with respect to physical conditions favoring the bacterial growth such as temperature, pH and gaseous environment. The successful isolation and cultivation of bacteria requires an awareness of all these factors.

4.2. Selective methods of isolation

A particular bacterial species is often present in small numbers compared to the total populations of a mixed culture, moreover the species may be one that grows less rapidly on

an ordinary culture media than other species. In order to achieve its isolation in to pure culture it is helpful and often necessary to first achieve an increase in the relative number of the species. Preferably to the point where the species becomes numerically dominant then other microorganisms present in the sample. This can be accomplished by the use of selective methods. These selective methods favor the growth of the desired species while discouraging or even killing the other organisms present in mixed culture. Chemical and Physical or Biological methods are used in order to achieve selection of a particular kind of bacterium.

4.3. Serial dilution of the sample

The purpose of serial dilution is to reduce the number of microorganism so that isolation becomes easy. The method is based on the principle that when material containing bacteria is cultured every viable bacterium develops in to a visible colony on a nutrient agar medium. The number of colonies is same as the number of organisms contained in the sample. When the microorganisms have to be isolated from different sources such as water, soil, milk etc the samples are subjected to dilution because it would be containing large number. of microorganisms and isolation of a specific microorganism from these samples becomes difficult so the number of organisms are reduced by diluting the samples for isolation to become easy.

In this procedure a small measured volume is mixed with a large volume of sterile water or saline called the dilute or dilution back. Dilutions are usually made in multiples of ten.

A single dilution is calculated as follows

$$\text{Dilution} = \frac{\text{Volume of the sample}}{\text{Total volume of the sample and the dilutent}}$$

Serial dilutions are later prepared by transferring a known volume of the dilution to the second dilution blank and so on. Once diluted the specified volumes of the dilution sample from various dilutions are added to a sterile petridish containing the medium.

The no. of microorganisms / ml is calculated as follows:

$$\text{No. of cells/ml} = \frac{\text{Number of colonies}}{\text{Dilution volume plated}}$$

Procedure for serial dilution

a) Label the dilution blank as 10^{--1}, 10^{--2}, 10^{--3}, 10^{-4}, 10^{--5}, 10^{--6}, $10^{—7}$ and so on.
b) Prepare the initial dilution by adding 1 ml or 1 gram of the sample in to a 9 ml of dilution blank labeled as $10^{—1}$ thus diluting the original sample 10 times (1/1+9 = 1/10 = 1:10 or $10^{—1}$).
c) From the first dilution transfer 1 ml sample to the second dilution blank. Marked as $10^{—2}$ with sterile and fresh 1ml pipette, diluting the original specimen to 100 times (1/10 x 1/10 = 1/100 = $10^{—2}$).

d) From 10^{-2} suspension transfer 1 ml of suspension to 10^{-3} dilution blank. Thus diluting the original sample 1000 times (1:1000 or 10^{-3}).
e) Repeat the dilution of the original sample up to 10,000,000 (10^{-7}) thus using each time a fresh pipette.(fig 4.1)
f) From appropriate dilution (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}) transfer 1 ml or 0.1 ml of diluted sample on to the Petri plates and count for no. of colonies as per the calculations.

Pure cultures that form discrete colonies on solid media may be most simply obtained by one of the plating methods. These methods involve the separation and immobilization of individual organisms on a nutrient medium solidified with agar. Each viable organisms give rise through growth to colony from which transfers can be readily made. Three different kinds of plating methods have been used. Streak plate method Spread plate method and Pour plate method.

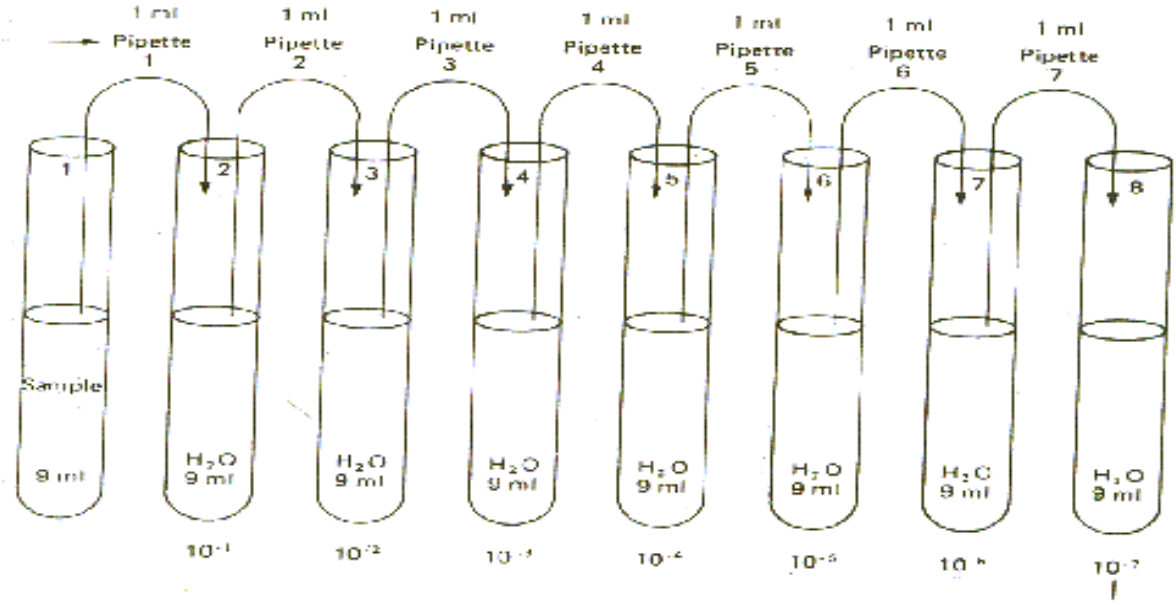


Figure 4.1: Procedure for serial dilution of the sample. (Courtesy, practical microbiology, Anaja).

Streak plate method

In the streak plate technique, for isolating pure cultures of bacteria, a loop full of bacterial cell suspension diluted is streaked across the surface of a sterile solidified nutrient agar medium contained in a petriplate. Many different streaking patterns can be used to separate individual bacterial cells on the agar surface and the plates are then incubated under favorable conditions to permit the growth of the bacteria. The key principle of this method is that by streaking, a dilution gradient (number of cells decreases as they move across the agar and away from the point of inoculation) is established across the face of the plate as bacterial cells are deposited on the agar surface. Because of this dilution gradient, confluent growth occurs on part of the plate where the bacterial cells are not sufficiently separated. In other regions of the plate where the bacteria are well separated and the bacteria grow as individual cell giving rise towel separated isolated colony. Separate microscope colonies develop that can easily be seen with naked eye.

Each well isolated colony is assumed to arise from a single bacterium and therefore to represent a clone of a pure culture. If this important premise is not sustained, for example because two bacterial cells are deposited at the same location on the same plate the method fails to produce a pure culture. Assuming that each colony comes from a single cell, sample of the isolated colonies can be picked up using a sterile inoculating loop and re streaked on to a fresh medium to ensure purity.

Spread plate

In the spread plate method a loop of liquid containing a suspension of microorganisms is placed on the centre of agar plate and spread over the surface of the agar using a sterile hockey stick shaped glass rod (L shaped). The glass rod is normally sterilized by being dipped in alcohol and ignited to burn off the alcohol,. When the suspension is spread over the plate individual microorganisms are separated from others in the suspension and are deposited at discrete locations. To accomplish this separation it is often necessary to dilute the suspension before application to the agar plate to prevent over crowding and the formation of confluent growth rather than the desired development of isolated colonies. After incubation isolated colonies are picked up and streaked onto a fresh medium to ensure purity.(fig 4.2)

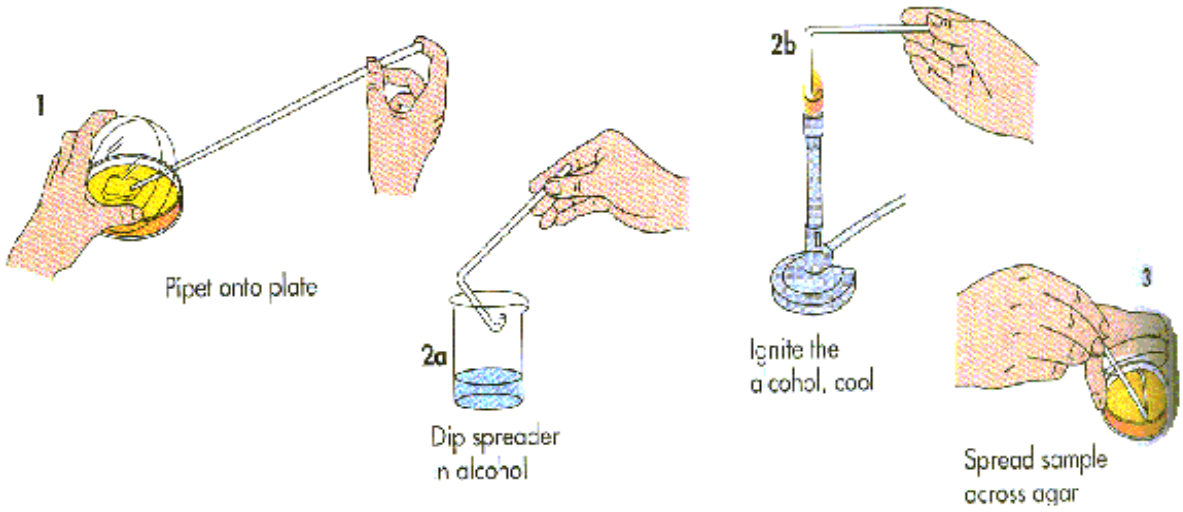


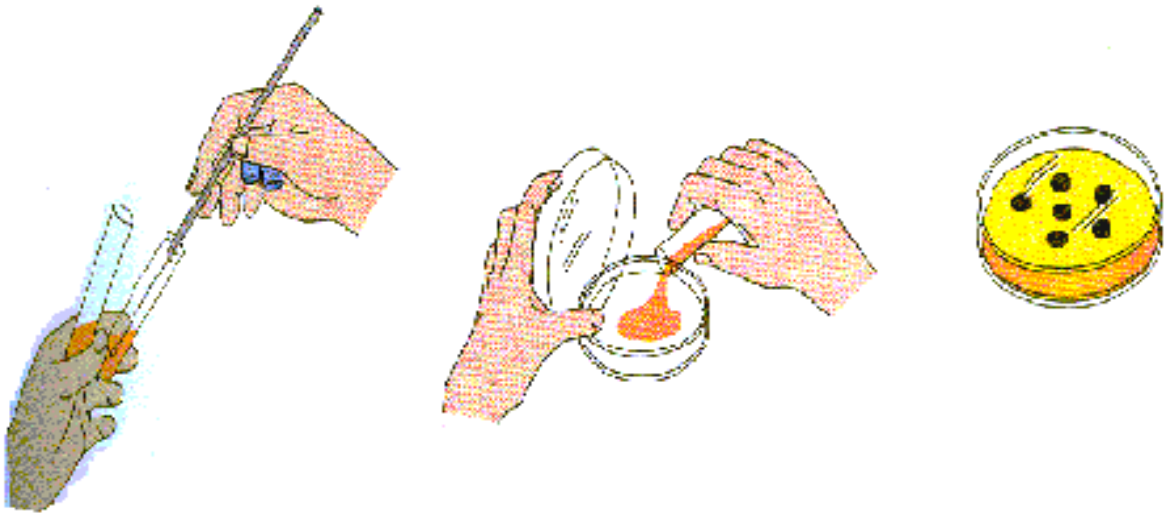
Figure 4.2: Spread plate method. (Courtesy Microbiology, Ronald M. Atlas).

Pour plate

In the pour plate technique, suspension containing microorganisms is added to the tube containing melted agar cooled to approximately 42^0 to 45^0C. The bacteria and the agar medium are mixed well and the suspension is poured in to a sterile petridish under aseptic conditions. The agar is allowed to solidfy by gently tapping. The bacteria are located at separate discrete positions within the medium, while the medium holds bacteria in place and is still soft to permit the growth of bacteria and the formation of discrete isolated colonies within the gel and on the surface of the agar.

As with the other isolation methods individual colonies are then picked up and streaked onto another plate for purification. In addition to its use in isolating pure cultures the

pour plate technique is used for quantification of numbers of viable bacteria. The facts that agar solidifies below 42^0C and that many bacteria survive at these temperature ensures the success of this isolation technique. Because in some cases such as in marine samples, significant numbers of bacteria are killed under these conditions. This method cannot always be used.(fig 4.3)



pipette 0.1 ml of bacterial suspension into molten agar

Pour

Colonies develop through out the plate

Figure 4.3: Pour plate method (Courtesy Microbiology, Ronald M. Atlas).

4.4. Chemical methods of selection

Use of a special Carbon or Nitrogen source

The application of chemical method is to provide in the culture medium a substrate i.e. a single carbon or nitrogen source that can be used only by the species being sought. This particular kind of selection is often referred to by a special name enrichment Enrichment technique by considering the metabolic capabilities of specific microorganisms it is possible to design growth media that favor the growth of a particular microorganism. This principle is the basis for the specific groups of microorganism and is based on designing of culture medium and incubation conditions that preferentially support the growth of a particular microorganism. The enrichment culture technique mimics many natural situations in which the growth of a particular microbial population is favored by the chemical composition of the system and by environmental conditions. Enrichment media tend to select the microorganisms that grow best among all the microorganism introduced in to the media.

For example to isolate microorganism, capable of metabolizing petroleum hydrocarbons; one can design a culture medium containing a kind of hydrocarbon as the sole source of carbon and energy, by doing so one establishes conditions where by only microorganism that are capable of metabolizing hydrocarbons can grow. Because other microorganisms can not reproduce in this medium, one there by prefentially selects for hydrocarbon utilizing microorganism. Similarly, a culture medium that favors the growth of autotrophic microorganism that derive their energy from the oxidation of ammonium ions and inorganic carbon could provide ammonium ions in the medium.

Example 2: If we wish to Isolate from soil bacteria capable of utilizing α – condendrin a constituent of wood, a very complex constituent as carbon source, we find that when we inoculate a medium, such as nutrient agar directly with the soil sample our chances of finding α – condendrin utilizing bacteria will be very limited. There are so many rapidly growing bacteria present that the more slowly growing kind we wish to obtain will be soon over grown. Consequently we prepare a liquid enrichment medium in which α – condendrin is the sole source of carbon. Under these conditions only organisms capable of utilizing this compound will be able to grow well, only organisms capable of utilizing this compound will be able to grow well. However it is important to recognize that other bacteria may be able to grow to some extant by utilizing organic compounds made by conidendrin utilizing organisms and the method is not completely specific. As another example if we wish to select for Nitrogen fixing bacteria Nitrogen gas (N_2) can be supplied as the sole nitrogen source. Since only nitrogen fixing bacteria will be able to grow well other bacteria may grow but to a lesser degree by using the nitrogenous products made by the nitrogen fixers.

Use of dilute media

Certain aquatic bacteria such as *Caulobacter* species are capable of growing with very low levels of carbon and nitrogen sources. Consequently one way to select for such bacteria, is to inoculate a mixed culture into a very dilute medium, example, A broth containing 0.01 percent peptone. The medium must have enough low levels of nutrients that other kinds of organisms will not be able to grow well in it.

Use of inhibitory of toxic chemicals

The addition of low levels of certain chemicals such as dyes bile salts of heavy metals or antibiotics to culture media can be useful for the selection of certain kind of bacteria. The following are some of the examples.

1) Many gram bacteria can grow in the presence of low concentrations of various dyes, that inhibit the growth of gram positive bacteria, similarly intestinal bacteria can grow in the presence of bile salts such as sodium deoxycholate but non intestinal bacteria are usually inhibited. Consequently a medium containing crystal violet dye plus sodium deoxycholate will allow gram-negative intestinal bacteria to grow but will inhibit most other kinds of bacteria. An example of such a medium is Mac Conkey agar which is widely used to select for gram negative intestinal pathogens such as *Salmonella* and *Shigella* species.

2) *Campylobacter jejuni* a frequent cause of diarrhea in humans, yet diarrheic stool sample contain many other kinds of bacteria that interfere with the isolation of the species. By incorporating certain antibiotics or other chemotherapeutic agents such as vancomycin, ploymyxin and trimethoprin into the culture medium, most of these containments can be inhibited without affecting the growth of *Campylobacter jejuni.*

Streak feeding (BAITING)

Some protozoa, slime, bacteria and slime molds may be isolated free from contaminating micro organisms by streaking by streaking their food organisms usually a bacterium (preferably dead) over the surface of an agar plate. A sample of liquid or soil containing these organisms is placed in the centre of the plate. The desired motile organism will find its food organism and following the streak as it feeds may migrate away from some if not all contaminants. The progress of baiting technique may be followed under a binocular dissecting microscope if the organisms are larger ones. The atmosphere over the agar surface

must be kept moist. These plates are incubated in a humid chamber or the plates may be sealed with paraffin.

4.5. Physical methods of selection

Heat treatment

To select for endospore-forming bacteria a mixed culture can be heated to 80^0C for 10minutes before being used to inoculate culture media. Vegetative cells will be killed by this treatment but endospores will survive and subsequently germinate and grow.

Incubation temperature

To select psycrophillic or psychotropic bacteria, cultures are incubated at a low temperature(0 to 5^0C). For selections of thermopiles a high incubation temperature is used (55^0C). Because based on the temperature tolerance bacteria are classified into mesophiles (bacteria which grow well in arrange of temperature from 20 to 45 degrees) thermopiles (which grow well at above 55 degrees temperature) and psycrophiles (grow well below 5 degrees temperature) mesophiles do not grow below 20 and above 45 degrees temperature so by maintaining conditioned temperatures the corresponding microorganisms can be isolated.

pH of the medium

To select for acid tolerant bacteria a low pH medium can be used, for example to select for the lactobacilli present in cheddar cheese. The pH of the medium is maintained at 5.35 with an acetic acid/acetate buffer; other organisms in the cheese can not grow well at such a low pH similarly to select for alkali tolerant organisms a high pH medium can be used.

For example : To select for the cholera-causing bacterium *Vibrio cholera* from stool sample we can use a medium with a pH of 8.5(alkali) most other intestinal bacteria are unable to grow at this pH and the organism which only can tolerate high pH only will grow

Cell size and motility

We can some times make use of a small cell diameter or of bacterial motility to achieve selection for instance. *Treponema* species form the human oral cavity can be selected by taking advantage of both these properties. A membrane filter having a pore size of 0.15 nm is placed on the surface of an agar plate and gingival scrapping is placed on the filter. The unusually small size of *Treponemes* allows them to penetrate the pores of the filter to reach the underlying agar. More over *Treponemes* have the ability to swim through solid agar media consequently they migrate away from the filter and grow to form a hazy zone with in the agar from which they can be sub cultured, other bacteria from the oral cavity are either too large to penetrate the membrane filter or if they can penetrate, they are unable to migrate away through the agar..

Centrifugation

Many micro organisms and their cysts, spores etc., may be separated from contaminating organisms by centrifugation. Depending on their density some are packed down, others float. This density difference may be more subtly exploited by centrifuging. The desired cell material in a density gradient in which the desired microorganism will seek a level corresponding to their own density But other microorganisms which may be only

slightly different in density are washed off and find their own density levels. The density gradient may be discontinuous or continuous and is usually made with biologically inert molecules i.e. sucrose, mannitol. The centrifuge method may also be used in conjunction with agents which kill or remove the contaminants such as antibiotics, disinfectants, antiseptics, mild detergents etc.

4.6. Biological methods of selection

A disease producing species occurring in a mixed culture can often be selected by taking advantage of its pathogen properties for example, sputum. sample containing Streptococcus *pneumonia* is ordinarily contaminated by many other bacterial species, how ever laboratory mice are extremely susceptible to infection by Streptococcus *pneumonia* and if sputum sample is injected into mice the pathogen will multiply extensively. Non pathogen bacteria present in the sample will either be inhibited or killed by defense mechanism of the animal. In a sense the animal serves as the selective medium.

4.7. Selection in nature

It is important to realize that the principle of selection is not limited to the laboratory it also commonly operates in nature. For instance the occurrence of high salt concentration in bodies of water such as the dead sea selects for extreme halophiles, such as those of genus *Halobacterium*. In lakes the anaerobic sulfide containing zone or above the sediment mud provides conditions that often favor the mass development of green purple sulfide oxidizing phototrophic bacteria. The nodules that occur on the roots of leguminous plant contain bacteria of the genus. *Rizobium* which are uniquely selected for nitrogen fixation in association with these plants. In many types of natural infections of humans or animals a single uncontaminated pathogen bacterial species can often be obtained from a blood sample blood from a healthy animal or human is normally free of bacteria. Numerous other examples of selection conditions in nature exist.

Micromanipulator technique

A device called the micromanipulator can be used in connection with microscope to pick a single bacterial cell from a mixed culture. The micromanipulator permits the operator to control the movement of a micropipette or microprobe (fine needle) so that a single cell can be isolated. This technique requires a skilled operator and is reserved for studies in which clone must be obtained unequivocally.

4.8 Summary

The ability to examine and study the characteristics of microorganisms including obtaining organisms for microscopic visualization depends in large part on being able to grow microorganisms in laboratory . Pure culture contain only one kind of microorganisms. They are free from all other types, several different methods are used for the establishment of pure cultures of microorganisms.

The isolation of pure culture involves separating samples of microorganisms into individual cell that are then allowed to reproduce and form clones of single microorganisms . Each clone represents a pure culture. Isolation is achieved by physical separation of microorganisms , but the success of an isolation method also depends on the ability to maintain the viability and growth of a pure culture of microorganisms . The different physical

parameters such as temperature PH oxygen requirement would add to isolation of microorganisms .The different chemical and biological methods are employed for isolation of microorganisms .The success of isolation methods depends on the ability to grow the microorganisms that is to define growth medium and to establish the appropriate incubation conditions that permit is growth

4.9 Model Questions

Essay type Questions

1) What are pure cultures and why are they important and write different methods employed in isolating the microorganisms from different sources?
2) What is meant by dilution gradient? How are spread plates streak plates and pour plates prepared?
3) Why are samples have to be serially diluted and what is the procedure involve in serial dilution?
4) Describe the following kinds of media such as selective media, differential media and they importance in isolation of microorganisms.

Short Answer Questions

1) Baiting technique
2) Isolation of microorganisms based on size.
3) Incubation temperature
4) Pour plate
5) Dilution gradient

4.10 Reference Books

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Lesson 6

MAINTENANCE AND PRESERVATION OF MICROBIAL CULTURES

6.0 Objective

To understand the different methods of maintenance and preservations of microbial cultures which is essential from research and application points of view.

6.1 Introduction

Microbiologists are concerned at some time with the maintenance and preservation of the cultures with which they work. It is necessary to have convenient methods to keep cultures alive, to keep them in a genetically stable form. The choice of method depends on the nature of the microorganism and on the preservation objectives. The method to be

used depends on whether the culture is to be preserved for a few days until a positive identification is made, for the duration of a research project or for future long term reference. The choice of method also depends on the nature of the microorganisms and on the facilities available. The preservation method also reflects the difference in the biological properties of the bacteria, viruses, fungi, algae and protozoa and in their ability to survive in natural and artificial environments. Preservation methods have a common objective of reducing the organism's metabolic rate as low as possible while still maintaining viability. A high recovery or survival rate with a minimum of damage or change to the surviving organisms is also highly desirable.

Often, valuable cultures are deposited in centralized culture collections and it is important to deposit all the new microbial species in such culture collections to ensure their indefinite preservation and to make them available for scientific study. Some of the centers recognized for this purpose include—

1. American Type Culture Collection (ATCC), USA
2. Central Bureau Voor Schimmel Cultures, The Netherlands
3. Commonwealth Mycological Institute (CMI), England
4. Culture Collection of Algae and Protozoa, England
5. Institute Pasteur, France
6. Microbial Type-Culture Collection, Japan
7. USSR Antibiotic Research Institute, USSR
8. National Collection of Industrial Bacteria, Scotland
9. Microbial Type Culture Collection and Gene Bank (MTCC), India
10. Indian Type Culture Collection (ITCC), India

Culture collections occupy a central and essential position in microbiology because effective identification, research and training demand reliable sources of microorganisms. Culture collections consist of three main types viz., service collections whose function and organization is chiefly for the preservation and supply of cultures on demand; institutional collections whose supply is mainly for internal use in the institution concerned; and private collections chiefly for personal research purposes. These private

collections may be highly specialized and important to other scientists, and often are the only available source of particular strains or kinds of microorganisms.

6.2 Preservation Methods

6.2.1 Periodic Subculture

Periodic transfer or subculture is the traditional method used by microbiologists to maintain isolates in the laboratory. Apart for some cultures for which no long term preservation methods are yet available, periodic subculture is not recommended for long term preservation. Genetic change through selection of variants is likely to occur, the chances of contamination and mislabeling are high and the risk of culture loss is greater than in other methods. Many bacteria and fungi are able to survive for ten or more years on sealed agar slopes or in agar stabs at room temperature or in refrigerators by this method. Longevity of such cultures is influenced by the nature of the organism itself, the composition and pH of the medium, the degree of aeration, and the temperature of storage. Low temperatures are usually preferred. However, some strains of *Pseudomonas cepacia* prefer storage at room temperature. The degree of hydration is also an important factor and slow uncontrolled dehydration often leads to a loss of viability.

6.2.2 Preservation by Freeze-Drying

Freeze-drying or Lyophilization has been widely used as the preferred method for long term preservation for many years. The method is suitable for many types of microorganisms including most bacteria, yeasts, sporing fungi but generally unsuitable for non-sporing fungi, algae and protozoa. Many cultures are able to survive for periods of twenty to thirty years. Freeze-drying combines two of the most successful long term preservation methods, freezing and drying. The overall process involves the removal of water vapour by vacuum sublimation from the frozen state. The method thereby overcomes the problems associated with drying from the liquid state and the dried ampoules may be stored at room temperature in the dark, although long term survival is improved by storage in refrigerators.

Freeze-drying is the most technologically complex of the preservation methods in use, requiring the highest level of technical skill and high capital expenditure for equipment. Freeze-dryers use one of the two methods for freezing the cell suspension prior to the drying process. In the pre-freezing method the cell suspensions are frozen in the ampoules before being dried under vacuum, freezing being achieved using a mixture such as dry-ice in ethanol. The alternative method is known as centrifugal freeze drying where the cell suspension is frozen by evaporative cooling under vacuum while the ampoules are spun in a low speed centrifuge to minimize foaming. It is essential to use a suitable preservative suspended medium to protect the living organisms from damage during the freezing and drying stages. The functions of such preservatives include stabilization of protein, prevention of freezing damage and protection against over drying. The choice of preservation depends on the organism and it must maintain the organism in a viable state and allow good recovery from the dried state.

6.2.3 Preservation by Liquid Drying

Some strains of bacteria which are sensitive to freeze-drying can be preserved by drying from the liquid state rather than the frozen state. The method was developed by Annear and has been used successfully to preserve bacteria, yeasts, fungi and viruses. Small quantity of dense suspension of cells dispensed into ampoules. Then ampoules are constricted in a fine gas-air flame and attached to a vacuum pump. The lower half of the ampoules is immersed into a water bath controlled at -25^{o} C. Ampoules are degassed and sealed under vacuum.

6.2.4. Cryogenic storage

Most microorganisms including bacteria, yeasts, fungi, viruses, bacteriophages, some algae and protozoa can survive long term storage in the frozen state by markedly reducing their metabolic rate. This method is also suitable for the preservation of animal as well as human cells. Microorganisms have been stored in freezers at temperatures around -20^{o} C and -70^{o} C. The lower the temperature the less is the loss of viability of most microorganisms and temperatures higher than -70^{o} C should not be used for long term storage but may be satisfactory for periods of up to 1 year.

The use of ultra-low temperatures obtained by freezing in liquid nitrogen at –196^{o}C has been used successfully to preserve a wide range of microorganisms and mammalian cells with a much reduced viability loss and a high degree of genetic stability. Though many bacteria survive freezing in their growth medium, the addition of cryoprotectants such as 5-10% glycerol or dimethyl-sulphoxide affords some protection from the stresses of freezing. Other cryoprotectants such as methanol, sugars, starch and polyvinyl-pyrrolidone have been used by various workers.

Some cryoprotectants at concentrations required to afford protection during freezing and thawing may be toxic and affect the recovery of the organism unless they are diluted out or removed completely. The rate of cooling should be slow and controlled down to the temperature range of –20^{o} C to –40^{o}C and then rapid to the final freezing temperature. The rate of thawing should be as rapid as possible. Rapid freezing rates may lead to intracellular ice crystal formation and electrolyte imbalance and cause lethal cell damage. In general, bacteria, yeasts and fungi are less sensitive to freezing damage than algae and protozoa.

6.2.5 Storage under Mineral Oil

This is a simple method for preserving the cultures of many bacteria and yeasts by storing them on agar slopes covered with sterile mineral oil. The oil used is medicinal grade liquid paraffin. In this way of protection, the interval between sub-culturing may often be extended for several years, and up to 20 years for some fungi. Long term preservation is often further improved if the oil-layered cultures are stored at –4^{o}C. The microorganism to be preserved is simply grown on its usual maintenance medium on a slope, or in an agar deep or broth culture, and then covered with sterile paraffin oil to a depth of approximately 20 mm, or at least 10 mm above the top of the slope. Addition of the sterile paraffin should be carried out without disturbing the growth on the slope. To avoid the cross contamination, the sterile paraffin is either stored in individual layering quantities and poured onto the culture, or transferred by means of a sterile 10 ml pipette

after each transfer is made. Cultures are routinely grown on slopes but to economize the oil, slope cultures are often grown on “short” slopes.

This technique is simple, but for purposes of transport is not as convenient as the drying methods and for longevity and subsequent ease of handling, not as convenient as freeze-drying or freezing in liquid nitrogen. However, in the absence of these facilities, storage under oil affords protection to many bacterial and fungal cultures for periods of several years. A disadvantage with this method is that there is a slow diffusion of oxygen through the oil which allows growth to continue at a slow rate and therefore genetic stability may be poor.

6.2.6 Preservation in Sterile Soil

Many bacteria and fungi survive well in dried soil for long periods of time of 20 years or more when stored at room temperature. The method is particularly useful for fungi, *Streptomyces* and for spore-forming bacteria such as *Bacillus* and *Clostridium*. Excellent survival has also been experienced with *Rhizobium*. The advantages of the method are its low material cost, room temperature storage and genetic stability, which would be expected to be much improved when compared with storage under sterile mineral oil. A suitable selected loamy soil is pulverized and autoclaved at 121^{o}C and 15 lbs pressure for 1 hr on 3 successive days. Autoclaved soil is then heated to dryness in an oven at 105^{o}C. The cell suspension is prepared in 2% sterile peptone water and small quantities are added to the dried soil and stored in desiccator till the further use.

6.2.7 Storage in Sterile Distilled Water

Some bacteria, particularly the Gram-negative rods such as *Pseudomonas* may be preserved for considerable periods of time when stored as a dense suspension in sterile distilled water. Storage may be at room temperature or slightly reduced to 10-15^{o}C if facilities are available. The method is applicable to a narrow range of bacteria like *Pseudomonas*, *Agrobacterium* and *Corynebacterium* and fungi, and as slow growth may continue, genetic stability would not be expected in the long term. As storage is in liquid, contamination is often a problem and one which is compounded when working with a

range of similar strains. This method is best used as a source of working stock cultures in conjuction with stocks preserved by other more stable methods where contamination is less likely.

Distilled water in screw-capped bottles is sterilized by autoclaving. Dense cell suspension prepared is transferred to the sterilized distilled water in the screw-capped bottles and stored at room temperature or preferably at 10-15^{o}C.

6.2.8 Preservation on Porcelain Beads

This method is a simple one for preserving many microorganisms involving the drying of cell suspension on porcelain beads, using silica gel as the drying agent. A layer of silica gel is placed in the bottom of a screw capped bottle. The silica gel is covered by a layer of "slag wool" and porcelain beads are mixed with a dense suspension of cells and placed on top of the slag wool. The bottle is tightly capped and the moisture is removed from the beads by the silica gel. Excess silica gel maintains the beads in a dried state. The method is suitable for the long term preservation of many bacteria and fungi. Perforated glass beads may be used in place of porcelain beads. The culture may be absorbed directly onto silica gel without indicator. But when water is added to silica gel, heat is produced. So, care should be taken to keep the silica gel cool while the suspension is being absorbed in order to avoid damage to the cells.

6.2.9 Preservation in Gelatin Discs

A simple but very effective method for the preservation of bacteria is to store them in dried gelatin discs. The method involves preparing single drops of a dense suspension of cells in nutrient gelatin supplemented with ascorbic acid, and drying these by evacuation over phosphorus pentoxide. The method is suitable for the long term preservation of bacteria but little data is available on survival rates. Sterilized paraffin wax is allowed to set as a layer in a sterile glass petri dish. A dense cell suspension is prepared in 10% nutrient gelatin containing 0.25% ascorbic acid. Using a sterile Pasteur pipette single drops of the gelatin cell suspension on the sterile paraffin surface are prepared and allowed the drops to set. Petri dish with gelatin drops is placed in a vacuum desiccator

containing phosphorus pentoxide and evacuated until the drops form dry discs. Aseptically, the discs are transferred to sterile tubes and stored over phosphorus pentoxide at 4^{o}C.

6.2.10 Preservation over Phosphorus Pentoxide in vacuo

This method has been successfully used in the National Collection of Type Cultures to preserve a wide variety of bacteria. The culture is preserved by preparing a suspension in horse serum which is deposited in a small glass tube. This tube is then placed in an outer tube containing a small quantity of phosphorus pentoxide in the bottom. The top of the outer tube is constricted and then evacuated and sealed. The ampoules are stored at room temperature or in a refrigerator. The method is suitable for the long term preservation of bacteria, yeasts and fungi and good survival has been observed for periods of 5-28 years for some strains.

A loopful of dense cell suspension prepared in sterile horse serum is transferred into a small sterile glass tube. This small tube is inserted into a larger tube containing phosphorus pentoxide at the bottom. A constriction is made in the neck of the outer tube using a fine gas-air flame. Then it is attached to a vacuum pump and subjected to evacuation for 5 minutes. Outer tube is sealed and the ampoules are stored at room temperature or in refrigerator.

6.3 Summary

Once a microorganism has been isolated and grown in pure culture, it is necessary to maintain the viable culture, free from contamination, for some period of time. There are several methods available for maintaining and preserving pure cultures. The organisms may simply be sub-cultured periodically onto or into a fresh medium to permit continued growth and to ensure the viability of a stock culture. Although proper aseptic technique must be used each time the organism is transferred, there is always a risk of contamination. Furthermore, repeated sub-culturing is extremely time consuming, making it difficult to maintain large numbers of pure cultures successfully for indefinite

periods of time. Additionally, genetic changes are like to occur when cultures are repeatedly transferred.

Therefore various methods besides sub-culturing have been developed for preserving pure cultures of microorganisms. These methods include refrigeration at 0^o to 5^o C for short storage times, freezing in liquid nitrogen at -196^o C for prolonged storage, and lyophilization or freeze-drying to dehydrate the cells. In lyophilization, the culture is frozen at a very low temperature and placed under a high vacuum. Under these conditions, the water in the culture and microbial cells goes directly from the frozen solid state to the gaseous state through a process of sublimation thereby drying the cells without disrupting them. By sufficiently lowering the temperature or by removing water, microbial growth is precluded but viability in a dormant state is maintained, permitting preservation of microorganisms for extended period of time.

The choice of the preservation method depends on the nature of the culture and the facilities available. When freezing is used to preserve microorganisms, the rates of freezing and thawing must be carefully controlled to ensure the survival of the microorganisms because ice crystals formed during freezing can disrupt membranes. Glycerol is often employed as an antifreeze agent to prevent damage due to ice crystals and to ensure the ability to recover viable microorganisms when frozen cultures are thawed.

Culture collections have played a fundamental role in the development of microbiology by ensuring that most of the types of microbes that have been described have been safely maintained for the present and future generations. Culture collections thus provide permanent laboratories where strains can be preserved and made available to scientists who wish to repeat, compare or extend work described in the literature.

Questions

Q.1. Describe the methods usually employed in microbial culture preservation.

Q.2. Write an essay on Maintenance and Preservation of microbial cultures.

Q.3. Write a short note on Lyophilization.

Q.4. Write notes on periodic sub-culturing and cryogenic storage of cultures.

Q.5. Discuss the merits and demerits of various microbial preservation methods.

Books for reference:

1. Prescott, L.M., Harley, J.P., and Klein, D.A. --- Microbiology
2. Brock, T.D. and Madigan, M.T. ---- Biology of Microorganisms
3. Atlas, R.M. ----- Principles of Microbiology
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Lesson 7

CULTIVATION AND PURIFICATION OF VIRUSES

7.1. Objectives

For the study of any virus it is essential to understand the nature and properties of individual virus. Cultivation and purification are the initial steps in the study of a virus. Cultivation of viruses can be done only on living cells because all are obligate pathogens, need a living cell for their survival. Different methods, including the whole organism, organ and cell cultures are used for animal viral cultivation and tissue culture and protoplast culture are used for plant viruses. For the study of properties of viruses it is essential to purify the viruses by different methods including precipitation filtration and centrifugation.

7.2. Cultivation of Animal viruses

Animal Viruses can be isolated from an affected host by harvesting excreted or secreted material, blood, or tissue and testing for induction of the original symptoms in the identical host or for induction of some abnormal pathology in a substitute host or in cell culture. Historically, dogs, cats, rabbits, rats, guinea pigs, hamsters, mice and chickens have all been found to be useful in laboratory investigations although most animal methods have now been replaced by cell culture methods. Once the presence of a virus has been established, it is often desirable to prepare a genetically pure clone, either by limiting serial dilution or by plaque purification.

7.2.1 Laboratory animals and embryonated chicken eggs

Prior to the advent of cell culture, animal viruses could be propagated only on whole animals or embryonated chicken eggs. Whole animals could include the natural host- laboratory animals such as rabbits, mice, rats and hamsters. In the case of laboratory animals, newborn or suckling rodents often provide the best hosts. Today, laboratory animals are rarely used for routine cultivation of virus but they still play an essential role in studies of viral pathogenesis.

The use of embryonated chicken eggs was introduced by Goodpasture *et.al.* in 1932 and developed subsequently by Beveridge and Burnet. The developing chick embryo, 10 to 14 days after fertilization, provides a variety of differentiated tissues, including the amnion, allantois, chorion ,yolk sac, which serve as substrates for growth of a wide variety of viruses, including orthomyxoviruses, paramyxoviruses, rhabdoviruses, togaviruses, herpesviruses and poxviruses. Several viruses from each of the groups cause discrete and characteristic foci – pocks, when introduced onto the chorioallantoic membrane (CAM) of embryonated eggs, thus providing a method for identification of virus types, or for quantifying virus stocks or assessing virus pathogenicity. Although the embryonated eggs have been almost wholly replaced by cell culture techniques, they are still the most convenient method for growing high tier stocks of some viruses and they thus continue to be used both in research laboratories and for vaccine production. In addition, pock formation on the CAM still provides a specialized method for assay of variants of poxviruses - wild type rabbit pox and cowpox viruses cause red hemorrhagic pocks on the CAM, whereas viruses deficient in specific virulence genes cause white pocks as a result of the infiltration of the lesions with inflammatory cells.

7.2.2 Organ Cultures

Organ cultures use the whole organ for culturing which provides the natural conditions for the virus. They have the advantage of maintaining the differentiated state of the cell. However, there are technical difficulties in their large-scale use, and as a result they have not been widely used. Figure 7.2.1 shows the procedure used to prepare organ cultures from trachea. Ciliated cells

lining the trachea continue to beat in coordinated waves while the tissue remains healthy. Multiplication of some viruses causes the synchrony to be lost and eventually causes the ciliated cells to death. Virus is also released into fluids surrounding the tissue and can be measured if appropriate assays are available.

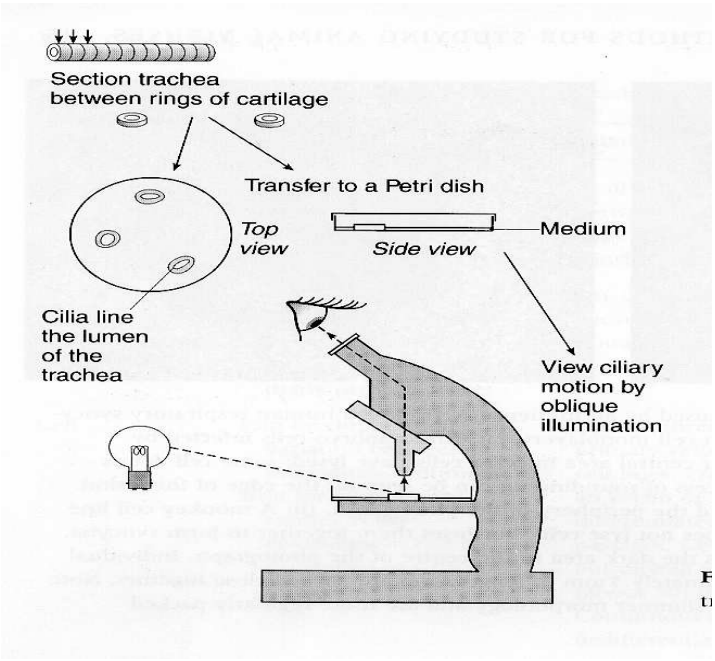

Fig. 7.2.1. Preparation of Tracheal organ culture
(redrawn from Dimmock *et al*, 2001)

7.2.3 Cell Cultures

In the 1950-60 period more than 400 viruses were isolated and cultured (Golden Age) by the cell cultures. Two discoveries greatly enhanced the usefulness of cell culture for virologists. First, the discovery and use of antibiotics made it possible to prevent bacterial contamination. Second, biologists found that proteolytic enzymes, particularly trypsin, can free animal cells from surrounding tissues without injuring the free cells. Cells in culture are kept in an isotonic solution, consisting of a mixture of salts in their normal physiological proportions supplemented with serum (usually 5-10%), and in such a growth medium most cells rapidly adhere to the surface of suitable glass or plastic vessels. Serum is a complex mixture of proteins and other compounds, without which mitosis does not occur. After cell division the cells form a mono layer in the vessels. Synthetic substitutes are now available but these are expensive and employed mainly for specialized purposes. All components used in cell culture have to be sterile and handled under aseptic conditions to prevent the growth of bacteria and fungi. Antibiotics have been invaluable in establishing cells in culture, and routine cell culture dates from 1950s when they first appeared on the market.

Cultured cells are either diploid or heteroploid (having more than the diploid number of chromosomes but not simple multiple of it). Diploid cell lines undergo a finite number of divisions, from around 10 to 100 whereas the heteroploid cells will divide forever. The latter are known as continuous cell lines and they originate from naturally occurring tumors or from some spontaneous event that alter the control of division of a diploid cell. Diploid cell lines are most easily obtained from embryos by reducing lungs, kidneys or the whole body to a suspension of single cells. Cell cultures are of three basic types: Primary cell cultures, Cell strains and Cell lines, which may be derived from many animal species, and differ substantially in their characteristics.

7.2.3.1 Primary cell cultures (Fig. 7.2.2.)

A primary cell culture is defined as a culture of cells obtained from the original tissue that have been cultivated in vitro for the first time, and that have not been subcultured. Primary cell cultures can be established from whole animal embryos or from selected tissues from embryos, newborn animals or adult animals of almost any species. The most commonly used cell culture in virology obtained from primates, including humans and monkeys, rodents including hamsters, rats and mice and birds most notably chickens. Cells to be cultured are obtained by mincing tissue and dispersing individual cells by treatment with proteases and/or collagenase to disrupt cell-cell interactions and interactions of cells with the extra-cellular matrix. With the exception of cells from the hemopoietic system, normal vertebrate cells will grow and divide only when attached to a solid surface. Dispersed cells are therefore placed in a plastic flask or dish, the surface of which has been treated to promote cell attachment. The cells are incubated in a buffered nutrient medium in the presence of blood serum, which contains a complex mixture of hormones and factors required for the growth of normal cells. The blood serum may come from a variety or sources, but bovine serum is most commonly used. Under these conditions, cells which attach to the surface of the dish will divide and migrate until the surface of the dish is covered with a single layer of cells , a mono layer, whereupon they will remain viable but cease to divide. If the cell mono layer is wounded by scraping cells from an isolated area, cells on the border of the wound will resume division and migration until the mono layer is reformed, whereupon cell division again ceases. Primary cultures may contain a mixture of cell types and they retain the closet resemblance to the tissue of origin.

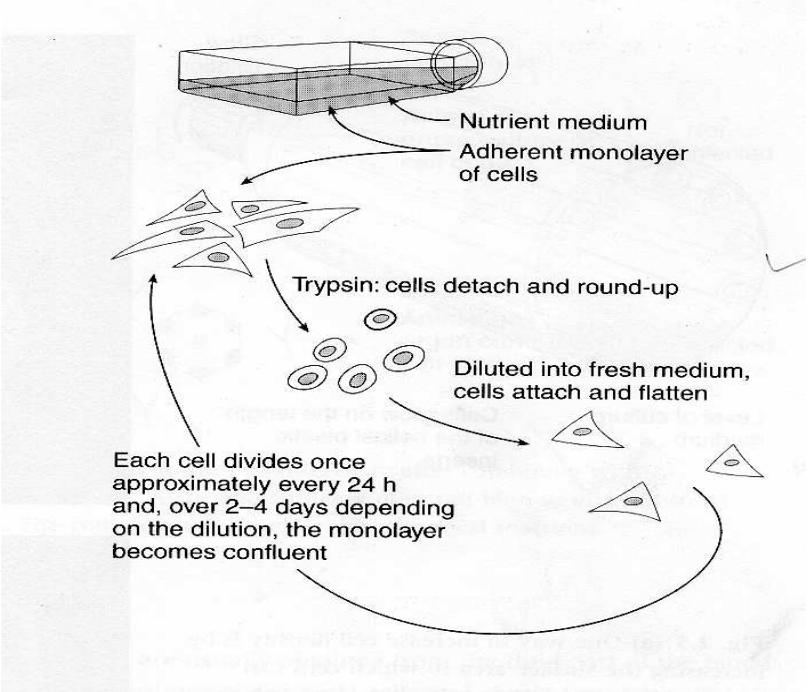

Fig. 7.2.2 Cell cultures (redrawn from Dimmock *et al* 2001)

7.2.3.2 Cell strains

Normal vertebrate cells cannot be generated indefinitely in culture. Instead, after a limited number of cell generations, usually 20 to 100 depending on the age and species of the original animal, cultured normal cells cease to divide and they degenerate and die, a phenomenon called crisis or senescence. Primary cell cultures may contain a mixture of cell types but only a few cell types survive after sub culturing and by subsequent generations, after second and third, typically only one cell type remains in the cell strain.

Cell strains are usually composed of one of two basic cell types, fibroblast like or epithelial-like , cells. Fibroblasts have an elongated, spindle shape, whereas epithelial cells have a polygonal shape. Although after only a few generations, only one cell type may remain in a cell strain, continued generations may select for faster growing variants, such that the characteristic of a cell strain may change with increasing generation number. Despite the fact that normal cell strains experience senescence in culture, they may be maintained for many years by expanding the culture to a large number of cells.

7.2.3.3 Cell lines

At any time during the culture of a cell strain, cells in the culture may become transformed. Transformation is a complex phenomenon, in the context of cell culture. The most important characteristic of transformation is that the transformed cells become immortalized. Immortal cell cultures are called cell lines or sometimes continuous cell lines to distinguish them from primary cultures and cell strains. Immortalization can occur spontaneously during passage of a cell strain or it can be induced by treatment with chemical mutagens, infection with tumorogenic viruses or transfection with oncogenes. In addition, cells cultured from tumor tissue frequently readily establish immortal cell lines in culture. Spontaneous immortalization does not occur in cultured cells from all animal species. Thus immortalization occurs frequently during culture of rodent cells, for example in mouse and hamster cell strains and in monkey kidney cells, but it occurs rarely in chicken or human cells. Like cell strains, cell lines are usually composed of cells that are either fibroblast-like or epithelial-like in morphology. These cell lines play a important role in the present day viral vaccine preparations.

7.2.3.4 Modern methods of cell culture

The methods described above are suitable for research and clinical or diagnostic laboratories, but it is difficult to scale up for commercial purposes which need increased cell densities. One of the earliest method to increase the cell density is to grow cells in suspension, and this has been refined, using hybridoma cells (which are immortalize antibody-synthesizing or B cells) that produces monoclonal antibodies (Mabs). As many cells grow only when anchored to a solid surface, the modified modern method is aimed to increase the surface area, by fitting the spiral inserts into the conventional culture bottles (Fig.7.2.3.). The added advantage of this method is by rotating the bottle slowly (5 rev/h) only a small volume of culture medium is enough for culturing. Another method is to grow cells on ‘micro carriers’- tiny particles about (200 μm diameter), on which cells attach and divide. The surface area afforded by 1kg of micro carriers is about 2.5 m^2 and the space take up(a prime consideration in commercial practice) is very economical. This method combines the ease of handling cell suspensions with matrix for the cell to grow on.

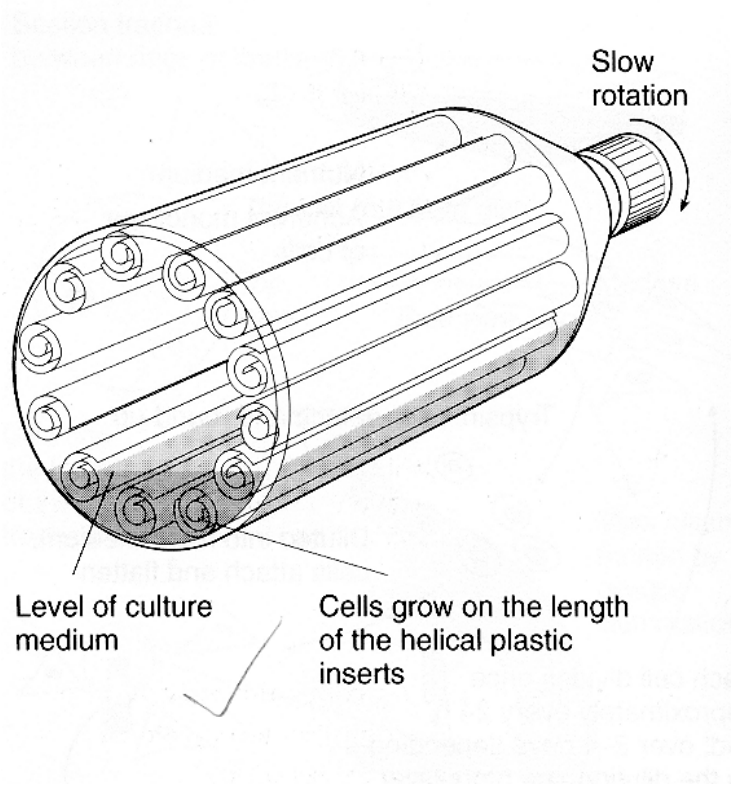

Fig. 7.2.3. Culture bottle lined with spiral plastic coils .

(redrawn from Dimmock *et al* 2001)

7.2.4.**The plaque assay**

The plaque assay is the most quantitative , and the most useful biologic assay for viruses. Developed originally for the study of bacteriophage by d'Herelle in the early 1990s, the plaque assay was adapted to animal viruses by Dulbecco and Vogt in 1953. This assay was relatively simple and permit a qualitative assay for individual virus variants that differ in growth properties or cytopathology.

The plaque assay is based simply on the ability of a single infectious virus particle to give rise to a macroscopic area of cytopathic effect on a normal monolayer of cultured cells. Specifically, if a single cell in a monolayer is infected with a single virus particle, new viruses resulting from the initial infection can infect surrounding cells, which in turn produce viruses that infect additional surrounding cells. Over a period of days, the exact length of time depends on the particular virus, the initial infection thus gives rise through multiple rounds of infection to an area of infection called a plaque.

The plaque assay can be used to quantify virus in the following manner. A sample of virus of unknown concentration is serially diluted in an appropriate medium, and measured aliquots of each dilution are seeded on to mono layers of cultured cells. Infected cells are overlaid with a semisolid nutrient medium usually consisting of growth medium and agar. The semisolid medium

prevents formation of secondary plaques through diffusion of virus from the original site of infection to new sites, ensuring that each plaque that develops in the assay originated from a single infectious particle in the starting inoculum. After an appropriate period of incubation to allow development of plaques, the mono layer is stained so that the plaques can be visualized. The staining technique depends on the cyto-pathology, but vital dyes such as neutral red are common. Neutral red is taken up by living cells but not by dead cells, so that plaques become visible as clear areas on a red mono layer of cells. In cases where the virus cytopathology results in cell lysis or detachment of cells from the dish, plaques exist literally as holder in the monolayer, and a permanent record of the assay can be made by staining the monolayer with a general stain such as crystal violet, prepared in a fixative such as ethanol. The aim of this assay is to identify a dilution of virus that yields 20 to 100 plaques on a single dish that is, a number large enough to be statistically significant. Usually a series of four to six 10-fold dilutions are tested. Dishes inoculated with low dilutions of virus will contain only dead cells or too many plaques to count. Whereas dishes inoculated with high dilutions of virus will contain very few plagues. Dishes containing an appropriate number of plaques are counted, and the concentration of infectious virus in the original sample can then be calculated by taking into account the serial dilution. The resulting value is called a titer, and it is expressed in plaque-forming units per milliliter or pfu/ml, to emphasize specifically that only viruses that are capable of forming plaques have been quantified. In this method an error of up to 100% is always possible because it mainly involves the multiple serial pipetting steps. However, a critical benefit of the plaque assay is that it measures infectivity, but it is important to understand that infectivity does not necessarily correspond exactly to the number of virus particles in a preparation.

7.3. Cultivation of Plant Viruses

Although viruses can not be grown in a synthetic medium , the cell, in which they live can be propagated. This procedure is called tissue culture. Plant viruses can be cultivated either by tissue cultures or by protoplast culture method. In tissue culture method, the ex-plants pieces of tissues are used, while in protoplast culture the cells from the host plants are used. In tissue culture various plant parts- roots, endosperm, pollen, nucelli and pieces of stem are commonly used.

7.3.1. Plant Tissue Culture

White (1934) was the first to examine the possibilities of growing plant viruses in tissue-culture. He investigated the multiplication of tobacco and cucumber mosaic viruses in growing excised tomato root tips.

A tomato plant already systematically infected with the viruses was used and the stem was cut up into segments, these were thoroughly washed and were suspended by threads in 3-litre

conical flasks containing a little water. The pieces of stem were kept out of contract with the water on the sides of the flask, the flasks were then plugged with cotton – wool and allowed to stand till roots developed. After 11 days, the root tips were removed and placed in 125 ml conical flasks, each flask contain 50 ml of nutrient medium as follows :

$Ca(NO_3)_2$	0.60 millimols
$MgSO_4$	0.30 millimols
KNO_3	0.80 millimols
KCl	0.87 millimols
KH_2PO_4	0.09 millimols
$Fe_2(SO_4)_3$	0.006 millimols
Sucrose	2 % by weight
Yeast extract	0.01 %

At the end of a week, the surviving cultures were cut into pieces of about 10 mm long. After further subculture, a single root tip was selected as parent stock for all subsequent subcultures. It was found that the two viruses continued to multiply actively in growing isolated root tips for at least 25 to 30 weeks.

Using the above technique, tobacco mosaic (TMV) and tobacco necrosis (TNV) viruses can be cultured in root tips of tobacco plants also. In addition to root tip cultures, plant viruses can also be cultured in callus tissues. In general, the tissues grew on a wide range of concentrations of the salts tested, but best growth was apparent when concentrations were increased over those of the basal medium (given above). Further it was found that increased phosphate concentrations increase the growth of tobacco-callus tissue. After callus development, infecting the callus with virus is also a difficult task in this method.

Since it is difficult to infect tissue cultures with viruses *de novo*, it is better to start the culture with tissues from systemically infected plants. However it is not always possible to get such type of plants, then it is better to employ alternative methods. One possible way is to use the natural vector of a virus to infect cultured tissue. In case of tobacco necrosis, the virus was inoculated to tobacco callus tissues by zoospores of the fungus *Olpidium brassicae*. Two strains of tobacco necrosis virus and three isolates of *O. brassicae* were used. One day before inoculation, the callus tissues were transferred to small filter-paper cups pushed into vials containing 5 ml Hoaglands solution (1:20 dilution). The solution just touched the bottom of the paper cup, which was used to prevent the callus cells being lost in the liquid. The method of inoculation was to add to each vial 1ml of Hoagland's solution containing zoospores and 0.5 ml containing purified virus. Four or 5 days after inoculation, the virus in the tissues and in the fluid beneath them was assayed by infectivity tests on French beans, the test plant for TNV. All three isolates of *Olpidium* transmitted both strains of TNV to the tobacco callus tissue.

Similarly it is also possible to propagate the virus in tissue cultures of insect vectors. Tissue cultures derived from the vector insect *Agallia constricta* (Van Duzee) were infected with wound-tumor virus and the infection was detected by staining with fluorescent-conjugated antibody, and by infectivity tests. These experiments demonstrate multiplication of the wound-tumor virus in the inoculated tissues of the leaf-hopper.

Many viruses - chilli mosaic virus, sun hemp mosaic virus and ring spot strain of potato virus X and a type strain of TMV have also been successfully cultivated in normal callus tissue obtained from virus-affected White Burley tobacco plants.

7.3.2 Protoplast culture

Use of protoplasts and isolated cells in the study of viruses have many advantages over the inoculated leaves. In the latter case only a few cells are initially infected and the virus replication must be studied against an overwhelming background of uninfected cells. Moreover the cells in the inoculated leaf are in varying stages of virus synthesis ranging from uninfected cells to cells in which virus synthesis is completed. Replication of virus within protoplast may be demonstrated in various ways, staining with fluorescent antibodies, infectivity assays, electron microscopy, incorporation of radioactive precursors into viruses and serology.

Certain conditions must be satisfied in order to achieve successful protoplast infection.

1. Protoplasts should be freshly washed with 0.7 M mannitol immediately before adding the inoculum.
2. Poly-l-Ornithine should be used to the inoculum
3. pH and osmolarity during inoculation should be within acceptable limits.
4. After infection takes place the protoplasts should be washed to remove excess virus and inoculation medium and then re-suspended in the incubation medium.

It is necessary to use very large numbers of virus particles to establish infection in inoculated protoplasts. Infection of protoplasts can be 10 times more efficient than is infection in a leaf probably because of the easier accessibility of the protoplast.

A simplified method of obtaining tobacco protoplasts for infection with tobacco mosaic virus is - incubating the tobacco leaf tissue, from which the lower epidermis was peeled, overnight with 0.3-0.4 percent Macerozyme and 0.6-1.2 percent cellulase, depending on leaf condition, produced a good yield of protoplasts that were susceptible to infection by TMV. Highest concentration of virus can be attained, when the protoplasts were inoculated as soon as they were washed free from the enzymes.

Protoplast culture of viruses is an important tool in plant virus study and offers much scope for progress. The time course of virus replication and its kinetics can be measured, and

with protoplasts infected *in vitro* it is possible to get a picture of the generation time of plant viruses.

7.3.3. Abrasive method

This is one of the simplest methods for cultivation of plant viruses. The viruses that are sap transmitted are generally cultivated by this method. In this method carborundum was used an abrasive to make injury on the leaf, through which virus can make entry. First, a fine homogenate of the infected leaf was prepared by using K_2HPO_4/Na_2SO_3 solution in the ratio of 1.5 ml solution to 1 g leaf, in a pre-cooled mortar. In a healthy plant, the leaf to be inoculated is marked and dusted with carborundum. Then by using the folded square of muslin cloth, the filtered homogenate of the infected leaf was applied on the surface of the leaf which was dusted with carborundum. The square of muslin dipped in the sap was firmly stroked on the upper surface of the leaf until complete leaf was moistened. These treated plants were kept in a green house at 22-25^oC. Then plants were observed for the appearance of symptoms from 4 to 5 days after inoculation. By this method viruses like *Cucumber mosaic virus* can be cultured in *Chaenopodium quinoa,* the local lesion host.

7.4. Purification of viruses

Purification is essential for the study of structure, replication and other biological aspects of viruses. These procedures are mainly aimed at removal of all contaminants without the loss of viruses. Viruses are basically proteins which are often more stable than normal cell components. Because of this characteristics, many techniques useful for the isolation of proteins and organelles can be employed in virus isolation and purification. Many viruses are purified quite satisfactorily by differential centrifugation or by repeated precipitation. However, more selective separation techniques are necessary, where the contaminant material have similar properties to those of the virus. Preferred procedures for the isolation and subsequent purification of viruses, are – Differential centrifugation; Density gradient centrifugation (Rate zonal and isopycnic density gradient centrifugation), Precipitation with ammonium sulfate or polyethylene glycol and filtration.

7.4.1 Precipitation and Filtration

Many viruses can be precipitated simply by lowering the pH of the extracts until the virus is precipitated at its isoelectric point. The pH at which viruses can be precipitated is usually in the range of 3.4 –5.5. This is the simplest method for precipitating the viruses, however some viruses are inactivated during this procedure. Another simplest method is precipitation by salts - ammonium sulfate. Only salt tolerant viruses can be precipitated by this method.- most of the viruses are precipitated at saturated ammonium sulfate concentrations between 20% and 40%,

however some viruses require about 80% saturation as for broad bean mottle virus. In this method the crude virus sediment is re-dissolved in water or buffer and treated with gradually increasing amounts of water saturated with ammonium sulfate. When a precipitate appears, it is separated by low-speed centrifugation and more ammonium sulfate is added slowly to the supernatant until another precipitate forms, which is centrifuged off. Usually the salt, as saturated solution or as crystals, is added slowly to sap and left for several hours or overnight for better precipitation.

The same principle is used in stepwise precipitation with polyethylene glycol, which can be used, in contrast to ammonium sulfate, with salt-sensitive viruses. Precipitation with PEG is also commonly used as an early purification step to concentrate animal viruses or bacteriophage from large volumes of culture media into which the viruses were released from infected cells.

Some plant viruses could be preferentially precipitated in a single-phase polyethylene glycol (PEG) system, although some host DNA may also be precipitated. Since that time, precipitation with PEG has become one of the commonest procedures used in virus isolation.

In case of viruses which are stable in organic solvents, precipitation by adding solvents is preferable. Ethanol and methanol have been used to precipitate plant and animal viruses. However , these alcohols are known to denature some viruses especially when the precipitation were performed at unfavorable temperatures. The only advantage in this method is – it minimizes the time and cost, particularly when large samples are processed.

Filtration is another method for purification of viruses where it can be done through filter paper, or through filter paper supporting a pad of Celite is sometimes used to precipitate viruses. If Celite is used it will also act as an adsorbant and under certain conditions as very effective in removing green material, the common contaminant, in plant preparations. This method is a best substitute for slow speed centrifugation.

7.4.2 Differential centrifugation

Differential centrifugation consists of alternating cycles of high and low speed centrifugation at 20,000 to 50,000 rpm (e.g. a force of 1,00,000 g) for 1 to 3 hr sediments viruses together with other particulate material and leaves soluble components of the extract of less than 106 molecular weight in the supernatant. The sediment is then dispersed in water or in a suitable buffer, this must often be done by allowing the pellet to soften rather than by vigorous stirring. The extract is then again centrifuged at 8000 to 10,000 g to remove membranous and fibrous cell components, denatured proteins, and so on. Subsequent centrifugation of the supernatant solution at the higher speed again brings down viruses, and two or three repetitions of this procedure, discarding low-speed sediments and high-speed supernatants, yields many plant viruses including TMV in comparatively pure form.

7.4.3 Density Gradient Centrifugation

One of the most useful procedures for purification, particularly for less stable viruses is density gradient centrifugation. Sucrose is the most commonly used material for making the gradient. It is a relatively mild procedure, although there may be a loss of infectivity with some viruses. It can give some indication of the purity of the preparation. It allows a correlation between particles and infectivity to be made and it frequently reveals the presence of non infective virus like multi-particle viruses. Gradient centrifugation can be done in two different way, one by considering the size and density of the particles together (rate zonal centrifugation) and the second by considering only the density (isopycnic centrifugation).

7.4.3.1 Rate zonal density gradient centrifugation

This method utilize centrifugation through increasing concentrations of sucrose, glycerol, cesium chloride and so on. Sucrose gradients (e.g. 25 to 5 percent) are mostly preferred and easily prepared, and they afford good separations of viruses and other particles and molecules of various sizes and densities. The gradient was prepared by pouring the sucrose solution in a test tube such that its concentration smoothly and linearly increase between the top and the bottom of the tube, the highest density of which does not exceed the densest viral particle to be separated. The virus preparation is layered on top of the gradient and centrifuged. The particles are separated based on differences in their sedimentation rates i.e., based on both size and density. The virus was clearly visible in the test tube as a light scattered band, when a light beam is allowed to pass through the test tube. From the test tube the virus is then recovered either by pearcing the test tube at the scattered light region or by introducing the syringe from top of the test tube up to the zone. Pure virus preparation can be obtained by this method. Salt sensitive and low-density viruses are preferentially purified by this method.

7.4.3.2 Isopycnic gradient centrifugation

A different use of density gradient centrifugation is isopycnic equilibrium centrifugation. This method relies strictly on the different buoyant densities of viruses, proteins, nucleic acids and so on. Each type of molecule will move in an increasing density gradient to the level at which its density equals that of the gradient and stop there. Most commonly, salts of heavy metals (Cesium or Rubidium) at high density (above 1-2 g/ml) are used since these form gradients automatically under the g forces of the ultracentrifuge. Thus, centrifugation of viruses in such a salt solution of appropriate concentration will result in location of the virus as a sharp band at the particular level of the tube where the solution density equals to the buoyant density of virus. This centrifugation require a long time (36-48h) for equilibrium to be reached and can be used for salt-stable viruses only. Isopycnic density gradient centrifugation is particularly useful in the separation and characterization of nucleic acids, since these have higher density than viruses and other cell components and are not dissociated by salts. Very slight differences in the

density of nucleic acids due to different ratios of G-C as compared with A-T base pairs, or the presence of heavy elements (5-bromo-uracil-instead of uracil) or isotopes can easily be detected by this method.

7.5 Summary

Cultivation of viruses can de done different methods depending on the nature of the virus. In the past whole organism was used a s media for cultivation. Later, the organ and now it is the cell that is used a s media for cultivation. Obviously virus needs a living system for its multiplication. Animal viruses are mostly cultivated in mono cell culture consisting of either epithelial or fibroblast cells , though the embryonated chicken egg is the method of choice for many viruses. Plant viruses are usually cultivated by tissue culture methods and protoplast cultures. Protoplast cultures are advantageous over tissue cultures is that with protoplasts infected in vitro it is possible to get a picture of the generation time of plant viruses and course of virus replication and its kinetics can be measured. Purification of the viruses was achieved by different methods which include mainly protein purification. Depending on the tolerance towards the precipitating agent , viruses are precipitated using either by ammonium sulfate, polyethylene glycol or solvents. Sometimes they can be isolated by filtration through filter papers with Celite pads. Differential centrifugation is the first option for purification of majority of viruses. In Gradient centrifugation low density and high density viruses are conveniently purified by using sucrose and cesium chloride gradients, respectively.

7.6. Model Questions

1. Write critical account on methods of cultivation of animal viruses.
2. Enumerate the plant viral cultivation methods.
3. Give detailed account on purification methods of viruses.
4. Write short notes on

   a) Cell Cultures b) Plaque method c) Plant tissue culture method

   d) Cell lines e) Differential Centrifugation f) Gradient Centrifugation

   g) Precipitation h) Protoplast culture

7.7. Reference Books

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LESSON 8

CHROMATOGRAPHY

8.0. Objective: To study about various chromatography techniques that are employed to separate the components of sample mixture.

8.1. Introduction

Michael Tswett, a Russian biochemist, who separated chlorophyll from a mixture of plant pigments in 1906, first developed chromatography. Because of the nature of chlorophyll pigments in the sample, each band had a distinctive color. Thus the name of the process was coined from the Greek words i.e. Chromo means *Color* and graphy means *to write*.

In any chromatographic method two phases are common. They are, one is stationary phase while the other is mobile phase. The mobile phase either moves over the surface or percolates through the interstices of the stationary phase. The sample mixture, introduced into the mobile phase undergoes repeated interactions or partitions between the stationary and mobile phases while being carried through the system by the mobile phase. Different components of sample mixture interact with the two phases differentially on the basis of small differences in their physico-chemical properties. Since these different rates of interactions govern the migration of the sample components through the system, each one of the components migrate at a different rate. The compound, which interacts more with the mobile phase and least with the stationary phase, migrates fast. The component showing least interaction with the mobile phase while interacting strongly with the stationary phase migrates slowly. This differential movement of the components is responsible for their ultimate separation from each other.

8.2. Techniques of Chromatography

There are two basic techniques of chromatography

(a) Plane chromatography

(b) Column chromatography,

In plane chromatography the stationary phase is coated onto a plane surface. There are two types of plane chromatography (1) Paper chromatography (2) Thin layer chromatography. In paper chromatography the stationary phase is supported by cellulose fibers of the paper sheet. In thin layer chromatography the stationary phase is coated onto a glass or plastic surface. But in case of column chromatography as opposed to plane chromatography the stationary phase is packed into a glass or plastic column. Each of these techniques has their specific advantages, applications and mode of operation.

8.3. Paper Chromatography

A.J.P.Martin and Richard Synge first developed paper chromatography in 1941. This technique has played an important role in biochemical analysis due to its ability to separate small molecules such as amino acids and oligo peptides.

Nature of Paper

The paper commonly used consists of highly purified cellulose. Cellulose, a homo polysaccharide of glucose, contains several thousands of anhydro-glucose units linked through oxygen atoms. Treating the paper with 0.1 N HCl and drying it before chromatography is carried out may remove any organic and inorganic impurities present on the paper. This gives better results.

Apparatus And Paper Development

The apparatus required for paper chromatography are

(a) Support for the paper

(b) Solvent trough

(c) An Airtight chamber in which the chromatogram is developed,

Different Techniques Used In Paper Chromatography

The sample is applied to the paper as a small spot. The sample is applied to the paper before dipping the paper into the eluting solvent. Any device, which can transfer a small volume of sample, can be used for spotting. Generally used devices are platinum loop, capillary tube or a micropipette. Of these three devices platinum wire is most preferable because it can be reused with several substances after heating on a flame. A micropipette can also be reused after its tip has been disposed and a new tip applied. For some methods the sample may be applied as a narrow streak at right angles to the flow of solvent.

There are two main techniques, which may be employed for the development of paper chromatography.

(1) Ascending chromatography

(2) Descending chromatography,

In both cases the solvent is placed in the base of a sealed tank or glass jar to allow the chamber to become saturated with the solvent vapour. After equilibration of the chamber is achieved the development of chromatography may be started.

Ascending Technique

If the development is to be performed by the ascending technique, the paper is allowed to hang in or suspended in a manner that the base of the paper is in contact with the solvent at the base of the chamber. The sample spots should be in a position just above the surface of the solvent, so that as the solvent moves vertically up the paper by capillary action, separation of the sample is achieved.

Descending Technique

In the descending technique, the end of the paper near which the samples are located is held in a trough at the top of the tank and the rest of the paper allowed to hang vertically but not in contact with the solvent in the base of the tank. Adding the solvent to the trough starts development. Separation of the sample is achieved as the solvent moves downward under gravity.

Ascending technique has two advantages (a) The set up required for it is very simple (b) The resolution of sample by ascending technique is somewhat better as compared to the descending technique. This is because in ascending technique, two forces are acting on the solute; these are capillary force, which makes it move up and the gravitational force, which opposes this movement. Under the influence of these two forces, the sample components are resolved better than in the descending technique. The only disadvantage in ascending technique is it is very slow. The descending technique on the other hand is much faster than ascending technique.

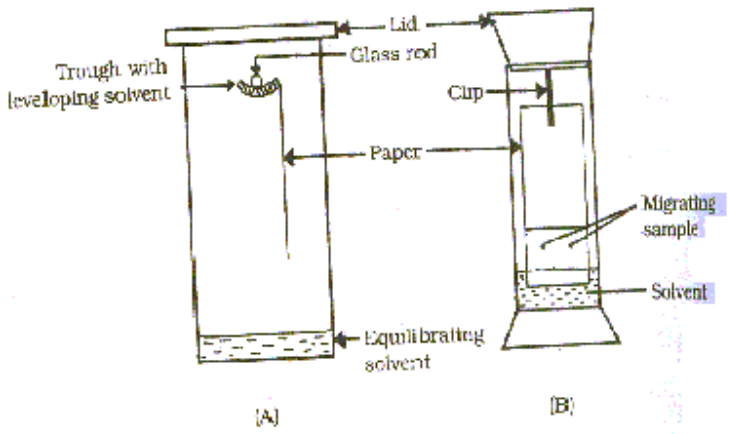


Figure 8.1. Methods of paper chromatography (A) Descending (B) Ascending (Courtesy from Upadhaya and Upadhaya Nath)

Radial Technique

In this method the sample is spotted at the center of a circularly cut disc of paper, which is placed horizontally. The center of paper is connected with a wick to the solvent, which is placed at the base of the jar. The solvent rises up the wick and then on to the paper through capillary action. The sample components now move outward radially forming concentric circles of increasing diameters. The resolution of components by this technique is sharper. But this is less used compared to ascending and descending techniques.

Two Dimensional Chromatography

The paper, with the sample applied as a spot close to the corner is developed in the normal fashion by either ascending or descending techniques. The development is continued until the faster moving component or solvent front approaches the end of the paper. The paper is removed and the solvent is allowed to evaporate. This paper is then turned 90^0 and developed a second time with another solvent having totally different eluting properties. Thus components that could not be separated serving one solvent above can be easily separated by this procedure.

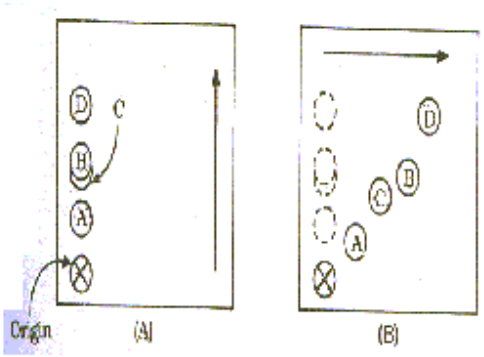


Figure 8.2: Two Dimensional chromatography (A) First development in the direction indicated by the arrow does not resolve B & C completely (B) Second development in a direction at right angles to the first using a different solvent system resolves all components completely.
(Courtesy from Upadhaya and Upadhaya Nath)

Choice of Solvent System

Usually in paper chromatography, the stationary phase is water since it is very well adsorbed by cellulose. The mobile phase, which is less polar, flows over the polar stationary phase. The mobile phases used in paper chromatography are usually a mixture of various solvents such as alcohols, acids, esters, ketones, phenols, amines and hydrocarbons etc. The solvents are selected in such a way that the resolution of sample components is satisfactory.

Aqueous + organic solvents used as a solvent system

Ex water : Butanol : Acetic acid (4:5:1ratio)

water : t-amylo alcohol : Pyridin (6:7:7) ratio

Detection

After the solvent has migrated to an appropriate distance the chromatography is stopped and the paper is removed from the solvent and dried. The separated materials on paper if not colored may be detected by various methods. Radioactively labeled compounds located by radiation detection methods. If the compounds are fluorescent they can be seen under UV light. Colourless compounds may be visualized by spraying chromatograms with color producing reagents.

Ex. Alpha amino acids form purple color with ninhydrin reagent but protein forms yellow color with the same reagent.

The identification of a given compound may be made on the basis of the distance traveled by the solute relative to the distance moved by the solvent. This rate, which reflects the distribution coefficient of the given solute, is known as the retardation factor (Rf).

$$Rf = \frac{\text{The distance moved by the solute}}{\text{The distance moved by the solvent}}$$

In case of Carbohydrates the term Rf is replaced by R_g for convenience.

$$R_g = \frac{\text{The distance moved by carbohydrates}}{\text{The distance moved by glucose}}$$

Since each compound has specific Rf (R_g) value, this value can be exploited to detect the unknown compound by matching its retardation factor value to those of the known compounds.

Applications

(1) The technique of paper chromatography has been widely used in identification and separation of biomolecules.
(2) It is also used in the control of purity of pharmaceuticals.
(3) It has been widely used in the detection of contamination in foods and drinks.
(4) It is used in the study of ripening and fermentation.
(5) It is also used in the detection of drugs and dopes in animals and humans.

8.4. Thin Layer Chromatography (TLC)

This technique is similar to paper chromatography technique. In "TLC" a thin layer acts as stationary phase and solvent acts as mobile phase. A thin layer of finely divided substance is deposited on to a flat glass plate. The sample to be separated is spotted at one end. The plate is

dipped into the solvent in a glass jar and the development carried out by ascending technique. After the development the layer can be dried and the components detected by various methods.

Thin layer chromatography may be either carried out by the adsorption principle i.e., thin layer is prepared by an adsorbent such as "Kieselguhr" or alumina or by the partition principle if a substance such as silica gel prepares the layer.

(a) Preparation of Thin Layer

The glass plate, which the thin layer is prepared, should be even and is thoroughly washed and dried before application of layer. The material of which the thin layer is to be made. Ex. Silica gel (or) Kieselguhr is severally mixed with water in such a proportion that a thick suspension is formed known as "slurry". This slurry is applied to a plate surface as a uniform thin layer by means of a "plate spreader", starting at one end of the plate and moving to the other in an unbroken uniform motion. Thickness of thin layer influences resolution power of "TLC". Thus for analytical separations, the thickness of layer is usually 0.25 mm; and for preparative separations the thickness of the layer might be about 5mm. Although thin layer technique can be used for many different types of chromatographic separations the most widely used technique is usually "adsorption type". While preparing stationary phase for adsorption chromatography calcium sulphate is mixed with the slurry. The binding helps in better adhesion of the stationary phase to the glass or foil plate. The plates are dried after application of the slurry. If adsorption chromatography is to be performed heating at 110° C for several hours activates the thin layer.

(b) APPARATUS AND PLATE ARRANGEMENT

TLC requires glass plate, TLC chamber, solvent, and substance for thin layer preparation, spreader and lid for TLC chamber.

Different solvent and adsorbent combinations are available for TLC.

Adsorbent	**Solvent**	**Compound separated**
Silica gel – G	Petroleum ether / diethyl ether **:** Acetone 90 : 10 : 1	Triglycerides
Silica gel – G	96% Ethanol / Water 70 : 30	Amino acids
Kieselguhr – G	Ethylacetate / propane - 1 - ol 65 :35	Mono and Di saccharides.

(C) DETECTION

After preparing the thin layer the sample to be separated is applied at one end and after drying, the plate is dipped in the solvent in a glass jar. After the plate development, the thin layer can be dried and the separated components can be detected by various methods. If the components are radio-labeled it is detected by autoradiography technique. If the components are colorless they can be visualized by spraying the chromatograms with color producing reagents. The techniques specific for detection of TLC are:

(a) Spraying the plate with 25 - 50% H_2SO_4 in ethanol and heating. This results in charring of most of the compounds, which show up as "brown spots".

(b) Iodine vapour is also used extensively as colour producing reagent in TLC.

As in paper chromatography in TLC also compounds are identified on the basis of rates between distances traveled by solute relative to the distance traversed by the solvent.

$$Rf = \frac{\text{The distance traveled by the solute}}{\text{The distance moved by the solvent}}$$

Each compound has a specific Rf value. This can be used to detect the unknown compounds by comparing its Rf value to Rf values of known compounds.

Applications

(1) One of the greatest advantages of TLC is the speed at which the separation is achieved. Generally 10-30 minutes are sufficient. However with certain compounds 90 minutes may be required. Compared to paper chromatography TLC is more convenient, faster and highly reproducible.
(2) It has been widely used to determine the complexity of mixture.
(3) TLC is also used to study the courses of reactions.
(4) TLC has been used to identify drugs, contaminants and adulterants.
(5) It has also been widely used to resolve plant extracts and many other biochemical preparations.

8.5. High Performance Liquid Chromatography (HPLC)

“HPLC” is the technique performed in column (it is an example of column chromatography) and the resolution power of “HPLC” is very high compared with other conventional chromatographic techniques. Other conventional chromatographic techniques require long time. To reduce the time of experiments, flow rate has to be increased. Although this reduces the time of the experiment, it also reduces the efficiency of chromatographic technique. In this the sample components undergo lesser number of equilibrations between mobile phase and stationary phase. To overcome this problem column chromatography technique performed in long columns. To increase the flow rate the pressure has to be increased on mobile phase. But commonly used supports in column chromatography cannot tolerate high pressures; there by causing flow rate abnormalities. All these problems were resolved with the development of high performance liquid chromatography. This method uses high pressures up to 8000 PSI. (Pressure for square inch). Therefore the flow rate is high and experimental time is shorted considerably and the resolution power is high. The technique may be used with small amounts of sample, (Pico or even femto gram) level. The technique is primarily stable for analytical purposes but can be used as a preparative technique also. It is particularly popular for the

separation of polar compounds such as drug metabolites. The greatest advantage of "HPLC" is that it may employ the principles of adsorption, partition, ion exchange, exclusion and affinity chromatography. This makes it an extremely versatile technique and explains its emergence as the most popular chromatographic technique.

Experimental Differences Between Conventional Chromatography And Hplc

Characteristics	**Coventitional chromatography**	**HPLC**
No. of plates / second	0.02	5
Pressure	Negligible	Upto 8000 psi
Time of experiment	Hours to days	Minutes to hours
Equipment	Simple: Column and accessories	Integrated chromatography
Purpose	Pre dominantly preparative	Pre dominantly analytical
Quantity chromatographed	mg to kg (milligrams to kilograms)	ng to mg (nano grams to milligrams)

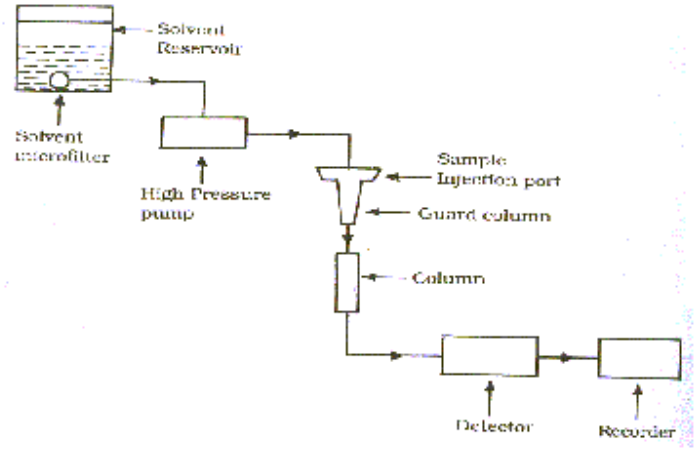


Figure 8.3: Schematic diagram of HPLC system.
(Courtesy from Upadhaya and Upadhaya Nath)

Instrumentations

Six major components needed to perform HPLC are:

(A) A solvent reservoir to store the mobile phase.
(B) High pressure pump to push the mobile phase through the column.
(C) A device to inject the sample into the mobile phase.
(D) A column in which the separation will take place.
(E) A detector used in detecting the concentration of the sample components as they come out of the column.
(F) A potentiometric recorder to produce a chromatogram.

Solvent Reservoir And The Solvents

Solvent reservoir in "HPLC" apparatus should contain enough volume of mobile phase for repetitive analysis. It must have a provision for degassing the solvents and it must be inert to the solvent. In "HPLC" apparatus solvent degassing is performed by heating (or) by application of vacuum (or) by treating it with ultrasonic sounds. Generally glass and steel containers of 0.5 – 2.0 liter capacity are suitable as solvent reservoirs.

The type of separation determines the choice of mobile phase. Isocratic separations perform with a single solvent (or) a fixed proportion mixture of two solvents. For gradient elution, the solvent composition changes continuously, during chromatography.

All solvents to be used in HPLC must be extra pure. Since even the smallest impurity interferes with the detection systems or they block the column. To provide extra pure reagents for HPLC, solvent reservoirs provided with a micro filter.

Pumping Systems

To provide high pressures in "HPLC" different types of pumping systems available. A good pumping system should give pulse less stable flow, constant volume delivery. It should provide solvent flow rates of 0.5 ml per minute for most HPLC models.

Commonly used pumping systems for HPLC are

1. Holding coil
2. Pneumatic amplifier
3. Moving fluid type
4. Reciprocating piston
5. Syringe pump,

Sample Injection

In HPLC sample is introduced on to the column as in conventional types of chromatographic techniques. Two methods are available for sample applications in HPLC.

(a) Stop plane injections: In this method sample is introduced with the help of micro syringe. Preferably the sample is injected when the pressure has dropped to almost one atmosphere, after switching the pump off. This technique is known as stop flow injection. Alternatively, the sample can be injected while the system is under high pressure.

(b) In the second method the sample applied directly on to the HPLC column with the help of a small volume metal loop. The sample is thus carried spontaneously with the eluant to the column.

Column

The columns for HPLC are made up of stainless steel, glass, aluminum, copper or PTFE (Poly tetra fluoro ethylene). But stainless steel columns are suitable; because they can withstand high pressures up to 8000psi.straight columns of between 20-50 cm in length are generally used.

Short columns are required for liquid adsorbent and liquid-liquid chromatography. Whereas other models of HPLC requires long columns. The internal diameter of the column is usually1-4 mm. The columns usually posses an internal mirror finish which allows, efficient packing. The packing material is supported by a porous stainless steel (or) teflan plug / disc at the end of the column.

In "HPLC" three forms of columns are available. They are

(i) Micro porous supporters

(ii) Pellicular supporters

(iii) Bonded phases,

Guard Column

Resolution power of HPLC is so high that sample preparation before chromatography is not necessary. Thus serum or other biological materials can be applied to the column without any pre treatment. This however, clogs the column after a free application as the column during separation retains many undesirable components of the biological sample. To overcome this problem a short column (2-10cm) precedes the main column. This short column is known as "guard column" and its function is to retain these biological components, which would otherwise clog the main column. The packing of the guard column can be replaced at regular intervals.

Column Packing Procedure

For HPLC, the packing must be uniform without any cracks (or) channels for obtaining optimum separations. Usually a method known as "high pressure slurry technique" is used for packing the column. In this method packing material is prepared in a suitable solvent. The column is sealed with a porous plug at the bottom. The slurry is now pumped into the column at high pressure. The column so packed, is then equilibrated for a long time by passing the developing solvent through it. The technique can be extended to hard gels. For soft gel, however this technique cannot be applied because pressure results in the fracture of gel particles. These gels therefore have to be filled into the column under gravity in a way similar to conventional chromatography.

Detectors

In HPLC a small quantity of sample is applied to the column. Therefore the sensitivity of the detector must be high and stable. U.V., visible photometers can be used for HPLC. These detectors are inexpensive and sensitive, and insensitive to normal flow and temperature fluctuations, and well suited for gradient elution. However they are sample selective. These detectors are suitable to detect macromolecules, which absorb at 250nm or 280nm. By receiving the signals from the detectors record chromatogram.

Applications

- Due to high speed of resolution and reproducible it has been widely used for separation of macromolecular mixtures.
- It is mainly used for separation of carbohydrates, proteins, lipids, fatty acids and nucleic acids.
- This technique has been widely used for purification of proteins from cell extracts.
- HPLC separates the microorganisms from culture supernatants.
- By using this technique we can separate different RNA's from mixtures of RNA's.
- It has been widely used to separate synthetic corticosteroids such as Hydrocortisone, and progesterone.
- HPLC is used in separation of drugs in horse plasma.
- It has been widely used in separation of sugars in food materials.
- It is also useful for separation of di and tri carboxylic acids such as succinic acid, Fumaric acid, Malic acid, citric acid, α – Ketoglutaric acid and oxalic acid.
- It is also used in separation of D and L isomers of amino acids.

8.6. Gas Liquid Chromatography (GLC)

The basis for the separation of the compounds in gas liquid chromatography is the difference in the partition coefficients of volatilized compounds in the liquid stationary phase and Gaseous mobile phase.

Gas liquid chromatography is a form of column chromatography where the stationary phase is a non-volatile liquid. Here, the stationary phase is liquid phase. This liquid phase is dispersed over a surface of an inert solid support. The solid support which is coated on to the inside surface of a long column is inert to the sample compounds and does not react with them in any way. Liquid phase interacts with sample components. A gas stream called carrier gas flows continuously through this column at a flow rate, which is controlled. When a small quantity of volatile sample is introduced in to the gas, the gas promptly carries it on to the column. In the column the sample components become distributed between liquid and the gas phases. These components therefore travel more slowly than the carrier gas because they are being retarded by virtue of their interaction with the liquid phase. The retarding effect is different for different components. The sample distributing more in the liquid phase is retarded more and the component, which preferred the gas phase, is retarded less. These separated components eventually elute out of the column and reach the detectors which reads the concentration of a given components present in the carrier gas and converts it to an equivalent electrical signal. The magnitude of the detector signal as measured by a continuous recorder when plotted against the time taken by the particular component to elute out of the column produces a pattern called a chromatogram. This chromatogram is made up of a number of peaks each of which is due to a component of the sample.

The characteristic position of peak is measured in terms of the volume of gas that has traversed through the column between the time that the sample was applied and the time at which the particular component emerged from the column. This gas volume has been given the term retention volume V_r. The area of the peak is usually proportional to the concentration of the component.

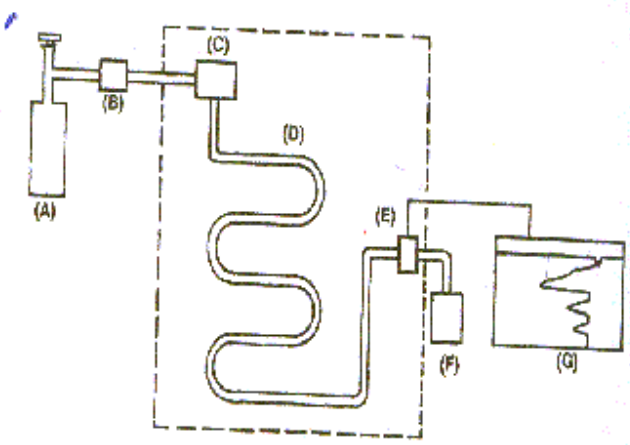


Figure 8.4: Schematic diagram of a gas chromatograph, (A) Carrier gas tank (B) Pressure regulator (C) Sample injection chamber (D) Column detector (E) Detector (F) Fraction collector and (G) Recorder.
(Courtesy from Upadhaya and Upadhaya Nath)

Carrier Gas

In GLC, the carrier gas constitutes the mobile phase and provides transportation for the sample components through the apparatus. The gas must be chemically inert and pure. Gas used at high density gives a better separation but takes a long time. The choice of gas usually depends on the requirements of the detector and also on the availability of the gas. Most commonly used gases are nitrogen and argon. Purity of carrier gas is critical because even a small impurity can give rise to noise in the detector. The usual contaminant is water vapor.

A soap film flow meter is used almost exclusively to measure the flow rate. The gas is usually passed through the column at a flow rate of 40-80 cm^3/minute.

Columns

Two distinct types of columns are commonly used, packed columns and open tubular columns. The open tubular columns are also known as capillary columns. Packed columns are stainless steel, copper or glass tubing 1.6, 3.2, 6.4 or 9.5 mm bore, and any where between 1-15 meter in length.

Open tubular or capillary column have an open unrestricted path for the carrier gas with in the column. These columns are about 15-30 meter in length with an inside diameter of about 0.25 mm. These open tubular columns are of two types. One is wall coated open tubular column and the second support coated open tubular columns (Slot).

Solid Support

An ideal support should be chemically inert. It should ideally have a high specific surface. In addition it must be thermally stable and mechanically strong. The most commonly used supports are derived from diatomaceous earth and Teflon. Deactivation of all diatomaceous supports is necessary for most applications. Acid washing is effective in removing mineral impurities. This treatment is needed for if the sample is non polar.

Liquid Phase

A good separation will occur only when the sample dissolves well in the liquid stationary phase. So since the gas phase is inert, the separation occurs only in the liquid phase. Thus to select the liquid phase it is necessary to match the polarity of the stationary phase and the sample components.

The requirements for a good liquid phase are

1) It must be non-volatile at the temperature it is to be used.
2) It should be thermally stable.
3) It should provide appropriate partition-coefficient values for the components of interest.
4) It should be completely inert towards the solutes.

Coating the Support

To prepare the column, correct amount of liquid phase dissolved in a low boiling solvent is added to the solid support. The mixture is then heated very slowly with continuous stirring in order to evaporate the solvent. The last traces of solvent are removed under vacuum. Columns are filled by pouring the packing into straightened columns. The column is gently shaken and tapped all the while to ensure an even packing. The pressure applied is 5 psi. Both ends are plugged with glass wool and the column is bent or coiled to an appropriate shape that will fit in the oven.

Sample Preparation And Introduction

If the samples are non polar or have a very low polarity they may not need any pretreatment. Sample should ideally be introduced in the vapour form. Size of the sample varies depends upon the column. Solid samples are much more difficult to introduce. The best method is to seal them into a thin vial which can then be introduced in to the injection port and then crushed from outside.

DETECTORS

These are located at the exit of the separation column. The detectors detect the presence of the individual components as they leave the column. The most commonly used detectors are

1) Flame Ionization Detector

This is most widely used detector. It measures all organic compounds and it can detect as low as one nano gram of any given compound. Hydrogen used as a carrier or introduced into the detector through elsewhere is burnt to give a nearly 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. The flame changes color, the moment a separated component comes out of the column and into the flame. The sample components become ionized in the flame and give rise to a current between the electrodes.

2) Electron Capture Detector

This detector has radioactive 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 a suitable voltage is applied. When a sample component, which has the ability to capture electrons, comes out of the column, it captures the ionized electron there by causing a drop in the current. This change in the current is measured and recorded. It is mostly used to measure polyhalogenated compounds, particularly pesticides such as DDT, dieldein and aldein. It is very sensitive and can detect as little as one pico gram of these compounds.

3) Thermionic Emission Detector

This detector employed fuel poor 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 1.25 cm above the plasma jet. This bead is electrically heated to about 600-800^{0}C. It is used to measure organo phosphorous pesticides.

Retention Time And Qualitative Analysis

Retention time aids in qualitative analysis in gas chromatography under standard conditions of temperature, gas flow, gas compressibility etc. The time taken for a compound to emerge from a column is constant and is known as the retention time.

The separation of two components of a sample is a function of the ratio of their retention times. This ratio is known as separation factor. The separation factor is a function of the stationary phase and can be varied in order to improve resolutions by varying the stationary phase.

Applications Of Gas Liquid Chromatography

Apart from the separation of components of tobacco smoke, atmospheric pollutants solvents plant extracts, essential oils, volatile vegetable oils and organic acids etc.

- Gas chromatography is being increasingly used as an analytical tool to study many parameters.
- It is widely used in the field of solution chemistry including study of polymers, Lewis acid base properties, liquid crystals, and adsorption.
- This is also an excellent tool to study thermodynamic properties of solutions.

- It is one of the most widely used to study reaction rates, energies and mechanisms.
- They are used to behave as isotope exchange vessels.
- GLC used to analyze such molecular properties as vapour pressure heat of vaporization molecular weight.

8.7. Summary

Chromatography is the technique used to separate the components of sample mixture. The differential movement of components in sample between stationary and mobile phases is responsible for their ultimate separation from each other. There are two basic techniques of chromatography, plane and column chromatography. There are two types of plane chromatography "Paper and TLC". In paper chromatography the stationary phase is supported by cellulose fibers of the paper sheet and solvent acts as mobile phase. The sample is applied to paper as a small spot. There are two techniques which may be employed for the development of paper chromatography. Those are ascending and descending chromatography. Separation of sample is achieved in ascending chromatography as the solvent moves vertically up the paper by capillary action. In the descending technique, the separation of the sample is achieved as the solvent moves downward under gravity. There are two other techniques in paper chromatography are radial and two dimensional chromatography. This technique has played an important role in biochemical analysis due to its ability to separate small molecules such as aminoacids and oligopeptides.

The "TLC" is similar to paper chromatography. In TLC a thin layer acts as stationary phase &and solvent acts as mobile phase. In "TLC" the stationary phase is coated onto a glass or plastic surface. The separation of components and detection is as similar to ascending paper chromatography. The advantage of "TLC" is the speed at which the separation is achieved.

HPLC is an example of column chromatography, and the resolution power of HPLC is very high compared with other conventional chromatography, techniques. This method has high pressures. Therefore the flowrate is high, and experimental time is short and resolution power is high. The greatest advantage of HPLC is that it may employ the principle of adsorption, partition, ion exchange exclusion and affinity chromatography. This makes it an extremely versatile and most popular technique.

In "GLC" the stationary phase is non volatile liquid and carrier gas constitutes the mobile phase. Liquid phase is dispersed over a surface of an inert solid support and is present in long column. When a small quantity of volatile sample is introduced into the gas, the gas carriers it into the column. In column the sample components become distributed between liquid and gas phases. The separated components elute out of the column and reach the detector which reads the concentration of a given component present in the carrier. Gas converts it to an electrical signal. The chromatogram is made of no of peaks each of which is due to components of sample. The area of peak is proportional to the concentration of component. The GLC is the most popular technique and having wide number of applications.

8.8. Model Questions

Essay Type Questions

1) Write about the principle, instrumentation, procedure and applications of high performance liquid chromatography.
2) Give an account on the principle, instrumentation, procedure and applications of gas liquid chromatography.
3) Define partition coefficient and explain the procedure for the separation of lipids by TLC.

Short Answer Questions

1) Explain the term chromatography and add a note on paper chromatography.
1) Thin layer chromatography.
2) Applications of HPLC
3) Radial paper chromatography.

8.9. Reference Books

1) Biophysical Chemistry by Upadhaya and Upadhya Nath, 3rd Edition, Himalaya Publications.
2) Analytical Chromatography by G.R.Chatwal, 1st Edition, Himalaya Publications.
3) Practical Biochemistry by Keith Wilson and Walker 5th Edition, Cambridge University Press.
4) A Biologist Guide to Principles & Techniques of Biochemistry, Keith Wilson and Goulding.

Usha Kiranmayi G.

LESSON – 10

ELECTROPHORESIS

10.0. OBJECTIVES

By the end of the lesson you will be able to understand

- the definition, principle and purpose of Electrophoresis and factors affecting electrophoresis
- different types of Electrophoresis

10.1. INTRODUCTION

Many important biological molecules such as amino acids, peptides, proteins, nucleotides and nucleic acids possess ionisable groups. These can be made exist in solution as electrically charged species, either as cations (+) or anions (-). Even typically non-polar substances such as carbohydates can be given weak charges by derivatisation, for example, as borates or phosphates. Molecules which have a similar charge will have different charge/mass ratios when they have inherent differences in molecular weight. These differences form a sufficient basis for a differential migration when the ions in solution are subjected to an electric field. This is the principle of electrophoresis.

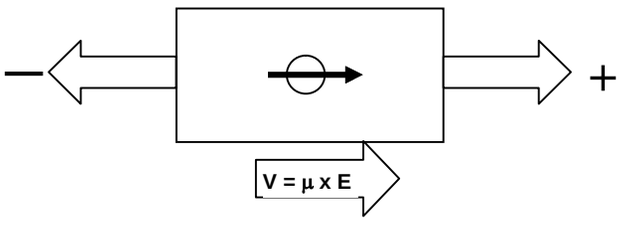
The migration of charged particles in a medium under the influence of an applied electric field. The usual purposes of carrying out electrophoresis are:

(a) to determine the number, amount and mobility of components or to separate them in a given sample
(b) to obtain information about the electrical layers surrounding the particles.
(c) determination of molecular weight of proteins and DNA sequence.

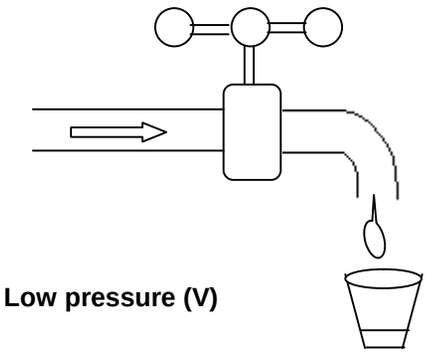
Electorphoresis is a Greek word means, ‘born by electricity’. In 1807 a Russian Physicist, Alexander Reuss observed the electrophoretic movement of some colloidal particles when current was passed through glass tube filled with water and clay. Michael Faraday was also confirmed this discovery.

10.2 PRINCIPLES AND FACTORS AFFECTING ELECTROPHORESIS

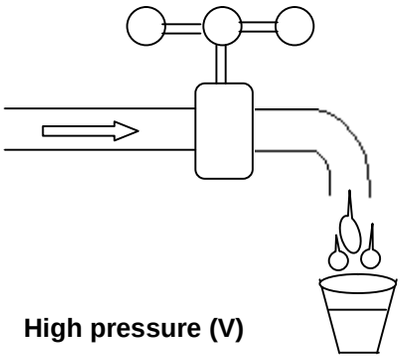
Principle of electrophoresis



$V=\mu \times E$ where,
V= Velocity of molecule (cm/second)
μ = Electrophoretic mobility (cm^2/volt/second)
E = Electrical field strength (Volt/cm)
⊖▶ = a molecule migrating in the electrical field
▬⇦ = migration of cations
Voltage is the fundamental driving force in electrophoresis. A volt is equal to the difference in potential needed to cause a current of one ampere to flow through a resistance of one Ohm. Mobility of molecule increases with increase in voltage. According to Ohm's law:
V = IR or I = V/R
V = volt, I = current and R = resistance

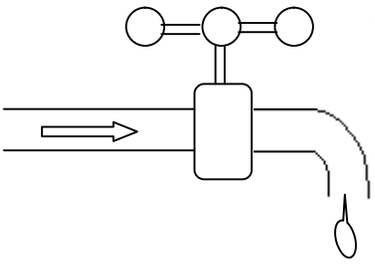


Low pressure (V)



High pressure (V)

Water tap is analogous to an electrophoresis system.



Voltage (V) = water pressure
Current (I) = water capacity in the pipe
Power (P) = water collected
Resistance (R) = tap valve

The equipment required for electrophoresis consists of two items

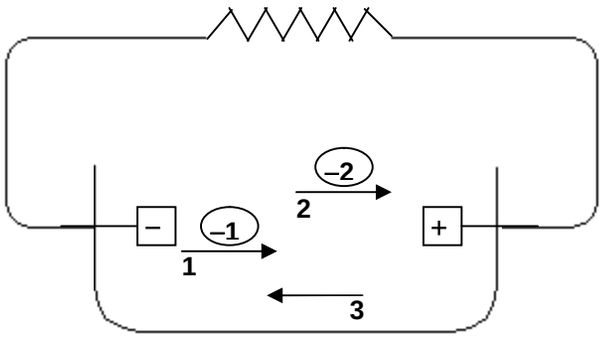
(a) a power pack and
(b) an electrophoretic unit

In an electrophoretic unit, the power pack supplies a direct current between the electrodes. The sample in an aqueous solvent acquires either positive or negative charge. Cations move to the cathode (-) and anions move to the anode (+). The acquisition of such charges depends on the nature of the particle/molecule and the solvent. The sample must be dissolved or suspended in buffer for electrophoresis and any supporting medium must be saturated with buffer to conduct the current. A buffer is also important to maintain a constant state of ionization, because changes in pH would alter the charge on molecules being separated. For example, the net charge density of a protein depends upon the number of ionizable amino and carboxyl groups and velocity depends on the sign and quantity of net charge density.

The net charge density depends upon chemical groups and their number present in the molecule is modified by the nature of the solvent. The current is maintained throughout the circuit by electrolysis taking place at the electrodes, both of which dip into buffer reservoirs. During electrolysis, OH^- ions and H^+ ions are produced at the cathode and O_2 and H^+ ions are produced at the anode.

$2e^- + 2H_2O \xrightarrow{\text{Cathode}} 2OH + H_2$

$H_2O \xrightarrow{\text{Anode}} 2H^+ + ½\ O_2 + 2e$



The hydroxyl ions produced at the cathode cause an increase in dissociation of the weak acid component (HA) of the buffer mixture. This results in the formation of more A to conduct the current to the anode. At the anode, A ions combine with H^+ ions to reform HA and the electrons are fed into the electric circuit. Therefore most of the current between the electrodes is conducted by the buffer ions in solution.

A charged particle (1) migrates toward the oppositely charged electrode (+). Arrow 2 indicates faster movement of a particle bearing a net charge density of –2. Arrow 3 indicates the direction of frictional force.

Factors affecting electrophoresis

There are certain factors like the charge, size and shape of the sample, composition, concentration or ionic strength and pH, adsorption, electro-osmosis, and molecular sieving of the supporting medium and voltage, current, resistance relationships in electric field.

(a) The sample: Electrophoretic mobility of the sample will be dictated by charge/ mass ratio of the sample.
Charge = positive or negative
Mass = size and shape of the molecule

1. Charge: Increase in the charge increases the electrophoretic mobility. It depends upon pH of the medium.

2. Size: Bigger the molecule, greater is the frictional and electrostatic forces exerted upon it by the medium of suspension. Larger particles have a smaller electrophoretic mobility than the smaller particles.

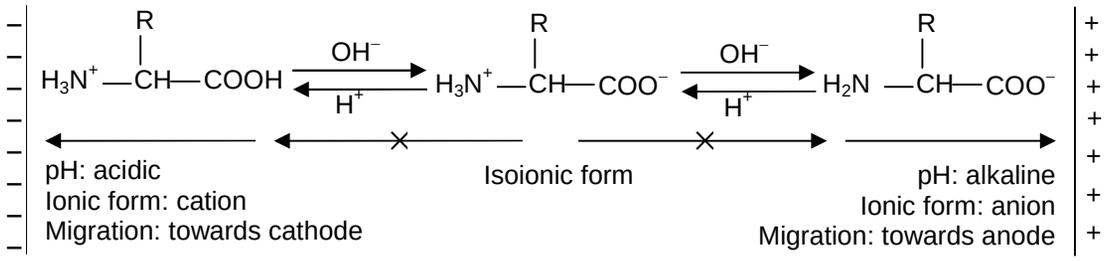
3. Shape: Round particles move faster than fibrous or other shapes.

(b) The buffer: The pH of the supporting medium will be determined and stabilizes by the buffer.

1. Composition: The commonly used buffers are formate, acetate, citrate, barbitone, phosphate, Tris, EDTA and pyridine. The rates of migration will be altered if the buffer binds with the compounds being separated. So buffer should not bind with the compounds being separated. In some cases binding can be advantageous, for example, borate buffers are used to separate carbohydrates. Some diffusion of the sample is inevitable, because buffer acts as a solvent for the sample, for example, amino acids and sugars. This diffusion can be minimized by avoiding over loading, using a high voltage for a short time and by rapid removal of the supporting medium after the separation has been completed.

2. Concentration (ionic strength): Increased ionic strength of the buffer means a large share of the current being carried by the buffer ions and a meager proportion carried by the sample ions. This situation leads to slower migration of the sample components. The overall current will also increase, there will be heat production. A decrease in ionic strength, would mean larger share of the current being carried by the sample ions leading to a faster separation. Since the overall current will be low, less heat will be produced. In low ionic strength buffers, diffusion tends to be high with concomitant loss of resolution. The chosen ionic strength of the buffer is usually between 0.05-0.10 M.

3. pH: pH shows more effect of ionization of organic compounds whereas only little effect on fully ionized compounds like inorganic salts. The ionization of organic acids increases as pH increases and the ionization of organic bases decreases as pH decreases. Therefore migration depends on pH. Both the above effects the amino acids that have both acidic and basic properties.



The direction and migration of ampholytes are pH dependents and buffers from pH 1-11 can be used to produce the required separation. Generally the buffer in both reservoirs is normally the same buffer, this is called continuous buffer system. In some forms of gel electrophoresis like SDS PAGE, where the buffer acts as part of the supporting medium and a different buffer may be used in the gel to that in the reservoirs. This is called discontinuous buffer system.

(c) The supporting medium: The supporting medium may cause adsorption, electro-osmosis and molecular sieving.

1. Adsorption: This is nothing but attachment of the component on to the surface of supporting medium. It causes tailing of the sample so that it moves in the shape of a comet rather than a compact band. Thus the rate and resolution of the electrophoretic separation can be reduced by adsorption.

2. Electro-osmosis: This results from a relative charge being produced between H_2O molecules in the buffer and the surface of the supporting medium. The charge may be caused by surface adsorption of ions from the buffer and the presence of stationary carboxyl groups on paper/sulphonates on agar. This generates a motive force for anions to the anode and cations to cathode carrying along the neutral substances by solvent flow.

3. Molecular sieving is the intertwined molecular chains, which are distributed throughout the gel, acts as a sieve like structure. The movement of large molecules is stopped/hindered by decreasing the pore size in case of agar, starch and polyacrylamide gels, where as in case of Sephadex gel, small pores shut out/exclude (leave) larger molecules, therefore they move outside the pores, where as small molecules are tightly held with in the pores.

(d) The electric field

1. Voltage: The distance of the electrodes is d meters and potential difference between them is V volts, therefore
Potential gradient = V/D volts m^{-1}
Ions bear a charge q coulombs. The force on this ion is Vq/d newtons.
This force causes migration and the rate of migration is proportional to Vq/D. Increase in potential gradient will increase the rate of migration.

2. Current: When a potential difference is applied between the electrodes, a current is generated. Current is measured in coulombs sec^{-1} or amperes. Current is directly proportional to the voltage. The current in the solution between the electrodes is conducted by the buffer ions. Increase in voltage increases the total charge/sec conveyed towards the electrode. The distance migrated by the ions is proportional to both current and time.

3. Resistance: Ohm's law expresses the relationship between current I (measured in Amperes A), voltage V (measured in volts V) and resistance R (measured in Ohms Ω) in which:

$$V/I = R$$

The current and rate of migration are inversely proportional to the resistance. Resistance will increase with the length of the supporting medium but will decrease with its cross-sectional area and with increasing buffer ion concentration. During electrophoresis the power (comes out from the supporting medium) dissipated in the supporting medium (W, measured in watts) is:

$$W = I^2 R$$

Increase in temperature will cause fall in resistance. Part of this is due to an increase in the mobility of the ions. This heating will produce evaporation of the solvent from the supporting medium causing a decrease in resistance. Increase in buffer ion concentration will result in slower migration of the sample.

Stabilized power packs are used which can automatically maintain either a constant voltage/constant current. When a constant voltage is applied, the current will increase due to a decrease in resistance of the medium with the rise in temperature. Consequently, more heat will be produced, more evaporation of solvent and a decrease in resistance. When a constant current is applied, these problems will be solved, but may lead to a drop in voltage due to decreased resistance, reduced rate of migration. If a number of supporting media are run in parallel from one power supply, the total resistance will decrease:

$$I/R = 1/r_1 + 1/r_2 + 1/r_3 + \ldots\ldots\ldots\ldots 1/r_n$$

The voltages used can be low (100-500) or high (500-10,000 v). High voltages are used for the separation of low molecular weight compounds. In low voltage constant voltage or current may be applied, as the heat generated is small and is easily dissipated (paper electrophoresis). In case of cellulose acetate and other gels, a constant current is used to reduce heat production. Direct current must be used. Enclosing the apparatus under an airtight cover can minimize evaporation.

10.3. TYPES OF ELECTROPHORESIS

10.3.1. Gel Electrophoresis

For the separation of high molecular weight substances like proteins and nucleic acids gels are used as supporting medium. Suitable gels may be prepared by using powdered solids like starch, agar and polyacrylamide. Molecular sieving property of the gel helps to separate proteins, which have similar charge but different sizes and shapes.

Starch gels

Starch gels are of two types, weak high porosity and strong low porosity gels. Weak high porosity gels are prepared by incorporating less than 2 % (W/V) starch in an appropriate buffer and strong low porosity gels are prepared by adding 8 to 15 % starch. In both the cases, the branched chains of the amylo pectin component of starch to intertwine and form a gel.

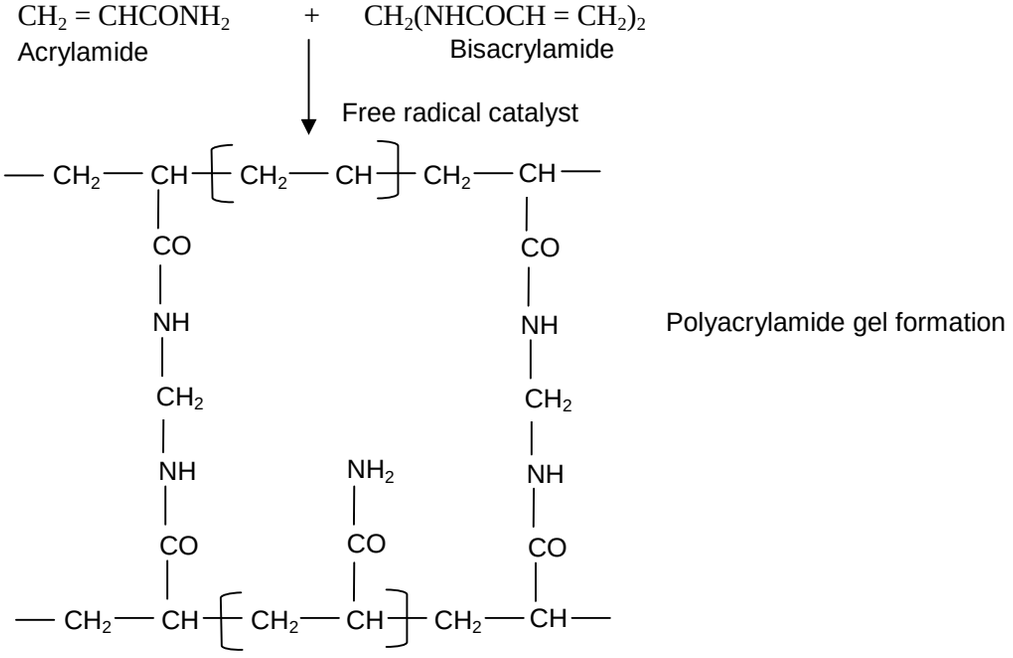
Agar / agarose

Agar is a cheap, non-toxic, complex powdery mixture containing two galactose-based polymers, agarose and agaropectin. 1% (W/V) gel in buffer has a high water content, good fibre structure, large pore size, low fractional resistance and sets at 38^0 C. Electro-osmosis is severe, because of sulfate content. It should be removed prior to purification. Purified agarose gel is now used for the separation of DNA restriction fragments and DNA because of lack of molecular sieving and electro-osmosis.

Polyacrylamide

Highly toxic synthetic chemicals are used for the preparation of polyacrylamide gels. The chemicals used are:
Acrylamide and bisacrylamide, APS (Ammonium per sulfate), TEMED (N,N,N'-tetramethylenediamine). Acrylamide monomer is copolymerized with a cross linking agent, bisacrylamide. Freshly prepared (0.1-0.3% W/V) APS is used as catalyst. TEMED is an indicator. It speeds up the rate of gel polymerization.

Degasing of the solution is required since molecular oxygen inhibits chemical polymerization. The relative proportions of acrylamide monomer to cross-linking agent determine porosity of a gel.



Gels may be defined in terms of the total percentage of acrylamide present. Low percentage gels have larger pore size, less resistance to the passage of larger molecules. Most protein separations are carried out using gels from 5 to 15 % acrylamide. These gels are useful for macromolecule separation because of their minimal adsorption capacity, lack of electro-osmosis and various types of histochemical analysis.

Equipment

Gels can be run as horizontal slabs or vertical slabs. Tube gels are also used. Power packs used are similar to that of low voltage electrophoresis. Slab gels can carry more samples than tube gels.

Preparation

The gels are cast between two clean glass plates, which are clamped together but held apart by plastic spacers. For agarose and starch 12 x 25 cm with 3 to 6 mm thick gels are prepared. For polyacrylamide, 12 x 14 cm with 1 to 3 mm thick gels are prepared (Fig.10.1).

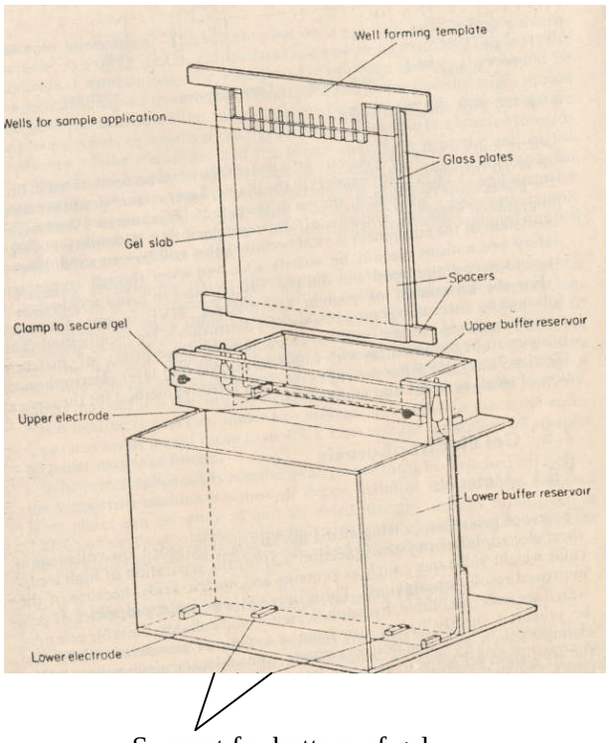


Fig. 10.1. Vertical gel electrophoresis unit

Sample application

Sample solutions are injected with a microsyringe into the wells of a gel. Wells are prepared by inserting a comb into the gel before it sets. For making sure that the sample should sink in the well, sample must contain sucrose or glycerol (10-15%). For monitoring migration, bromophenol blue dye is added. Urea or SDS may be added to facilitate solubilization of proteins and disulfide reducing agents like β-mercaptoethanol or Dithiothreitol may be added. Only microgram (μg) quantities of proteins and DNA are used.

Running the sample

The horizontal gel should be submerged in the buffer so that buffer allows current to pass directly through it. In vertical systems, gel will be sandwiched between glass plates, is placed in

the lower reservoir, with the top of the gel in contact with the buffer in the upper reservoir. The voltage and time required to obtain optimal separations will depend on the nature of the sample and the type of gels used. Marker dyes like bromophenol blue for proteins and ethidium bromide for nucleic acids enable the progress of the run to be monitored.

Sodium dodecylsulfate (SDS) Polyacrylamide gel electrophoresis

SDS is an anionic detergent. It binds strongly to proteins and causes denaturation. It gives negative charge to the proteins. Protein-SDS complexes will move toward the anode. Their mobilities are inversely proportional to their molecular weight are also run, the molecular weights of the sample proteins can be determined.

Apparatus and methods

Standard SDS polyacrylamide gels are run vertically. The protein samples are generally dissolved in Tris buffer pH 6 to 8, SDS, 2-mercaptoethnol, glycerol/sucrose (to increase density) and bromophenol blue. Resolution of the protein band is increased by using both stacking and separation/resolving gels. Differences in pH and composition between these two gels cause the samples to be concentrated into narrow bands before separation occurs. Separation is primarily based on differences in size of the proteins.

10.3.2. Gradient gels

Gradient gels contain a *concentration gradient* of acrylamide increasing from around 5 to 25 %, with a corresponding decrease in pore size, causing improved resolution of protein bands. The gradients are formed by running high and low concentrations of acrylamide solutions between the gel plates via a gradient mixer. If the pore size is too small, narrow bands will be produced and thus increased resolution. Separation is primarily based on differences in the size of the proteins. This means that samples can be run in which there are wide ranges of molecular weights. When molecular weights are similar, better separation is achieved than in a uniform gel. Though gradient gels can be used without SDS and stacking gels, these should be utilized if optimal separations are required.

Two dimensional gels

Two-dimensional gels enable the protein components of a complex mixture to be separated with still greater resolution. The samples are first partially separated by isoelectric focussing on the basis of differences in isoelectric points, using a cylindrical column of gel. This gel is then applied along the top of the stacking gel for separation to be completed on the basis of differences in molecular size. Either uniform or gradient SDS gels may be used, depending on requirements. It can resolve a mixture containing 5000 particles into individual species.

Procedure

The mixture is first subjected to isoelectric focussing on a 1 mm diameter gel in a capillary tube. At the conclusion of the isoelectric focussing, the gel is extruded from the column and placed on to the top of the slab gel. The sample is now subjected to SDS acrylamide gel electrophoresis, which separates the proteins according to their molecular weight. Isoelectric point and molecular weight of a protein are in no way connected to each other and thus the technique exploits two properties of protein for separation and thus has a great resolution power.

Detection, recovery and estimation

Gels from a column are removed by forcing water from a hypodermic syringe around the walls of the column, allowing the gels to be extruded under gentle pressure. Slab gels are removed by introducing a thin metal plate between two gel plates and remove the plates.

Before staining, the gels are immersed in a fixative (7 % acetic acid) to guard against diffusion of separated components. The sample is stained with Coomassie Brilliant Blue. The starch gel becomes opaque after staining and so very difficult for direct densitometry. Direct UV absorbency may also be performed. However, polyacrylamide absorbs in the UV range. Inorder to get accurate quantitative results, the compound has to be removed from the supporting matrix. Compounds from the starch gel can be removed by slicing the appropriate portion of the gel, macerating it and then treating it with amylase which solubilizes the starch and leaves the compounds in solution. If the sample has been radioactive labelled, prior to electrophoresis, autoradiography may be performed to detect the portion of the separated components in the gel.

Fluorescent staining: - Fluorescent staining includes flouresamine is applied to proteins before electrophoresis, if SDS-containing gels are to be used. In other cases stain can be applied before or after electrophoresis. This method does not give quantitative results because fluorescence decreases with time. Proteins can have varied content of lysin and so the dye would give high results.

Densitometry: - After staining, the quantitative estimation of individual components can be carried out by densitometry. Depending on the intensity of the colour the results are made.

10.4. ISOELECTRIC FOCUSSING

Isoelectric focussing was discovered by H. Svensson in Sweden and has high-resolution power. A simple comparison would help to establish the methods supremacy over other methods, when paper electrophoresis resolves plasma proteins into 6 bands, isoelectric focussing resolves it into atleast 40 bands.

Protein molecules have a net positive charge in an acidic solution because most amino groups carry a positive net charge and most carboxylic groups are protonated and electrically uncharged. With a gradual increase in pH, the number of carboxylic groups carrying a negative charge increases, while the number of positively charged groups decreases. At a certain pH value, the isoionic point, the net charge of the protein molecule is zero. The isoionic point of a molecule is determined by the number of types of proteolytic groups and their dissociation constants. Although there is considerable variation in the isoionic points of proteins, they are generally in the pH range of 3-11. In conventional electrophoresis, the pH between anode and cathode is constant and the positively charged ions migrate towards the cathode and the negative ions migrate to the anode. In isoelectric focussing on the other hand, a stable pH gradient is arranged, the pH increases gradually from anode to cathode. Protein introduced into this system at a point where the pH is lower than the isoionic point will possess a net positive charge and will migrate in the direction of cathode. Due to the presence of the pH gradient, the protein will migrate to an environment of successively higher pH values which in turn, will influence the ionization and net charge of the molecule.

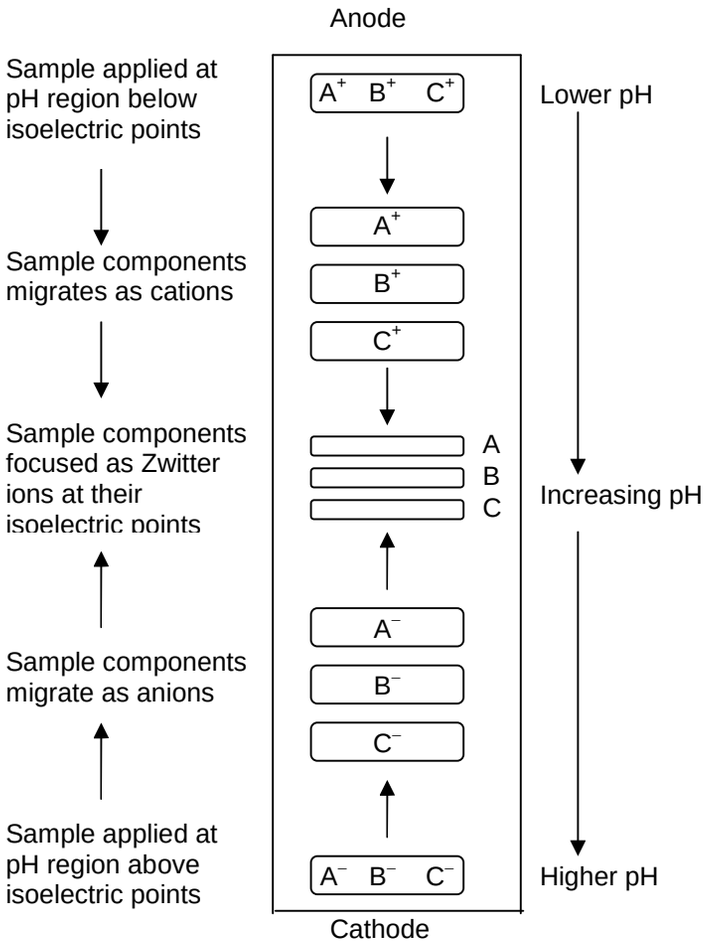


Fig.10.2. The Prnciple of Isoelectric Focussing.

Finally, the protein will encounter a pH where its net charge is zero and will stop migrating. This is the isoelectric point of the protein. The consequence of this is that, every protein will migrate to and focus at its respective isoelectric point in a stable pH gradient, irrespective of its origin in the apparatus at the time the current was applied. Thus, the point of application and the volume of the protein solution are not critical. Diffusion, which is an obstacle with every other method of electrophoresis, is not a problem with electrofocussing, because focussing effect works against diffusion. Thus, once a final stable focussing is reached, the residual will be retained even if the experiment is continued for a long time (Fig.10.2).

Establishing the pH gradient carrier ampholytes

The pH gradients may be obtained by electrofocussing special buffer substances known as carrier ampholytes. The carrier ampholytes must have the properties:

1. Since carrier ampholytes must dictate the pH course, they should have a certain buffering capacity at their isoelectric point.
2. They should have conductance at their isoelectric point.
3. They should have low molecular weight so that macromolecules can be separated from them easily after electrofocussing.
4. They should be soluble in water. This hydrophilic character will also prevent their binding to hydrophobic regions of proteins.
5. Ideally they should have a low light absorption at 280 nm. This would permit the detection of proteins after electrofocussing by measuring at 280 nm. Carrier ampholytes are isomers and homologs of aliphatic poly amino, poly carboxyl acids. The general formula for a carrier ampholyte is:

$$R –N –(CH_2)_n –N – (CH_2)_n – COOH$$

where R can be

$$- (CH_2)n – COOH, H \text{ or } (CH_2)_n – N - R$$

η is usually less than 5. Carrier ampholytes are available commercially in mixtures covering a wide pH band or various narrow bands. The pH range of the carrier ampholytes should be chosen such that the pI (isoelectric point) values of the proteins under study lie well within the corresponding pH range. When making the first run with the protein sample it is often advisable to work with the pH range 3-10. Generally an amount of carrier ampholyte which gives a final average concentration of 1% (W/V) in the column is used. For electrofocussing in gels, an average concentration counteracts electroendosmosis. When pH range is outside 6-8, ampholyte concentration of upto 10% have been used in order to obtain a more even distribution of conductivity between electrodes.

Stabilization against convection

As for all other electrophoretic techniques described so far, electrofocussing also needs provision for stabilization of separating protein zones against convecting flow in the solution. Three ways are in use:

1. Density gradient
2. Gel
3. Zone convecting electrofocussing (not popular)

1. Density gradient

Density gradients suitable for electrofocussing can be made with many uncharged solutes which are dissolvable in water to a concentration that will increase the density sufficiently. The compounds should not react with proteins and should have a low content of heavy metals. They should be of high purity. Sucrose is the most ideal compound for the formation of density gradients as it has a protective action on proteins. It has been used with 50% (W/V) as the densest solution. Maximum solute concentration and thus the maximum density are placed at the bottom of the column. There is a linear decrease in the concentration of the solute as a function of the column height giving rise to approximately linear density gradients. However, non-linear density gradients have also been used. Sucrose cannot, however, be used at all pH ranges since it is destabilized at pH range 10. Glycerol is generally used at such pH ranges other compounds which can be used in formation of density gradients are mannitol, sorbitol, ethylene glycol, dextran and ficoll. The gradient should be unchanged so that they won't effect the pH. They should not react with sample, should not contain heavy metals, and should be pure.

2. Gels

In electrofocussing, the gel serves only as an anticonvectent and not as a molecular sieve. Obviously, the gel concentration should be low to provide larger diameter pores. For large proteins, molecular weight exceeding 200 KD, lower concentration of acrylamide is the preferred for electrofocussing. Agarose and starch gels are not preferred as in these gels, pH gradient drifts considerably during prolonged experiments.

3. Zone convection electrofocussing

The apparatus is made up of two rectangular boxes, the upper one being the cover. The upper surface of the lower box is with ridges, with a height of about 10 mm, separated by depressions and is facing the upper box which has corresponding ridges (Fig. 10.3).

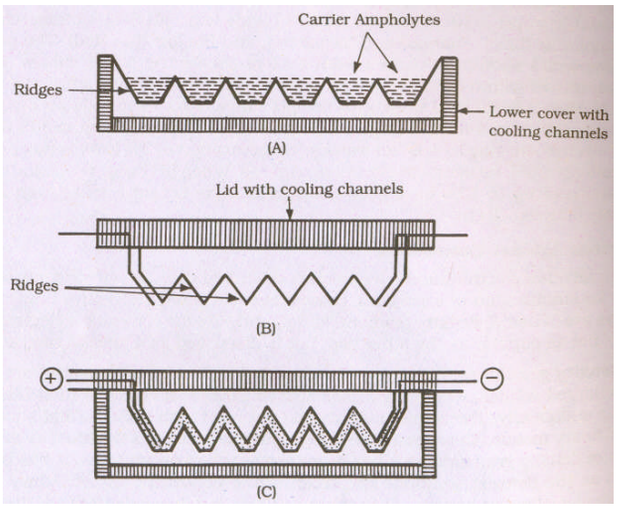


Fig. 10.3. Schematic diagram of Zone convection electrofocussing apparatus. A) Bottom part filled with carrier ampholytes; B) The lid with corresponding ridges; C) The apparatus assembled for electrofocussing.

The ridges and depressions of the two halves fit together leaving a space of few milli meters in between. Thus from one end to the other there will be a narrow wave like channel between the two parts which can be described as series of inter connected broad U-tubes. The carrier ampholyte solution is filled in this space with electrodes situated at the two ends. Both the lid and the bottom part are hollow with channels built into them through which the liquid streams to maintain a constant temperature. When the current is on, a density gradient is formed in each depression by the solute. When proteins become immobile at their isoelectric pH, the density increases locally and the proteins settle down in the depression of the bottom part. When the experiment is over, the cover is lifted and the liquid collects in the depression. Each depression will contain a fraction separated by a ridge. The fractions can now be collected without any possibility of contamination by neighbouring fraction.

Procedure: Separation can be carried out in a vertical column or on a horizontal gel plate, but in both cases purpose made equipment is required (Fig.10.4).

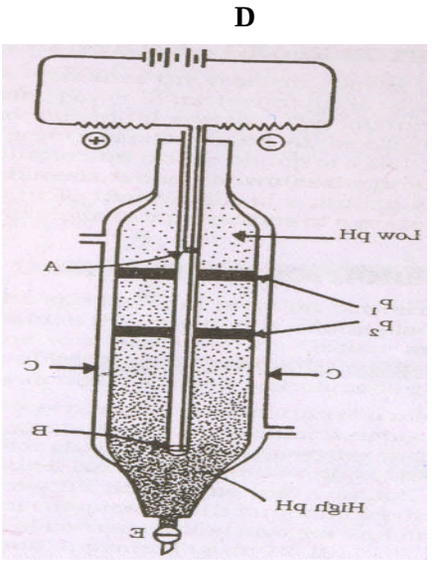


Fig. 10.4. Schematic design of an Isoelectrofocussing apparatus.
A) Anode platinum ring B) Cathode platinum
B) ring C) Water jacket to maintain the
temperature. D) Power pack , E) Valve to empty the column , P_1, P_2) Protein bands.

The column system in modern days being superceded by the plate system. For preparative purposes, the column mode is still preferred. Water-cooled vertical glass columns are commercially available. These are filled with a mixture of carrier ampholytes suspended in a density gradient solution (sucrose, glycerol). The anode end of the column is connected to a reservoir containing an acidic solution ($H_3 PO_4$) and the cathode end is connected to a reservoir filled with an alkaline solution (NaOH). The valves of the reservoir are opened to allow the acidic and the alkaline solutions to diffuse through the column. This results in the formation of a pH gradient between the anode and cathode. The valves are closed and the power is switched on. The carrier ampholytes now migrate until they become immobile upon reaching the regions of their corresponding isoelectric pH. These compounds remain fixed in these regions and because of their buffering capacity, the pH gradient is stabilized. Now the sample is applied at the upper end of the column. The charged components of the sample migrate in the electric field till their net charge becomes zero, that is, till they reach their isoelectric pH. The component proteins of the sample remain focussed at the regions of their isoelectric pH. The whole process might take 1-3 days. Once the experiment is over, the power is switched off and the sample components are allowed to run off through a valve at the base of the column into a faction collector. The fractions can be analyzed further.

Instead of density gradient, polyacrylamide gel impregnated with carrier ampholytes, can be used in the vertical columns. The time required for electrofocussing in the gel is considerably less than that required in the density gradients (2,3Hours). The carrier ampholytes are mixed with the unpolymerized gel solution and the mixture is allowed to polymerize. The rest of the process remains the same as for the density gradients. Gels are however, used more with the plate mode rather than the column mode. Upto 24 samples can be simultaneously analyzed using the plate system.

Separation of protein from carrier ampholytes

Dialysis against a buffer would effectively remove at least 99% of the ampholytes, but it is a slow process. Gel filtration would give effective separation in a very short time. Sephadex G-50 is the general choice for the process, others to be ammonium sulfate precipitation of proteins, ion exchange chromatography and partition chromatography by counter current.

10.5. PULSED FIELD GEL ELCTROPHORESIS

Agarose gel electrophoresis can not separate linear double stranded DNA molecules that have a radius of gyration which is larger than the pore size of the gel. These large liner duplex DNA molecules migrate through agarose gels at the same rate irrespective of their size. Agarose stops to perform any molecular sieving effect on these large DNA duplexes. This behavior of large DNA molecules is because of a phenomenon known as Reptation, which means the molecules migrate ‘end-on’ through the matrix.

The problem of separating large sized DNA can be removed to some extent by increasing the pore size of the gel. These large pore gels are capable of sieving larger size DNA molecules. Thus, extremely low concentration of agarose (0.1% to 0.2%) have been used to resolve extremely large DNA molecules. This low percentage of gels present two problems of their own:

1. They are very fragile and have to be handled extremely carefull.
2. They have to be run very slowly failing which the resolution might be poor.

They are absolutely incapable in separating linear DNA molecules whose size is in excess of 750 kb. The major importance is Histo compatability locus of mammals and therefore, the need to study DNA is well known. This locus occupies several thousands of DNA. Naturally, inorder to study this locus, it is desirable if one can separate it from other genetic material. In order to solve this problem, Schwartz and Cantor (1984) developed pulsed-field gel electrophoresis. It uses pulsed, a Hernating orthogonal electric fields.

When such a field is applied to a gel, large DNA molecules become trapped into their separation tubes every time the direction of the electric field is changed. These molecules remain immobilized till they reorient themselves along the direction of the new electric field. The different DNA molecules adopt a behavior consonant with their respective sizes. Large DNA molecules take a larger time to reorient themselves and are consequently retarded more in the new electric field as compared to the smaller DNA molecules. The molecules of DNA whose reorientation times are less than the period of electric pulse can be fractionated in a size-dependent manner. The resolution of pulsed-field gel electrophoresis depends on

1. The absolute periods of electric pulses.
2. The angles at which the two electric fields are applied to the gel.
3. The relative field strengths of two electric fields and the degree of uniformity of the two electric fields
4. The ratio of the periods of the electric pulses employed to generate the two electric fields. By this method DNA molecules upto 2000 kb in size can be separated.

Instrumentation

The original apparatus used alternately pulsed electric fields are perpendicular orientations and linear electrodes. This type of apparatus does not give proper resolution. The electric field generated is never uniform and so the speed and direction of DNA, which depends on the position at which they are loaded into gel, so no proper resolution at the edges of the gel.

1.Recent innovation is by Carle *et al.*, :- The apparatus designed by them does not use orthogonal or perpendicular arrangement of electric fields. Instead, the apparatus utilizes periodic inversion of a single electric field. So, this method is also called as Field-Inversion Gel Electrophoresis (FIGE). This arrangement produces an electric field, which is uniform in both the directions. The pulse in the forward direction is slightly longer than the one in the reverse direction. This arrangement ensures the migration of the DNA along an absolutely straight track.

The ratio between forward and reverse pulses is always maintained as a constant, the absolute lengths of the individual pulses might be varied to improve the resolution. This innovation (FIGE) makes it relatively easy to resolve DNA fragments of upto 2000 kb with fairly good resolution.

2 The innovation by Gardiner *et al*., :- In the apparatus designed by them, a vertical gel apparatus with platinum wire electrode placed on opposite sides of the gel. The electric field is continuously switched between the two electrodes and the DNA moves alternately toward one and then toward another electrode in a movement, which can be described as zigzag. However, the net result of such a zigzag movement is a straight line and the DNA moves from the place of application to the bottom of gel. All the lanes in the gel experience equivalent electric field. This condition prevents any horizontal distortion of the resolved DNA bands.

The usual arrangement of the electrodes is at an angle of 90^{o}. The length of the electric pulses varies depending upon the size of the DNA to be separated. 10-second pulses used for DNA between 50-500 kb in length, 60 second pulses used for DNA larger than 1000 kb. Extremely useful for DNA molecule upto 9000-kb length can be separated from each other satisfactorily.

As pulsed field gel electrophoresis is a relatively new technique, no single type of apparatus has gained broad popularty till date. Different laboratories use apparatus of different designs depending on their needs. There are several companies, which are marketing such apparatus.

10.6. Summary

- Electrophoresis is the migration of charged particals in a medium under the influence of an applied electric field. The equipment required for electrophoresis consists of two items; power pack and an electrophoresis unit. Power pack supplies direct current between the electrodes. The positive charged samples move to the cathode (-) and negative charged samples move to the anode (+). The buffer that is used to dissolve the sample and in electrophoretic apparatus must be the same. Buffer plays an important role in maintaining constant ionization, as changes in pH would alter the charge on molecules being separated.
- Voltage is the fundamental driving force in electrophoresis.Volt is equal to the difference in potential needed to cause a current of one ampere to flow through a resistance of one Ohm. Mobility of the molecule increases with increase in voltage.
- There are certain factors like charge, size and shape of the sample, composition and ionic strength of the buffer, pH, adsorption, electro-osmosis and molecular sieving of the supporting medium, voltage, current and resistance of the electric field, influence electrophoretic mobility.

- For separation of high molecular weight substances like proteins, nucleic acids gels are used as supporting medium like starch gel, agar or agarose and polyacrylamide gels. Each type of gel has separate compositions, preparations and apparatus are required. Weak high porosity gels are prepared with less than 2% starch and strong low porosity gels are prepared with 8 to 15% starch in starch gels. 1% agar or agarose is appropriate for the separation of DNA restriction fragments and DNA. Toxic synthetic compounds like acrylamide, bisacrylamide, APS and TEMED are used for polyacrylamide gels. Most protein separations are carried out using gels from 5 to 15% acrylamide. Only μg quantities of protein and DNA are used.
- Sample should be added with glycerol (10-15%), which makes the sample to sink in the well, bromophenol blue for monitoring migration, urea or SDS may be added for solubilization of proteins and disulfide reducing agents like β-mercaptoethanol or dithiothreitol may be added. Marker dyes like bromophenol blue for proteins and ethidium bromide for nucleic acids are used to know the progress of the run to be monitored.
- For studying denaturation proteins, SDS-polyacrylamide gels are used. SDS causes denaturation of proteins and binds to them and gives negative charge to the proteins. Resolution of proteins is increased by using stacking and resolving gels.
- Gradient gels contain a concentration gradient of acrylamide increasing from around 5 to 25%, with a corresponding decrease in pore size, causing improved resolution of protein bands. Separation depends on differences in size of the sample. Samples can be run where there are wide ranges of molecular weights.
- Two dimentional gels are used to separate proteins with greater resolution. Either uniform or gradient SDS gels may be used and it can resolve a mixture of 5000 particals into individual species.
- Staining the gel with Coomassie Brilliant Blue or UV detection of DNA, fluorescent staining or by densitometry are used for the detection of the sample.
- A resolution electrophoresis is isoelectric focussing. Proteins carry a net positive charge. At a certain pH, the ionic point, the net charge of the protein molecule is zero. The ionic point of a molecule is determined by the number of types of proteolytic groups and their dissociation constant.
- Diffusion, is a problem in every electrophoretic methods, is not a problem because focussing effect works against diffusion.
- Electrofocussing needs density gradient, gel and zone convecting electrofocussing to separate protein zones against convecting flow in the solution. Gel filtration is an effective separtion. Sephadex G-50 is the general choice for the process.
- Pulsed field gel electrophoresis is used to separate large sized DNA, by increasing the pore size of the gel. 0.1 to 0.2% agarose gels are used.
- The resolution depends on the absolute periods of electric pulses, angles at which the two electric fields are applied to the gel and the relative field strength of two electric fields and the degree of uniformity of the two electric fields, the ratio of the periods of the electric pulses.
- Pulsed field gel electrophoresis is a new technique. There are several companies designed depending on the needs.

10.7. Model questions

1. Write the principle behind electrophoresis and what are the factors that influence electrophoresis.
2. Discuss briefly the Isoelectric focussing .
3. Write an essay on different types of electrophoresis
4. What is pulsed field gel electrophoresis.

10.8. Reference Books

1.Biophysical chemistry principles and techniques 4th ed., Keith Wilson and John Walker, Cambridge University Press

2.Biologists' guide to principles and techniques of practical biochemistry 3rd Ed. by Keith Wilson and Kenneth H. Goulding, Cambridge University Press.

3.Biophysical chemistry : Priciples and techniques by Upadhyay, Upadhaya and Nath. Himalaya publishing house

Dr.P.KIRANMAYEE.

LESSON-11

S P E C T R O S C O P Y

11.0 Objective

This lesson mainly deals with properties of electromagnetic spectrum, various spectroscopic techniques (UV-vis, Infrared and Mass spectrophotometry), their principle and applications.

11.1 Introduction

Spectroscopy deals with interaction of electromagnetic radiations with matter. Spectroscopic techniques now occupy a key position in biochemical studies and some of them are employed routinely and quite extensively. These techniques are extremely useful in characaterization of chemical compounds in a sample on the basis of their spectral properties. In the beginning, the spectroscopy was largely confined to use light (visible radiation) for quantitative estimation and in some cases even for identification of biomolecules. With new developments and refinements in instrumentation, the scope of spectroscopic techniques broadened tremendously and now, in addition to visible light, it encompasses examining the behaviour of chemical substances upon their irradiation with regions of electromagnetic radiations such as X-rays, γ-rays, UV-rays, infrared light, microwaves and even radio frequencies. The spectrophotometric methods offer a rapid and convenient means of qualitative identification and quantitative estimations of biomolecules even in relatively impure samples.

11.2 Electromagnetic radiation properties

The electromagnetic spectrum is composed of a continuum of waves with different properties (Fig. 11.1). Several regions of the electromagnetic spectrum are of importance in biochemical studies including X-rays (upto 7 nm), the ultraviolet light (UV, 180-340 nm), the visible (VIS, 340-800 nm), the infrared (IR, 1000-100,000 nm), and radio waves (NMR, 10^6-10^{10} nm).

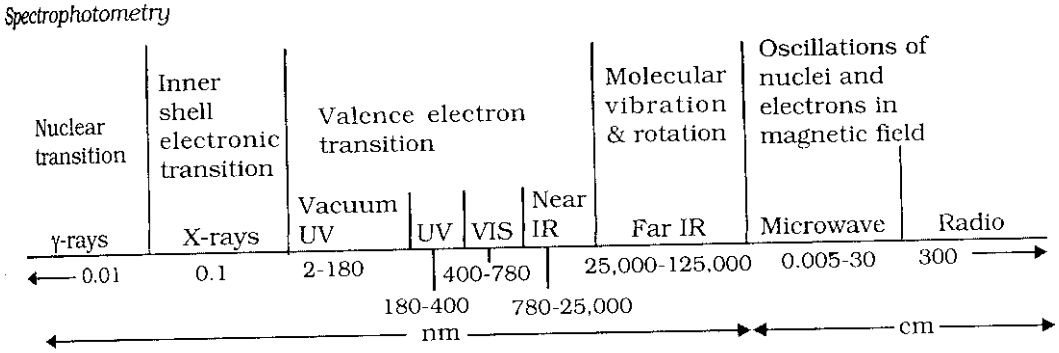


Fig. 11.1 The electromagnetic spectrum – the main regions and their wavelengths. Physical events involved in their production are also indicated.

The electromagnetic radiation (Fig. 11.2) is composed of an electrical field component E, and a magnetic field component H, which oscillate in planes at right angles to each other and both at right angles to the direction of propagation. The light propagated either in wave form or corpuscular (or photons or quantum) form. The wave length (λ) of light, defined by equation 11.1, is the distance between adjacent wave peaks as shown in Fig. 11.2. Wavelength may be measured in centimeters (cm), micrometers (μm), nanometers (nm), or angstrom units (A^o), where 1 nm = 10^{-3} μm = 10^{-6} mm = 10^{-7} cm = 10^{-9} m, and $1A^o = 10^{-8}$ cm. Sometimes a term, frequency, V, is used rather than wave length to describe a particular radiation. The number of waves passing through a fixed point on the time axis per second is known as the frequency, V, of the radiation, usually expressed in hertz (HZ).

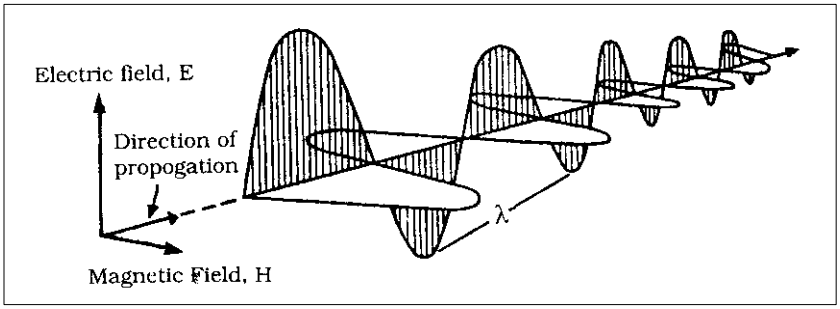


Fig. 11.2 The electromagnetic wave. The magnetic vector (unshaded) and electric vector (shaded) are perpendicular to each other and to the direction of propagation. Wavelength (λ) is the distance between two crests or troughs.

$$\lambda = \frac{C}{V} \quad \text{... Equation 11.1}$$

Where

λ = Wave length
V = Frequency
C = Velocity of the light, 3×10^8 m/sec.

Frequency shares an inverse relationship with the wavelength so that

$$V = {}^{C}/_{\lambda} \quad \text{… Equation 11.2}$$

Radiation in the infrared region is characterized by another term known as '**Wave number'** and denoted by the symbol $\bar{V}$. Wave number of light, defined by Equation 11.3 is the number of complete cycles occurring per centimeter.

$$\bar{V} = {}^{1}/_{\lambda} \quad \text{… Equation 11.3}$$

The amount of energy, E, associated with energetic particles (or photons) is given by Equation 11.4.

$$E = hv = h\,{}^{c}/_{\lambda} \quad \text{… Equation 11.4}$$

where h is plank's constant, c is the velocity of the light in vacuum. Wave length and frequency share an inverse relationship; this means that as the wave length increases, the energy of the radiation decreases, while the energy increases with the increase in frequency.

When a photon of specified energy interacts with a molecule (matter), one of two processes may occur. The photon may be scattered, or it may transfer its energy to the molecule, producing an excited state of the molecule. The former process, called **Rayleigh scattering**, occurs when a photon collides with a molecule and is diffracted or scattered with unchanged frequency. Light scattering is the physical basis of several experimental methods used to characterise macromolecules.

The other process mentioned above, the transfer of energy from a photon to a molecule, is **absorption**. For a photon to be absorbed, its energy must match the energy difference between two energy levels of the molecule. Molecules possess a set of quantized energy levels, as shown in Fig. 11.3. In Fig. 11.3, different electronic states are shown, a ground state, G, the first excited state, S_1, and second excited state, S_2. These states differ in the distribution of valence electrons. When electrons catapult from a ground state (G) orbital to an orbital of higher energy in S_1, an **electronic transition** occurs. The energy associated with UV and visible light is sufficient to promote the molecules from one electronic state to another, that is, to move electrons from one orbital to another. The atoms in molecules may vibrate and rotate about a bond axis, which gives rise to

vibrational and rotational sub-levels. This situation is shown diagrammatically in Fig. 11.3. The electronic transition for a molecule from G to S_1, has high probability of occurring if the energy of the photon corresponds to the energy necessary to promote an electron from energy level E_1 to energy level E_2:

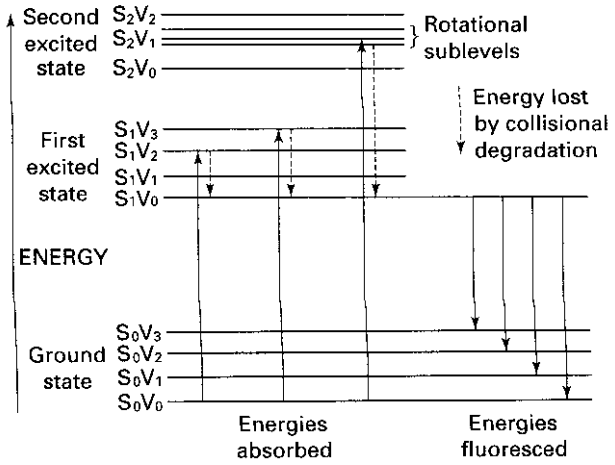


Fig. 11.3. Energy levels and transitions of electrons: in a fluorescent organic molecule. Note for clarity, rotational sublevels have been indicated only for vibrational sublevel S_2V_1.

Where $\Delta E = E_2 - E_1 = hv$,

because of $V = {}^{c}/_{\lambda}$ $\quad \therefore \Delta E = \dfrac{hc}{\lambda}$ … Equation 11.5

Where ΔE is the change in energy state of the electron or the energy of electromagnetic radiation absorbed or emitted by an atom or molecule.

E_1 = energy of electron in original state (G)
E_2 = energy of electron in final state (S)

11.3 The laws of absorption

The absorption of light by any absorbing material is governed by two laws. The first of these laws is known as Bouger-Lambert Law. It 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. It is an exponential function and may be expressed as

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

I = the Intensity of the transmitted light,
Io = the intensity of the incident light,
b = path-length (the absorbing thickness)

and K = the linear absorption coefficient of the absorbing material. The power term in the above relationship can be removed by converting to the logarithmic form. Thus,

$$\text{In}\ \frac{I}{Io} = -Kb, \quad \text{or} \quad \text{In}\ \frac{Io}{I} = Kb$$

changing to common logarithms we get,

$$2.303 \log_{10} \frac{Io}{I} = Kb \qquad \ldots \text{Equation 11.6}$$

The second law of absorption is known as the **Beer's law**. This law states that the amount of light absorbed by a substance is proportional to the number of absorbing molecules i.e., the concentration of absorbing solution. This can be mathematically expressed in the form of an equation similar to the one above.

$$2.303\ \text{Log}_{10}\ \frac{Io}{I} = K'C \qquad \ldots \text{Equation 11.7}$$

Where K' = absorptivity constant, and

C = the concentration of the absorbing material

We can combine the Equations 11.6 and 11.7 for the **Bouger–Lambert Law** and the **Beer's Law**. Here, K and K' merge to become a single constant **a**. The combined equation is

$$\text{Log}_{10}\ \frac{Io}{I} = abc \qquad \ldots \text{Equation 11.8}$$

Equation 11.8 has been alternately referred to as the Beer-Lambert Law, **the Bouger-Beer Law**, or more simply, Beer's-Law. This combined law states that "the amount of light absorbed (absorbance or extinction) is proportional to the concentration of the absorbing substance and to the thickness of the absorbing material (path length). The ratio, $^{I}/_{Io}$, of the intensities of transmitted and incident light gives the transmittance, T (the amount of light which escapes absorption and is transmitted). The reverse, $^{Io}/_{I}$ is known as the absorbance (A) or the optical density (O.D).

Absorbance shares a linear relationship with sample concentration. On the other hand, the relationship between transmittance and sample concentration is a non-linear one. It is therefore easier to use absorbance as an index of sample concentration. The two terms are mathematically commutable and so one can be calculated from the other. For this purpose, we rewrite the equation so that

$$A \text{ (absorbance)} = \log I_o - \log I$$

but I_o is always set at 100% and $\log 100 = 2$, so

$$A = 2 - \log I \qquad \ldots \text{ Equation 11.9}$$

11.4 Ultraviolet – visible absorption Spectrophotometry

Electron transitions in atoms or molecules (Fig. 11.3) give rise to the electronic spectra generally observed as absorption, emission or fluorescence phenomena in the ultraviolet and visible regions of the electromagnetic spectrum. UV and visible regions of the electromagnetic spectrum and associated techniques are probably the most widely used for routine analytical work and research in biological problems.

The instruments that are used to study the absorption or emission of electromagnetic radiation as function of wavelength are called **Spectrometers** or **Spectrophotometers** (colorimeters, if the instrument applies wavelengths only in the visible range).

The spectrophotometer is used to measure absorbance experimentally. This instrument produces light of a preselected wavelength, directs it through the sample, and measures the intensity of light transmitted by the sample. More or less similar optical principles are employed in these instruments. There are, however, some important differences in the specific components used in the various regions of the spectrum.

The essential components of a spectrophotometer include: (i) light source, (ii) a monochromator (including various filters, slits and mirrors), (iii) a sample chamber (cuvettes) and (iv) detector and an associated read out system (meter or recorder). All spectrophotometers represent variations of the block diagram shown in Figure 11.4.

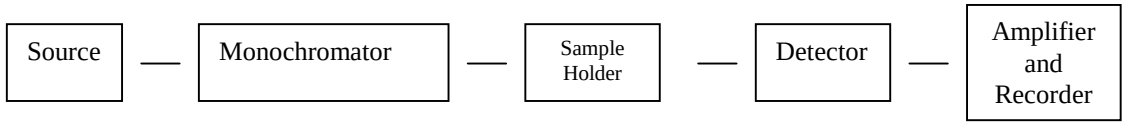


Fig. 11.4. Block diagram of a Spectrophotometer

11.4.1 Light source

(a) Sources of ultraviolet light: For absorption measurements in the ultraviolet region, a high pressure hydrogen or deuterium lamp is used. These lamps produce radiation in the range of 200 to 340 nm. Xenon lamp may also be used for ultraviolet radiation, but the radiation produced is not as stable as the hydrogen lamp.

Sources of visible light: The light source for the visible region is the tungsten halogen lamp. It emits the radiation with a wavelength range of 350-800 nm. Carbon arc, which provides more intense visible radiation is used in a small number of commercially available instruments.

11.4.2 Wave length selectors

All the sources discussed above produce continuous radiation over wide range of wavelengths. However, the laws of absorption in the strict sense apply only to monochromator radiation. Thus, absorption of narrow band width will show greater adherence to Beer's law. Narrow band widths are made possible by using wavelength selector(s). Wavelength selectors are of two types namely filters and monochromators.

(a) Filters: Filters operate by absorbing light in all other regions except certain limited range of wavelengths, which they reflect. This limited range is known as the band width of the filter. Gelatin filters are made of a layer of gelatin, coloured with organic dyes and sealed between glass plates. Most modern filter instruments use tinted-glass filters. Filters resolve polychromatic light into a relatively wide band-width of about 40 nm and are used only in colorimeters. The disadvantage of glass filters is their low transmittance of 5-20%.

(b) Monochromators (Fig. 11.5): The name itself indicates a monochromator resolves polychromatic light into its individual wavelengths and isolates these wavelengths into very narrow bands. The essential components of a monochromator are: (a) an entrance slit which allows polychromatic light from the source, (b) Lens or mirror which collimates the polychromatic light onto the dispersion device, (c) a wavelength resolving device like a prism or a grating which breaks the radiation into component wavelengths, (d) a focussing lens or mirror, and (e) an exit slit which allows the monochromatic beam to escape. A monochromator employing a prism for dispersion. The effective band width of the light emerging from the monochromator depends mostly upon the dispersing element (prism or a diffraction grating) and the slit widths of both the entrance and the exit slits. Narrow slit widths isolate narrow bands. However, the slit width also limits the radiant power which reaches the detector. Prisms or diffraction gratings are the effective resolving units.

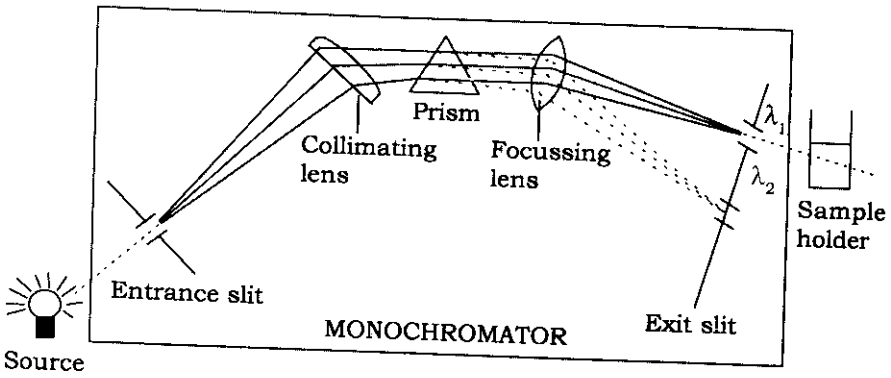


Fig. 11.5 Prism monochromator

(c) Prism: A prism disperses polychromatic light from the source into its component wavelengths by its ability to refract different wavelengths to a different extent. Because of prism disperses the short wavelengths more and long wave lengths less, the wavelengths at the red end of the spectrum are not fully resolved. This is a major disadvantage of a prism. Two types of prisms, namely 60^o cornu quartz prism and 30^o Littron prism are usually employed in commercial instruments. Simple glass prisms are used for visible range. For ultraviolet region silica, fused silica or quartz prisms are used. Flourite is used in vacuum ultraviolet range.

(d) Gratings: Gratings (Fig. 11.6) are often used in the monochromators of spectrophotometers operating in UV, and visible regions. The grating possesses a highly aluminized surface etched with a large number of parallel grooves which are equally spaced. These grooves are also known as lines. The principle behind dispersion of radiation by grating is that it resolves light into its component wavelength by virtue of constructive reinforcement and destructive interference of radiation reflected. Very often, the monochromator consists of both, a prism and a grating. The prism placed before the grating is known as the fore prism. It preselects a portion of the spectrum which is then allowed to be diffracted by the grating. The major advantage of diffraction grating monochromators is that their resolving power is far superior to that of prisms. In addition, they yield a linear resolution of spectrum which is not possible when prisms are used.

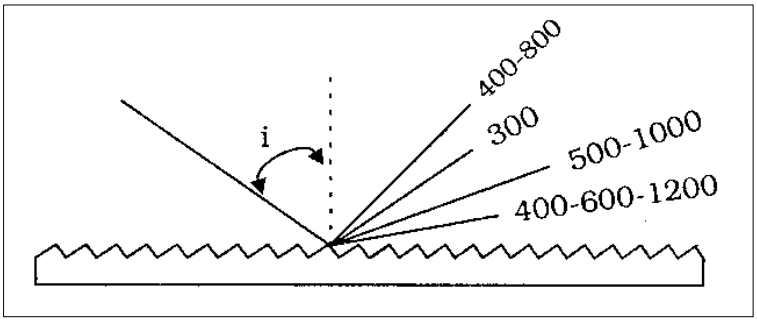


Fig. 11.6 Diffraction grating and the dispersion of polychromatic radiation

11.4.3 Sample chambers

Samples to be analysed in the UV or visible region are usually solutions or gases and are put in cells known as **Cuvettes**. Most of the spectrophotometric studies are made in solution. Cuvettes meant for visible region are made up of either ordinary glass or some times quartz. Since glass absorbs in the UV region, quartz or fused silica cells are used in this region. Standard path length of these cuvettes is usually 1 cm. The surface of the cuvettes must be kept clean; finger print smudges and traces of previous samples, by causing interference in the optical path, might cause serious errors in quantitative measurements. The most important factor in choosing the solvent is that the solvent should not absorb (optically transparent) in the same region as the solute. Oftenly used solvents in the UV and visible regions are water, methyl-, ethyl-, isopropyl-alcohols, chloroform, hexane etc.

11.4.4 Detection Devices

The intensity of the light that passes through the sample under study depends on the amount of light absorbed by the sample. In the UV and visible regions there are three basic kinds of light sensitive detectors involved in the measurement of intensity of the light that passes through the sample. They are photocells, phototubes and photomultiplier tubes (PMT).

(a) Photovoltaic or barrier layer cells: It employs semi-conductor materials. Although a number of materials are used in photocells (cadmium sulphide, silicon, selenium) selenium based photocells are most common. A typical photocell consists of a thin coating of selenium over a thin transparent silver film on a steel base. This arrangement ensures that electrons pass easily from selenium to silver but not in the reverse direction. Due to the inability of electrons to move away from the silver film, the silver acts as the collecting electrode for electrons liberated from selenium by the incident radiation. The steel plate functions as the other elecrode. The current flowing between the two electrodes is then measured by a microammeter. Fig. 11.7 showed selenium based photovoltaic cell. Photocells have a long life and are inexpensive and reliable. They are widely used in colorimeters but their use in spectrophotometers is becoming limited.

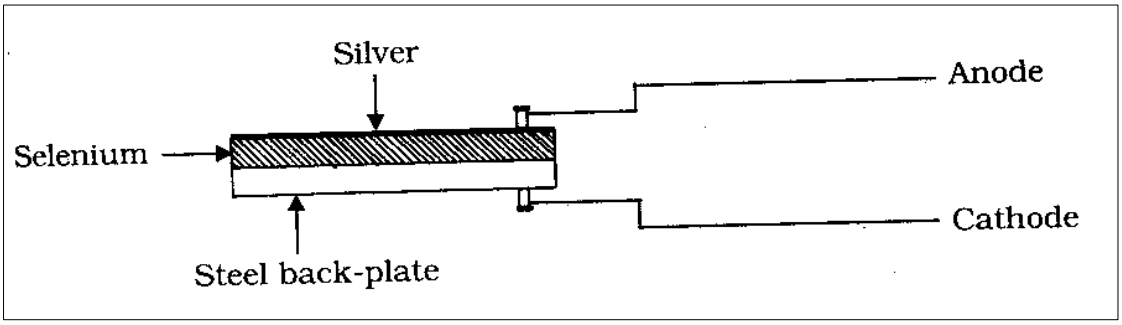


Fig. 11.7 Selenium based photo voltaic cell

(b) Phototubes or photoemissive tubes: The essential components of phototube include (a) an evacuated glass envelope (with a quartz window), (b) a semicylindrical cathode, and (c) a centrally located metal wire anode. The quartz window allows the passage of radiation which strikes the photoemissive surface of the cathode. The energy of the photon is transferred to the loosely bound electrons of the cathode 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. Phototube and its circuit is shown in Fig. 11.8. Phototube currents are small and require amplification.

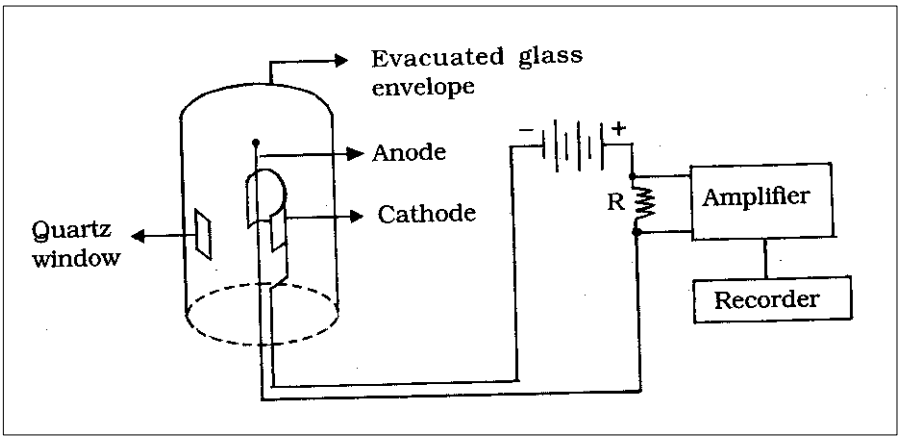


Fig. 11.8 Diagram of a photoemissive tube; R stands for resistance

(c) Photomultiplier tubes (PMT) (Fig. 11.9): These detectors are designed to amplify the initial photoelectric effect and are suitable for use at very low light intensities. A PMT consists of (a) an evacuated glass tube which contains the anode and cathode, and (b) Dynodes. High voltage (1000 volts) difference exists between anode and cathode. As the radiation strikes the photocathode, electrons are liberated and the applied potential difference accelerates the electrons towards the first dynode. Each successive dynode is at a higher electrical potential and thus acts as an amplification stage for the original photon. The applied voltage causes sufficient electron acceleration to knock out other electrons from each dynode surface. The liberated electrons are dragged on to the next dynode. By the time electrons arrive at the collecting anode, the initial photo electric current is amplified by a factor of approximately 10^6. In practice, PMT's are used only for low light intensities. At higher light intensities photomultipliers exhibit great instability. Photomultipliers are widely used in all modern spectrophotomers.

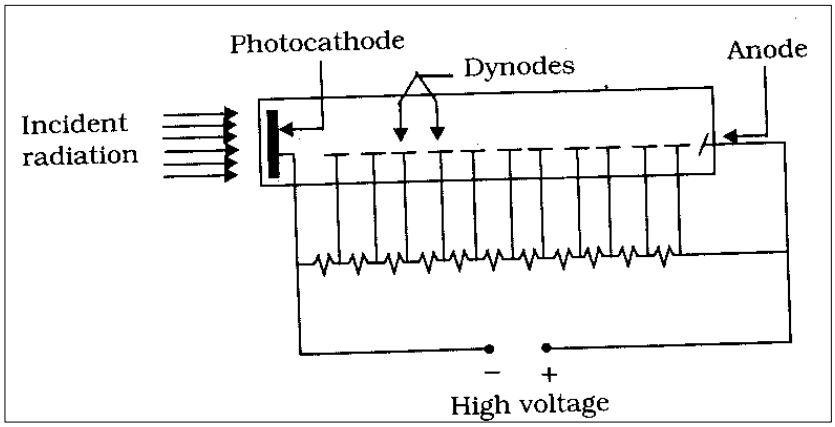


Fig . 11.9 A photomultiplier tube

A new technology has been introduced during the past few years that greatly increases the speed of spectrophotometric measurements. New detectors called photodiode arrays are being used in modern spectrometers; Photodiodes are composed of silicon crystals that are sensitive to light in the wavelength range of 170-1100 nm. Upon photon absorption by the diode, a current is generated in the photodiode that is proportional to the number of photons. Linear arrays of photodiodes are self-scanning and have response times on the order of 100 milliseconds; hence, an entire UV-VIS spectrum can be obtained with a brief exposure of the sample to polychromatic light.

11.4.5 Amplification and read out

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

Modern, research-grade spectrometers are completely under the control of a computer. Hence, by operating the button, one can obtain the UV-VIS spectrum of a sample displayed on a computer screen in less than 1 second. In addition, these modern instruments with computers used to carry out several functions, such as subtraction of solvent spectrum, spectral overlay, storage, differentiate spectra, derivative spectra and calculation of concentrations and rate constants. Components of UV-visible spectrophotometers have been summarized in Table 11.1.

Table 11.1 A summary of components of spectrophotometers and colorimeters

	Region of electromagnetic spectrum	
	Ultraviolet	**Visible**
Radiation source	Hydrogen or deuterium lamp	Tungsten filament lamp, carbon arc (less used)
Optical system	Prism or diffraction grating, or a foreprism grating double monochromator	Tinted glass filters or interference filters
Material used in the Optical system	Quartz or fused silica	Glass
Sample holders	Quartz or fused silica, rectangular cells	Round glass cells
Detector	Photomultiplier	Photovoltaic cell

11.5 Double beam operation

Voltage fluctuations inducing fluctuations in the source intensity can cause large scale errors in spectrophotometer operation. To overcome this situation, double beam spectrophotometers have been designed (Fig. 11.10). In the double beam device, the source beam is split in to two parts prior to the sample container and after the monochromator, one of the split beam passing through the blank, or reference cell, at the same time, the other passes through the sample. This approach obviates any problems of variation in light intensity as both reference and sample would be affected equally. The resultant measured absorbance is the difference between the two transmitted beams of light recorded by the matched detectors. Double beam devices are more sophisticated mechanically and electronically as compared to the single beam devices, and are also more expensive.

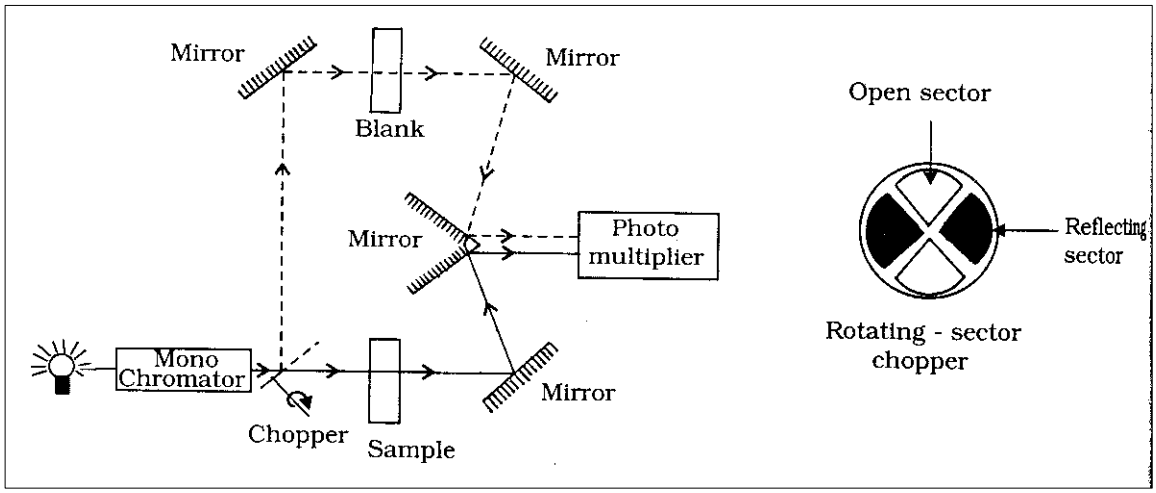


Fig. 11.10 Optical arrangements of a Double beam instrument.
A rotating sector chapter is also shown.

11.6 Dual wavelength Spectrophotometer

Dual wavelength spectrophotometry refers to the photometric measurement of substance by passing radiation of two different wavelengths through the sample before reaching the detector. Light from two different sources is allowed to be resolved into two different wavelengths are made to pass through the sample by a complex arrangement of a large number of mirrors (Fig. 11.11). In this device, only a single detector is used. It is always photomultiplier tube.

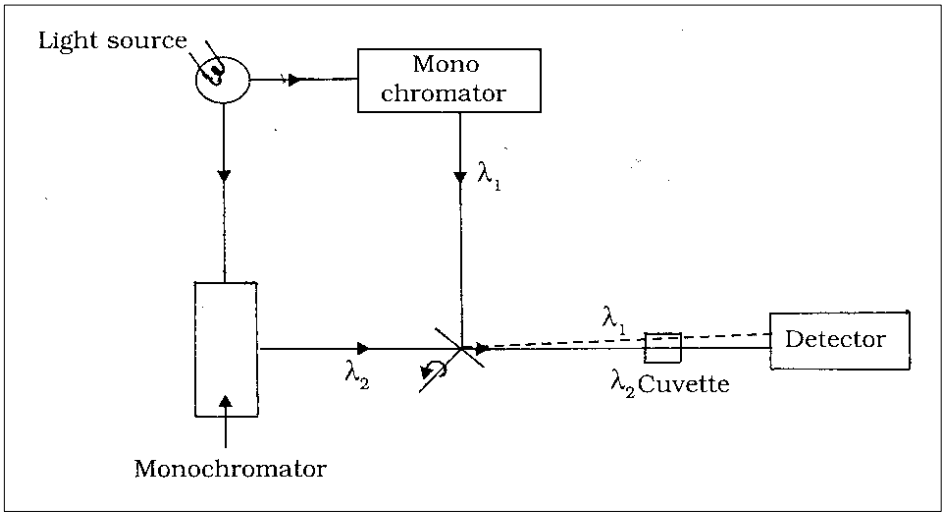


Fig. 11.11 Optical arrangements in a dual wavelength spectrophotometer

11.7 Applications of UV-vis Spectrophotometer

Although many different types of operations carried out on UV-vis spectrophotometer all applications fall in one of two categories:

(a) Measurement of absorbance at a fixed wave length.

(b) Measurement of absorbance as a function of wavelength. Measurements at a fixed wavelength are most often used to obtain quantitative information. Absorbance measurements as a function of wavelength provide qualitative information.

11.7.1 Qualitative analysis

(a) UV and visible spectra may be used to identity certain classes of compounds both in pure state and in biological mixtures. This is usually done by plotting absorbing spectrum curves. Since these curves specific for a class of compounds, a knowledge of the absorption spectrum can help in identification of a substance in biological mixtures, i.e., proteins, nucleic acids, cytochromes and chlorophylls.

(b) The UV-vis spectrum of biomolecules reveals much about its molecular structure e.g., Nucleotides, pigments and proteins.

11.7.2 Quantitative analysis

(a) UV and VIS spectrophotometers are useful in determining the unknown concentration of given species. **Eg:** Nucleic acids, proteins, pigments, carbohydrates.

(b) Quantitative assay of enzyme activity. **Eg:** Lactate dehydrogenase, pyruvate kinase.

(c) The molecular weight (M) of the compound can be calculated on the basis of its absorption data.

$$M = awb/OD$$

Where w = is weight of the compound in grams per litre, and
b = is the path length
a = extinction coefficient

Molecular weights of amine picrates, sugars and many aldehyde and ketone compounds have been determined by this method.

(d) Spectrophotometry is one of the best methods available for measuring the rates of biochemical reactions.

11.7.3 Study of Cis-Trans isomerism

UV-visible spectrophotometry useful in differentiate the cis- trans isomers of compound.

11.7.4 Other physicochemical studies

UV-vis Spectrophotometry has been used to study such physicochemical phenomena as heats of formation of molecular addition compounds and complexes in solution, determination of empirical formulas, formation of constants of complexes in solution, hydration equilibria of carbonyl compounds, association constants of weak acids and bases in organic solvents, tautomeric equilibria involving acid base systems, protein-dye interactions, chlorophyll-protein complexes, vitamin A aldehyde-protein complex, determination of kinetics biochemical reactions, association of cyanide dyes, determination of labile intermediates and dissociation constants of acids and bases.

11.7.5. Control of purification

This is one of the most important uses of UV-VIS spectrophotometry e.g., detection of carbon disulphide impurity in carbon tetrachloride.

11.7.6 Difference spectroscopy

It provides a sensitive technique for detecting small changes in the environment of a chromophore. It may also be used to demonstrate ionization of a chromophore leading to identification and quantification of various components in a mixture. It is also useful in compare the absorption spectra of two samples which differ only slightly in their physical states. This technique provides information in regarding the sequence of electron transport proteins. Difference spectroscopy has also been utilized in toxicology laboratories for analysis of many toxic drugs.

11.7.7 Turbidmetry

Turbidmetry oftenly used to measure the number of bacterial cells in a given suspension.

11.7.8 Nephlometry

Nephlometry is commonly used for waste water analysis as well as to estimate the concentration of microorganisms. It is also useful in quantifying haze present in the preparations of beverages and pharmaceuticals.

11.8 Infrared Spectroscopy

It is evident from the electromagnetic spectrum, infrared radiation is of much higher wavelength as compared to the ultraviolet and the visible region. Consequently, electromagnetic radiation of this region has considerably lower energy. Infrared radiation is not associated with electronic transitions; rather, it is associated with vibrational transitions of molecules. Vibrational transitions represent changes in the stretching and bending of covalent bonds. Vibrational transitions are low energy transitions. Infrared region, of the electromagnetic spectrum, is characterized by the wave number ($\bar{V}$). Infrared spectra are typically presented as per cent transmission (transmittance x 100) versus wave number.

11.8.1 Vibrational Frequencies

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

$$V \alpha \sqrt{\frac{\text{bond strength}}{\text{mass}}} \quad \text{or} \quad V = \frac{1}{2\pi} \sqrt{\frac{K}{m_1 . m_2 (m_1+m_2)}} \qquad \ldots \text{Equation 11.6}$$

Where V is the frequency, K is the force constant of the bond and m_1 and m_2 are the masses of the two atoms involved in bond formation. The quantity $m_1 m_2 / (m_1 + m_2)$ is often expressed as m, the reduced mass of the system. Above equation 11.6 states that the vibrational frequency of a bond should increase with the strength of the bond and indirectly proportional to the mass of the atom.

11.8.2 Instrumentation

The essential components of device are, infrared radiation source, wavelength selector, sample container, detectors and meter or recorder.

11.8.2.1 Sources of Infrared radiation

Nernst Glower and Globar are the most important sources of infrared radiation. The globar consists of a silicon carbide rod which when heated to approximately $1200^{o}C$, emits radiation in the 1 to 40 μ region. Nernst Glower employs a hollow rod of zirconium and yttrium. It emits the radiation in the range of 0.4 – 20 μ and less stable than the globar.

11.8.2.2 Wavelength selectors

(a) Prism: Ionic crystalline materials are used in the infrared region. Some examples are NaCl, KBr, CSBr and the mixed crystalline material called KRS-5.

11.8.2.3 Sample containers

Sample containers are the glass cells. These glass tubes possess NaCl, KBr or CaF_2 windows for the passage of infrared radiation. The cells have varying path length; from few centimeters to several meters. Liquids are studied as thin films or solutions between NaCl, KBr or CaF_2 plates. Samples of solids are either prepared in mulls such as nujol and held as layers between salt plates such as NaCl or pressed into KBr discs.

11.8.2.4 Detection Devices

(a) Near infrared detectors: are usually photo inductive cells which detect infrared radiation in the range of 0.8 – 3.0 μ. The sensing element is a semi-conductor made up of germanium, lead sulphide or lead telluride. Upon illumination with radiation of appropriate wavelength, the electrons of the semi-conductors are raised to conduction bands. This causes a drop in electrical resistance. Consequently, a large increase in current can be noted. The resistance of the system is such that the current may be amplified and finally indicated on a meter or recorded.

(b) Middle and far infrared detectors: Thermocouples, holometers and gas thermometers (pneumatic or Golay cells) are used as detectors in this region. Thermocouples used in the infrared receivers typically consist of blackened gold lead-tellurium metal pin junction which develops a voltage that is temperature dependent. Thermocouple prevents temperature fluctuations by avoiding heat loss.

11.8.2.5 Amplification and Readout

Radiation detectors generate electronic signals which are proportional to the transmitted light. These signals need to be translated into a form that is easy to interpret. This is accomplished by using amplifiers, ammeters, potentiometers, and potentiometric recorders. Components of infrared spectrophotometers have been summarized in Table 11.2.

Table 11.2 A summary of the components of infrared spectrophotometers

	Region of electromagnetic spectrum		
	Near-Infrared	**Mid-Infrared**	**Far-Infrared**
Wave number (cm^{-1})	12,500 ←→	4000 ←→ 200	←→ 10
Source of radiation	Tungsten filament lamp	Coil of Nichrome wire, or Nernst Glower, or Globar	High pressure mercury are lamp
Optical system	Quartz prisms or prism grating double monochromator	Diffraction gratings with a fore-prism monochromator	diffraction gratings
	Optical elements are made up of ionic crystalline materials like NaCl, KBr, CsBr, or KRS-5.		
Detector	Lead sulphide, or lead telluride photo-conductive cells	Thermocouple, thermistor, or pyroelectric	Pneumatic or Golay cells, or bolometers

11.8.3 Applications

Infrared spectroscopy used mainly in biochemical research for intermediate-sized molecules such as drugs, metabolic intermediates and substrates.

(a) **Identification of compounds:** IR spectroscopy involves in identification of macromolecules possess a large number of atoms and as such may have numerous fundamental vibrations. **Eg**. Penicillin and its derivatives, small peptides and environmental pollutants.

(b) **Rate of reactions:** Infrared spectroscopy provides the rate of reactions by measuring the rates of appearance or disappearance of stretching vibrations.

(c) **Study of conformation:** IR spectroscopy is of considerable use in studies of protein conformation. Macromolecular conformation obtained by using the infrared linear dichorism phenomenon.

(d) **Interaction between molecules:** Hydrogen bonds present in the polypeptide chains and in nucleic acids, molecular interactions such as protein-ligand binding enzyme-co-enzyme interactions studied by using IR spectroscopy.

(e) It is an ideal and rapid technique for measuring certain contaminants in foodstuffs and it is also frequently used for the analysis of drug metabolites.

11.8.4 Disadvantages of Infrared spectroscopy

It suffers from two major disadvantages: (a) The problem is that the data obtained in related to any molecule or situation in CCl_4, D_2O, Chloroform solvents may not necessarily be true for aqueous solution, because of water also absorbs IR radiation intensely. (b) It is very difficult to obtain quantitative data of biomolecules with infrared spectrophotometry.

11.9 Mass Spectroscopy

Mass Spectroscopy is an extremely useful technique as it can provide fairly detailed information about the structure of the molecules with very small amounts of the substance (10^{-6} to 10^{-9} g).

11.9.1 Principle of Mass spectrometer

This technique essentially involves ionization of the parent molecule to give parent molecular ion and fragmentation of these ions to give fragment ions. This is achieved by bomboarding the sample with a beam of electrons. All the ions produced generally have positive charges (rarely negative charges). These ions, which possess a certain amount of kinetic energy, if subjected to a magnetic field will be deflected to different degrees depending upon their mass/charge (m/z) values. This is the basis of separation by mass spectrometry. If the charge is kept constant, the ions are deflected by angles which are inversely proportional to the square roots of the masses of the ions.

All mass spectrometers (Fig. 11.12) are essentially composed of three parts:

(i) ionization chamber or source
(ii) a mass analyser
(iii) a detector

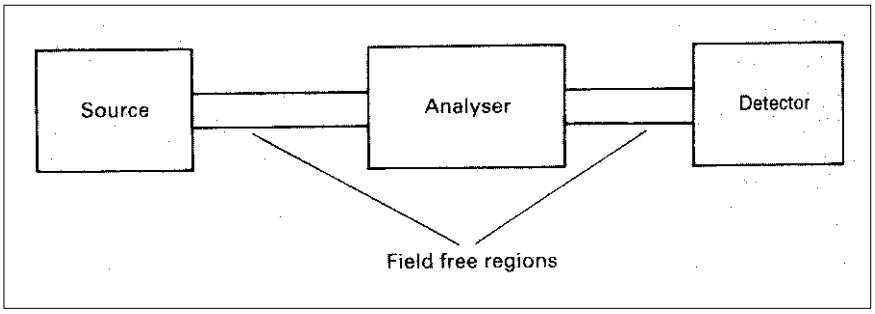


Fig. 11.12 Diagram of a mass spectrometer. The whole system is kept under high vacuum of the order of 10^{-5} torr.

11.9.2 Ionisation techniques

Several ways are involved in the production of ions in a mass spectrometer. They are (a) Electron Impact ionisation (EI), (b) Chemical ionisation, (c) Field ionisation, (d) Field desorption ionisation, (e) Fast atom bombardment ionisation (FAB), (f) Plasma desorption ionisation, (g) Laser desorption ionisation, (h) Thermospray ionisation source, (i) Electrospray and ionspray ionisation, whichever ionistion method is chosen a successful ionisation process requires, the energy is put in to the material under investigation. This turns out to be extremely important, because the amount of energy input and the way it is applied dramatically affect the thermodynamic conditions and ultimately the type of mass spectrum produced. It also indicates that there is no universal ionisation source and the type used is determined by the information required from the experiment.

11.9.2.1 Electron impact ionisation (EI)

Source: Of all the ionisation processes EI is probably widely used ionisation source in mass spectrometry. In many biological problems involving metabolic studies, drug studies, pollutants, etc., it would probably be the method of choice. Many metals, when heated to a sufficiently high temperature (approx. 2000 K) lose electrons by diffusion from their surface. This arises from the structural nature of metals and two that are particularly useful are rhenium and tungsten because they can be readily drawn into thin filaments. Tungsten filaments are sometimes coated with thoria (thorium oxide) to increase the ease with which electrons are emitted. If such heated filaments are subjected to an appropriate potential gradient then electrons are removed from the surface more rapidly and gain an energy directly related to the potential applied. These electrons stream across an evacuated chamber into which molecules of the substance to be analysed are allowed to diffuse. In the electron impact source the substance to be analysed must be in the vapour state, which obviously limits the applicability to biological materials, although it should be remembered that most substances may have their natural volatility increased by chemical modification.

The stream of electrons from the filament interacts with the molecules of the substance to be analysed (which are neutral and in random thermal motion). This interaction results in either loss of an electron from the substance (to produce a cation) or electron capture (to produce an anion). As the positive ionisation potential of most organic molecules is of the order of 20 eV, there is ample excess energy in the beam of bombarding electrons. The electron impact source is shown diagrammatically in Fig. 11.13.

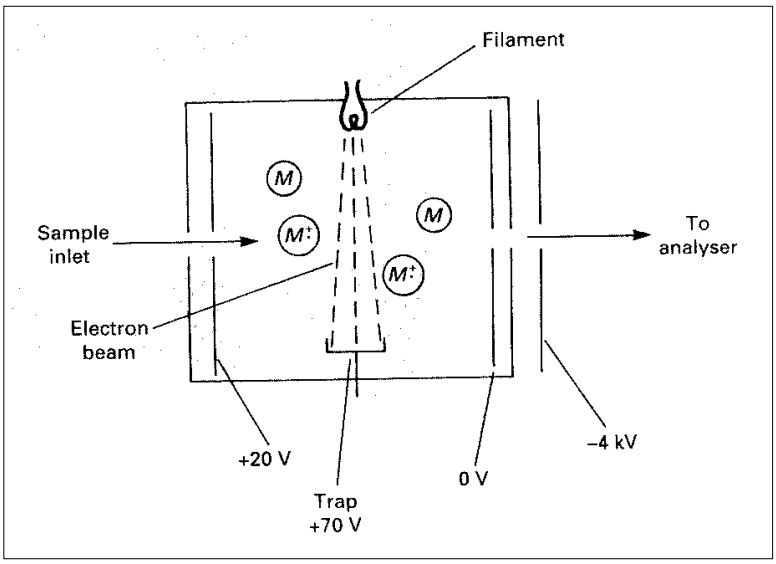


Fig. 11.13 Electron impact ionisation source

The possible events which may occur are shown below: M represents the neutral molecular species:

$M + e^{--}$ (bombarding electron) $\rightarrow$ $M^{+} - 2e^{--}$

(one bombarding electron and one electron removed from M)

$M + e^{--} \rightarrow M^{--}$

Chemical bonds in organic molecules are formed by the pairing of electrons. Ionisation resulting in a cation requires loss of an electron from one of these bonds effectively knocked out by the bombarding electrons), but it leaves a bond with a single unpaired electron. This is a radical as well as being a cation and hence the representation as M^{+}. , the plus sign (+) indicating the ionic state and the dot (.) a radical. Conversely, electron capture results both in an anion but also the addition of an unpaired electron and therefore a negatively charged radical, hence the symbol $M^{-\cdot}$. Such radical ions are termed molecular ions or parent ions and under the conditions of electron bombardment are relatively unstable. The imparted energy in excess of that required for ionisation has to be dissipated and this latter process results in the parent ion disintegrating into a number of smaller fragments. Also the fragments themselves may be relatively unstable and further fragmentation may occur. This gives rise to a series of daughter ions which are eventually recorded as the mass spectrum.

11.9.3 Analysers

In the simplest mass spectrophotometers, a single mass analyser is used that involves either magnetic or electric fields. The processes involved in separating entities of different masses are different in each of the cases (magnetic or electric fields). An alternative approach is to measure accurately the times taken for entities of different masses to travel a given distance in a space in the so-called “time-off flight” mass spectrometer (TOF). Analysers are of obvious importance in mass spectrometer.

(a) Magnetic sector analyser: The term magnetic sector arises because the beam trajectery traverses only a sector of the circular poles of the magnet. In this analyser the mixture of ions, precursor and product are separated according to m/z (mass/charge) and the mass spectrum produced. This design results in low resolution mass spectrometry using single sector or single focussing. This analyser is most widely used in mass spectra.

(b) Electric sector analyser: Ions emerge from the source with varying terminal velocities and hence varying momenta and kinetic energies ($\frac{1}{2}$ mv^2). In order to over come this variation it is necessary to energy analyse the emergent ion beam. This is achieved in the electric sector analyser, which consists of two stainless steel plates bent into segments of concentric circles. The ions follow a circular trajectory between these plates, whose radius Re is given by $Re = 2\ V/E$

Where V is the accelerating voltage (in the source), and

E is the electrostatic field in the analyser.

The electric sector is usually referred to as the electrostatic analyser (ESA) and packets of ions emerge form this with the whole range of masses but the same velocity. A given packet with the appropriate velocity then enters the magnetic sector analyser to undergo mass analysis. The ESA is solely an energy and not a mass analyser. This type of instrument is the two sector or double focussing device, which enables high resoltuion mass spectrometry to be performed.

(c) Quadrupole mass filters (Fig. 11.14): This type of mass analyser is known as a mass filter. It is smaller and cheaper device than sector system and is much lighter in weight, hence it is wide use in bench top type instruments. The disadvantages are the lower mass range and sensitivity. The device generally consisting of 4 solid cylindrical rods of circular-cross section, to which are applied both direct current (DC) and radio frequency (RF) voltages. Both the fixed (DC) and oscillating (RF) fields cause the ions undergo complicated motion in the X-Y plane (cross section). This, together with the component of motion in the Z direction, results in the ions following complicated trajectories through the quadrupole filter. For a given set of field conditions, only a certain trajectories are stable, allowing ions of specific mass to be transmitted through to the collector or detector.

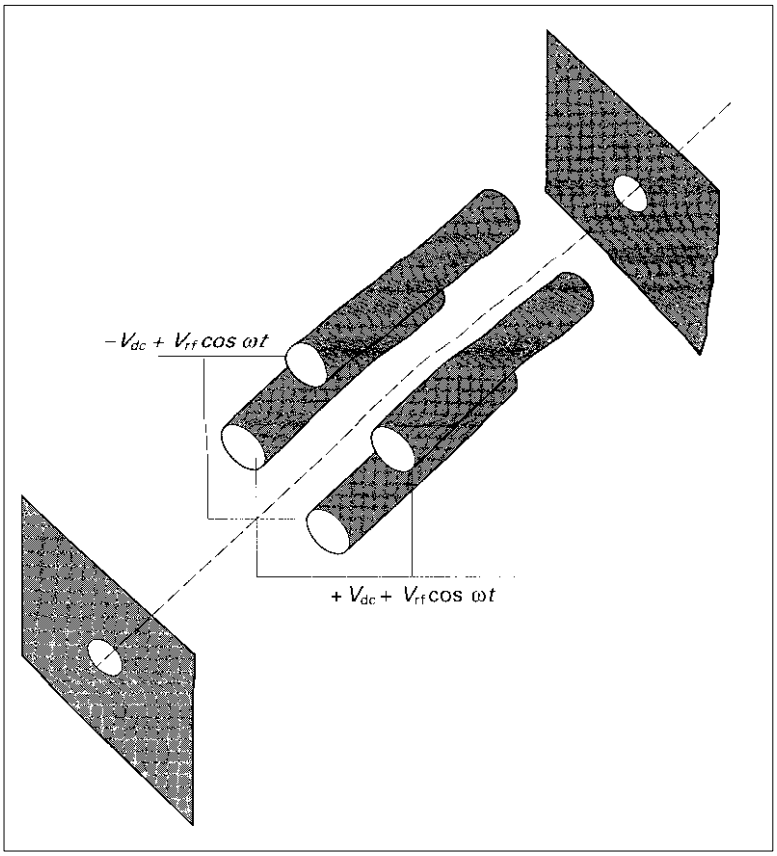


Fig. 11.14 Quadrupole mass filter. In the quadrupole mass filter one opposite pair of rods has a negative DC voltage, $-V_{dc}$, applied and the other pair a positive DC voltage, $+V_{dc}$.

11.9.4 Detectors

Most detectors are of the impact or ion collection type. All types of detectors require a surface on which the ions impinge and the charge is neutralized either by collection or donation of electrons. Hence, electron transfer occurs and an electric current flows that may be amplified and ultimately converted into a signal recorded on a chart or processed by a computer. The total ion current (TIC) is the sum of all the current carried by all the ions.

(a) The Faraday cup: This is probably the simplest device and shown in (Fig. 11.15). For example, a positive ion striking the surface of the cup abstracts an electron to neutralise its charge and if enough ions strike then a measurable current flows.

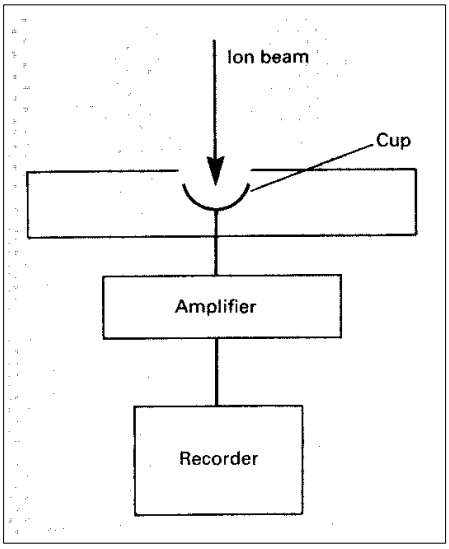


Fig. 11.15 Simplified diagram of the Faraday cup

(b) Electron multiplier: Greater sensitivity can be obtained with this detector and the degree of amplification is large. The original ions cause a shower of new electrons to be produced. These electrons impinge on a second dynode and produce more electrons. This process continues until a sufficiently large current for normal application is obtained. Modified photomultipliers may also be used, the advantage being that they are cheaper and more robust than electron multipliers.

(c) Array detectors: These types of detector are really many detectors arranged in an array or matrix, commonly having 1024 sites for ion collection. Most designs involve the use of microchannel plates (electron producing plates) as the ion-to-electron converter. Also most array detectors are electrooptical devices in which the electrons strike a suitable surface and produce photons that can be focussed and transmitted. Finally, the photons strike a photosensitive plate and an electric current is produced.

11.9.5 Types of the Mass Spectrometers

Mass spectrometers are of different types. Different mass spectra especially differ in their ionisation processes. Some of the important mass spectra are:

(a) Pyrolysis mass spectrometry: The principle is simple in that materials are subjected to a precisely controlled high temperature for a fixed and measured time span. Volatile substances are ejected from the material, under vacuum conditions and can then be ionised by EI, the mass spectrometric analysis being conducted in any of the usual ways.

Applications:

(i) A major application is the identification of microorganisms carried out on swab samples. This reduces the need to culture the cells by this way minimize the delays involved.

(ii) This is also used in the study of effects of antibiotics and other growth conditions on the structure and composition of cell walls of microbes.

(iii) Because of portable in size it is gaining in importance in field studies.

(iv) This technique is also useful in environmental science, and ecological studies.

(b) Fast Atom Bombardment Mass spectroscopy (FAB-MS): This involves fast atom bombardment ionisation process. FAB-MS has been applied to study the structure of several different kinds of biological macromolecules including proteins, peptides, nucleic acids and lipids.

(c) Tandem-Mass spectrometry: It is also increasingly used to obtain sequence data of biomolecules.

11.9.6 Applications of Mass spectrometry

Mass spectrometry has been widely used in biological field. The use of mass spectrometry in biological science has been mainly in the area of biochemistry.

(1) Determination of molecular masses of bio-molecules **e.g,** proteins, nucleic acids and cytochromes, pigments and short peptides.
(2) Elucidation of sequence of short peptides.
(3) Enumeration of microorganisms.
(4) Elucidate the structures of biomolecules, **eg.** proteins, carbohydrates etc.
(5) Widely used in diagnosis of diseases caused by micro-organisms.
(6) Widely used in the study of drugs and secondary metabolites.

11.10 Summary

Electromagnetic radiation is composed of both an electric vector and magnetic vector (which gives rise to the name), which oscillate in planes at right angles to each other and mutually at right angles to the direction of propagation. The interaction of electromagnetic radiation with matter (solutions or crystals of biomolecules) resulting processes like absorption and fluorescence (or emission). Both processes have led to the development of fundamental techniques like visible, UV and infraredspectrometry. UV-visible spectrophotometry is an important technique for molecular structure elucidation and quantification of compounds (biomolecules). Infrared-spectroscopy is widely used in biochemical research for intermediate sized molecules such as drugs, metabolic intermediates and substrates. In contrast to the spectroscopic techniques involves quantum principles, the mass spectrum is essentially dependent upon the thermodynamic stability of the ions produced and collected during a mass spectrometric experiments. Mass spectrometry is widely used to measurement of protein molecular weight and may also be used to sequence polypeptides of 25 residues or fewer.

11.10 Model Questions

1. What are the differences between the visible and UV spectrophotometry?
2. Explain the differences between a spectrophotometer that uses a phototube for a detector and one that uses a photodiode array detector.
3. Discuss and describe the principle and instrumentation for UV absorption spectrophotometry?
4. Why can you not use a glass cuvette for absorbance measurements in the UV spectra ranges?
5. Discuss the principle of mass spectrophotometry and its applications?

11.11 Reference Books

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LESSON-12

USE OF RADIOACTIVE SUBSTANCES IN BIOLOGY

12.0 Objective

Radioisotopes have an immense importance in the biological field. The use of radioactive isotopes in experimental biochemistry has provided us with a wealth of information of about biological processes. This lesson comprises the nature, properties, detection, measurement and applications of radioisotopes.

12.1 Introduction

The phenomenon of radioactivity was discovered by Henri Becquerel in 1896 and the radiations emitted were called ‘Becquerel rays’ or ‘Uranic rays’. Pierra and Marie Curie concluded that these uranic rays were due to atomic phenomenon, characteristic of the element and not related to its chemical and physical states. They introduced the term ‘Radioactivity’ for this phenomenon. Radioactivity results from the spontaneous nuclear disintegration of unstable isotopes. The stability of a nucleus depends on the ratio of neutrons to protons. Some nuclei are unstable and undergo spontaneous nuclear disintegration accompanied by emission of particles and/or electromagnetic radiation. Unstable isotopes of this type are called Radioisotopes.

12.2 Radioactive decay

The emission of radiation from radio active compound is known as radioactive decay. A radioactive compound may decay by any one or more of the several ways described below:

(a) α-Decay: Emission of α-particles results in a decrease in atomic number of two and a decrease in mass number of four. Isotopes that decay by α-emission are rarely encountered in biological work. Decay of ^{238}U is cited as an example:

$$^{238}_{92}U \longrightarrow {}^{234}_{90}Th + {}^{4}_{2}He^{-}$$

(b) Decay by Negatron emission (β-Decay)

In this case, a neutron is converted to a proton by the ejection of a negatively charged beta (β) particle called a negatron (β^{--}):

neutron → proton + negatron

An isotope frequently used in biological work that decays by negatron emission is ^{14}C.

$$^{14}_{6}C \longrightarrow {}^{14}_{7}N + \beta^{--}$$

(c) Decay by positron emission (β-Decay)

Some isotopes decay by emitting positively charged β-particles referred to as positrons (β^{+}). This occurs when proton is converted to a neutron:

Proton → neutron + position

An example of an isotope decaying by positron emission is ^{22}Na:

$$^{22}_{11}Na \rightarrow {}^{22}_{10}Ne + \beta^{+}$$

(d) Electron capture or EC-Decay

This is similar to positron emission in that proton is transformed into a neutron. The proton becomes a neutron and electromagnetic radiation (X-rays or γ-rays) given out. Atomic number is decreased by one during EC-Decay.

Proton + electron → neutron + X-ray / γ-ray

Example: $^{54}_{25}Mn + e^{-} \rightarrow {}^{54}_{24}Cr$ + X-ray / γ-ray

(e) Decay by emission of gamma (γ) rays

Emission of γ-particle in itself leads to no change in atomic or mass numbers.

Example:

$$^{60}_{29}Co^{*} \rightarrow {}^{60}_{29}Co + \gamma \text{ ray}$$

(f) Spontaneous Fission

Spontaneous fission is a decay process in which nucleus undergoes division into two fragments along with emission of 2-3 neutrons. This is prevalent in isotopes of heavy elements.

Example:

$$^{252}_{98}Cf \rightarrow F_1 + F_2 + F_3 \text{ neutrons}$$

Where F_1 and F_2 are fission fragments

12.3 Radioactive decay energy

The principle unit used in expressing energy levels associated with radioactive decay is the electron volt. One elctron volt (eV) is the energy acquired by one electron in accelerating through a potential difference of one volt and is equivalent to 1.6×10^{-19}J. It is a very small of amount of energy and usually kiloelectron volts (KeV) and mega electron volts (MeV) are used. Isotopes emitting α-particles are normally the most energetic, falling in the range 4.0 to 8.0 MeV, whereas β- and γ- emitters generally have decay energies of less than 3.0 MeV.

12.4 Kinetics of radioactive decay

Radioactive decay is a spontaneous process and it occurs at a definite rate characteristic of the source. Thus the number of atoms disintegrating at any time is proportional to the number of atoms of the isotope (N) present at that time (t). If the decline in activity of a radioactive isotope is plotted against time (Fig. 12.1), a typical exponential form is obtained:

This may be mathematically expressed as a simple first order process

$$-\frac{dN}{dt} = \lambda N \qquad \ldots \text{ Equation 12.1}$$

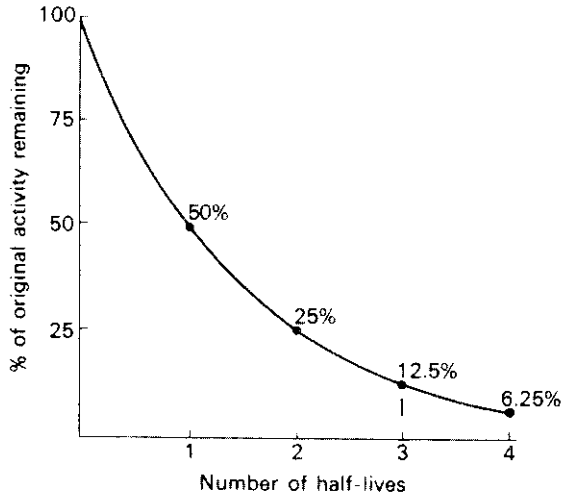


Fig. 12.1 Demonstration of the exponential nature of radioactive decay

Where $-\frac{dN}{dt}$ = the number of atoms decaying per small increment of time

N = the total number of radioactive atoms present at any given time

λ = disintegration or decay constant, a characteristic of a given isotope defined as the fraction of an isotope decaying in unit time and t = time.

The negative sign is essential because the activity is decreasing.

The above equation may be rearranged to get a term for λ

$$-\frac{dN}{dt} = \lambda N \text{ or } \lambda = \frac{-dN}{N.dt} \text{ or } \lambda = \frac{-dN/N}{dt} \quad \text{… Equation (12.2)}$$

By integrating the equation 11.2, it can be converted to a logarithmic form:

$$\text{In } \frac{N_t}{N_o} = -\lambda t \quad \text{… Equation 11.3}$$

Where N_t is the number of radioactive atoms present at time t, and N_o is the number of radioactive atoms originally present. In practice, it is more convenient to express the decay constant in terms of **half-life** (t ½). This is defined as the time taken for the activity to fall from any value to half that value (Fig. 12.1). If N_t in equation 1.3 is equal to one-half of N_o then 't' will equal the half-life of the isotope. Thus:

$$\text{In } ½ = - \lambda t ½ \quad \text{… Equation (12.4)}$$

or

$$2.303 \log (½) = - \lambda t ½ \quad \text{… Equation (12.5)}$$

or

$$t ½ = 0.693 / \lambda \text{ or } \lambda = \frac{0.693}{t ½} \quad \text{… Equation (12.6)}$$

The values of t ½ vary widely from over 10^{19} years for lead-204 (^{204}Pb) to 3 x 10^{-7}S for polonium-212 (^{212}Po). The half-lives of some isotopes frequently used in biological work are given in Table 12.1.

Table 12.1 Half-lives of some isotypes used in biological studies

Isotope	**Half-title**
^{3}H	12.26 years
^{14}C	5760 years
^{22}Na	2.58 years
^{32}P	14.20 days
^{35}S	87.20 days
^{42}K	12.40 h
^{45}Ca	165 days
^{59}Fe	45 days
^{125}I	60 days
^{131}I	8.05 days
^{135}I	9.7 h

12.5 Units of radioactivity

The international system of units (SI) uses the term *becquerel* (Bq) as the unit of radioactivity. This is defined as one disintegration per second (1 d.p.s.). However, this unit has still not been widely adopted and a commonly used unit is still the *Curie* (Ci). This is defined as the quantity of radioactive material in which the number of nuclear disintegrations per second is the same as that in 1 g of radium, i.e., 3.7 x 10^{10} (or 3.7 x 10^{10} s^{-1}). For biological purposes this unit is too large and the microcurie (μCi) and millicurie (mCi) are used.

Normally in radiotracer experiments the radioactive isotope is added mixed up with the stable isotope. It therefore becomes necessary to express the quantity of radioisotope present per unit mass. This is known as the **Specific activity** and may be expressed as (i) disintegration rate (ds^{-1} or d min^{-1}), (ii) count rate (ct s^{-1} or ct min^{-1}), or (iii) curies (mCi or μCi per unit mass of the mixture (μCi or mCi / mole or gram).

12.6 Detection and measurement of radioactivity

Radiation detection methods are based on measurement of the charge produced due to interaction of radiation as it passes through detector volume. The net result of the radiation interaction is the appearance of an electric charge within the active volume of the detector. The interaction times are so short that the deposition of radiation energy can be considered as instantaneous and charge appears in the detector at zero time. This charge must be collected to form the basic signal for radiation detection. Charge is collected by applying an electric field across the detector. Time required for charge collection greatly varies from one detector to another depending on the mobility of charge carriers in the detector and the average distance to be travelled. The charge collected is so small that it has to be amplified. The amplified signal is further processed to obtain intensity (total number) of radiations of all energies (gross counting) OR intensity as a function of energy (spectrometry).

Detectors are operated either in current mode or pulse mode. In pulse mode operation, interaction of each individual quantum of radiation is recorded. Thus only in pulse mode the information on the energy of each radiation is obtained. In the current mode, average current, produced in the interaction of radiation, is measured. It depends on the product of interaction rate and the charge per interaction. Thus in this mode, the average event rate and charge produced per event are obtained. This mode of measurement is highly useful where the event rate is high and pulse mode operation becomes impractical.

There are three commonly used methods of detecting and quantifying radioactivity. These are based on the ionisation of gases, on the excitation of solids or solutions, and the ability of radioactivity to photographic emulsions, i.e. autoradiography.

12.6.1 Methods based upon gas ionisation

As a charged particle passes through a gas, its electric field dislodges orbital electrons from atoms sufficiently close to its path and causes ionisation (Fig. 12.2). The ability to induce ionisation decreases in the order

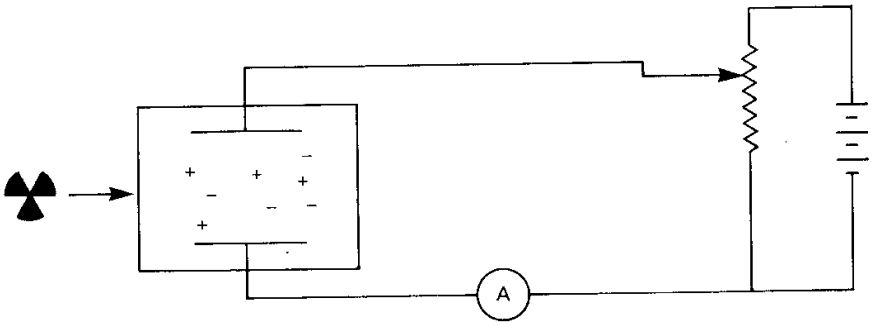


Fig. 12.2 Detection based on ionisation

$\alpha > \beta > \gamma$ (10,000 : 100 : 1)

Accordingly, α- and β- particles may be detected by gas ionisation methods, but these methods are poor for detecting γ- radiation.

(a) Ionisation chambers (Fig. 12.3): Ion chambers are the simplest of all gas filled detectors. Ionization chambers are not used in biochemistry on large scale. Principle of the ionization chamber is the measurement of the number of ions and electrons produced by the radiation in a gas filled chamber. This is a achieved by creating an electrical potential across the chamber by means of two electrodes. The electrons then rush towards the anode, while the positive ions travel towards the cathode. The migration of these charged particles gives rise to a current, which can be measured directly with the help of a galvanometer or can be amplified electronically.

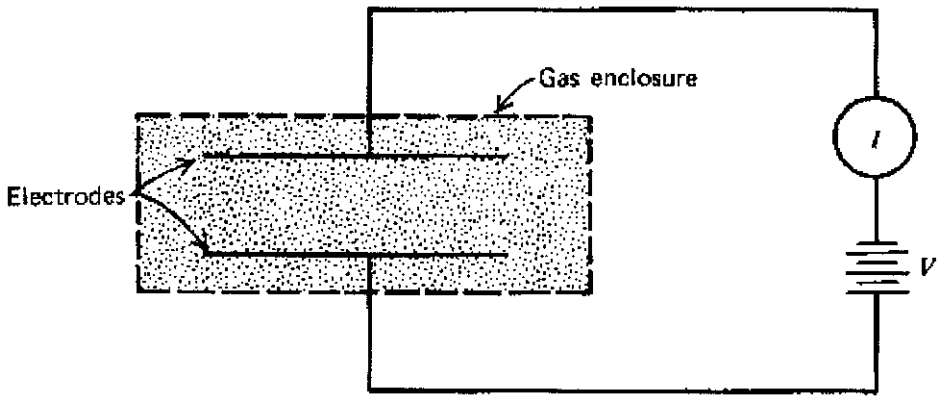


Fig. 12.3 Cross-sectional view of an ion chamber

These negative and positive ions formed in the gas chamber attract one another and can 'recombine' leading to formation of neutral atoms and molecules. To avoid this recombination a strong electric field is created which accelerates the particles towards the respective electrodes giving them lesser opportunity to meet and recombine. Above a certain minimum value of the field strength essentially no recombination takes place. The current through the chamber is then dictated solely by the number of ions formed per unit time in the sensitive volume of the gas. This 'saturation current', therefore, is an unequivocal reflection of the intensity of radiation and hence a measure of the activity, if the nature of radioisotope and the experimental conditions remain constant. When the current obtained is plotted against the applied voltage, the region of saturation current shows up as a long plateau in the value of current (Fig. 12.4). In practice, ionization chamber is always used in the region of this plateau.

Important applications of ion chambers are calibration of radioactive sources, radioactivity dose monitors, radiation survey instruments and measurement of radioactive gases. Ionization chambers are useful in charged particle spectroscopy, e.g., measurement of α-particles and fission fragments. These chambers are used to measure low α-active samples in presence of large amount of β-radiation and low fission rates in presence of large α-particle fluxes taking advantage of pulse height discrimination.

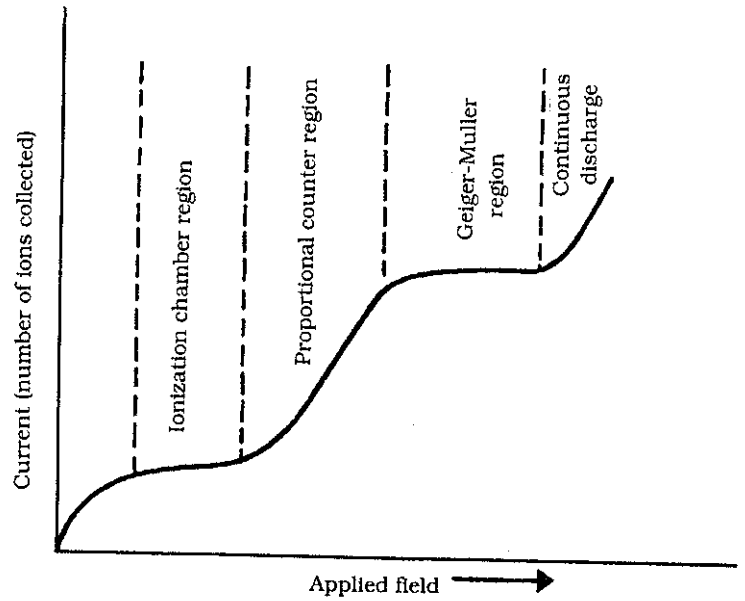


Fig. 12.4 Relationship between the voltage applied and the number of ions collected (current). The regions at which different counters operate are indicated

(b) Proportional counters: Proportional gas ionisation detectors (proportional counters) operate at a higher voltage gradient than ion chambers. Proportional counters are almost always operated in pulse mode. They rely on the gas multiplication to amplify the charge created by interaction of radiation with the fill gas. Multiplication is a consequence of increasing the electric field within the gas to a sufficiently high value. There is a threshold value of the electric field for the onset of gas multiplication. In typical cases, it is about 10^6 V/m at atmospheric pressure. Therefore, the geometry of the detector becomes very important. A cylindrical tube with a thin anode wire is the general configuration of a proportional counter and a cross sectional view of such a proportional counter for measuring radiation that penetrates into the tube is shown in Fig. 12.5. For less penetrating radiations like α, window less flow proportional counter is used. In this counter, source is introduced normally with a leak tight rotating platform into the detector volume and the fill gas flows through the detector. Efficiency of this detector is nearly 50% for α-counting.

The fill gas should not form anions and should not contain components with high electron attachment co-efficient. Nobel gases like argon meet this requirement optimally. Gas mixtures like P-10 having 90% argon and 10% methane is most commonly used fill gas. Methane exchanges the excitation energy from excited atoms of argon and discharges the energy by non-radiative processes. Thus it inhibits the deexcitation of excited argon atoms by emission of UV light which may create an electron by photoelectric absorption and that electron under applied voltage may trigger an avalanche.

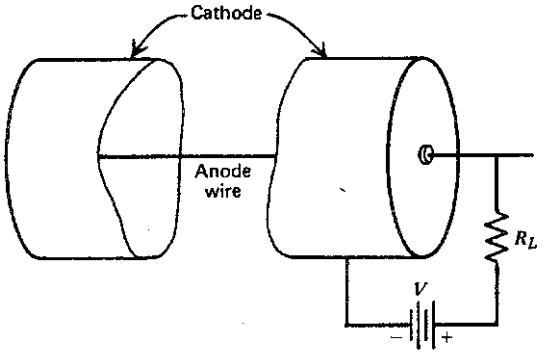


Fig. 12.5 Cross-sectional view of a proportional counter

Proportional counters have good efficiency for α, β and other charged particles. For γ and X-ray measurement, detection efficiency is low. The spectroscopy of low energy X-rays is one of the most important applications of proportional counters. These detectors also useful for measurement of energy in addition to their intensity of the radiations. Parallel plate avalanche counters, position sensitive detectors, are different types of proportional counters with special applications.

(c) Geiger Müller (G.M.) counters: The most common device that uses the gas ionisation technique is the Geiger- Müller tube (G-M tube). When β particles pass through a gas, they collide with atoms and may cause ejection of an electron from a gas atom. This results in the formation of an ion pair made up of the negatively charged electron and the positively charged atom. If this ionization occurs between two charged electrodes (an anode and a cathode), the electron will be attracted to the anode and the positive ion to the cathode. This results in a small current in the electrode system. If only a low voltage difference exists between the anode and cathode, the ion pairs will move slowly and will, most likely, recombine to form neutral atoms. Clearly, this will result in no pulse in the electric circuit because the individual ions do not reach the respective electrodes. At higher voltages, the charged particles are greatly accelerated toward the electrodes and collide many times with un-ionized gas atoms. This leads to extensive ionization and a cascade or avalanche of ions. If the voltage is high enough (1000 volts for most G-M tubes), all ions are collected at the electrodes. A Geiger-Müller counting system uses this voltage region for ion acceleration and detection. A typical G-M tube is diagramed in Figure 12.6. It consists of a mica window for entry of β particles from the radiation source, an anode down the center of the tube, and a cathode surface inside the walls. A high voltage is applied between the electrodes. Current generated from electron movement toward the anode is amplified, measured, and converted to counts per minute. The cylinder contains an inert gas that readily ionizes (argon, helium, or neon) plus a quenching gas (Q gas, usually butane) to reduce continuous ionization of the inert gas. Beta particles of high energy emitted from atoms such as ^{24}Na, ^{32}P, and ^{40}K have little difficulty entering the cylinder by penetrating the mica window. Particles from weak β emitters (especially ^{14}C and ^{3}H) cannot efficiently pass through the window to induce ionization inside the chamber. Modified

G-M tubes with thin mylar windows, called flow window tubes, may be used to count weak β emitters.

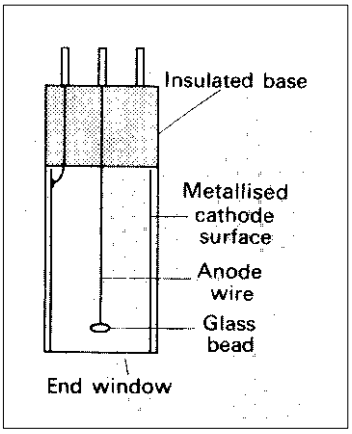


Fig. 12.6 The Geiger-Muller (G-M) tube

G-M counting has several disadvantages compared to liquid scintillation counting. The counting efficiency of a G-M system is not as high. The response time for G-M tubes is longer than for photomultiplier tubes; therefore, samples of high radioactivity are not efficiently counted by the G-M tube, G-M meters are seldom used when careful, accurate measurements are required. They are most useful in the biochemical laboratory as survey meters to monitor and detect radioactive contamination on lab benches, glassware, equipment and laboratory personnel.

12.6.2 Photographic Methods

Ionising radiation acts upon a photographic emulsion to produce a latent image much as does visible light. For a photograph, a radiation source, an object to be imaged and photographic emulsion are required. For an autoradiograph, a radiation source (i.e. radioactivity) emanating from withint he material to be imaged (the object) is required, along with a sensitive emulsion. The emulsion consists of a large number of silver halide crystals embedded in a solid phase such as gelatin. As energy from the radioactive material is dissipated in the emulsion, the silver halide becomes negatively charged and is reduced to metallic silver, thus forming a particulate latent image. Photographic developers are designed to show these silver grains as a blackening of the film, and fixers to remove any remaining silver halide. Thus, a permanent image of the location of the original radioactive event remains.

This process, which is known as **autoradiography**, is very sensitive and has been used in a wide variety of biological experiments. These unusually involve a requirement to locate the distribution of radioactivity in biological specimens of different types. For instance the sites of localisation of a radiolabelled drug through out the body of an experimental animal can be determined by placing whole-body sections of the animal in close contact with a sensitive emulsion such as an X-ray plate. After a period of exposure, the plate, upon development, will show an image of the section in tissues and organs in which radioactivity was present (Fig. 12.7). Similarly, radioactive metabolites isolated and separated by chromatographic or electrophoretic techniques during metabolic studies can be located on the chromatograph or electrophoretograph and the

radioactive spots can subsequently be recovered for counting and identification. The techniques of autoradiography have become more important with recent developments in molecular biology.

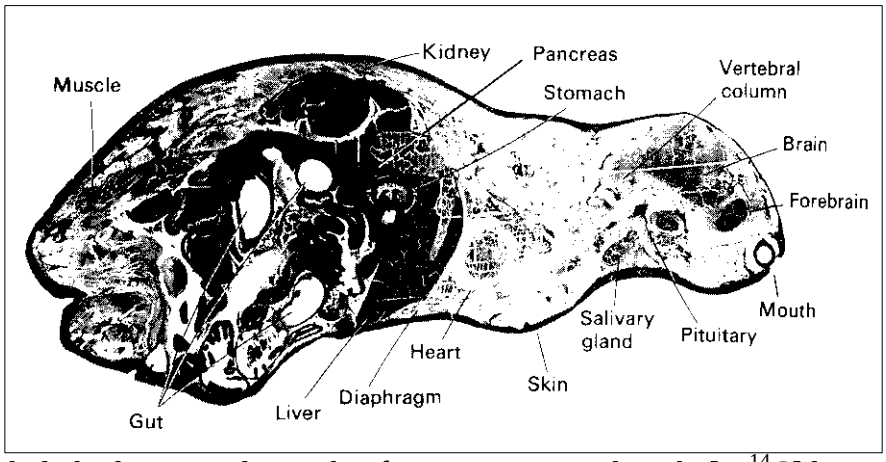


Fig. 12.7 Whole-body autoradiograph of a mouse treated with [L-^{14}C]dopa. The dark areas indicate the presence of radioactive isotope and show high concentrations in the liver, pancreas, kidney, skin and forebrain.

12.6.3 Methods based upon excitation

Radioactive isotopes interact with matter in two ways, causing ionisation, which forms the basis of Geiger-Müller counting, and excitation. The latter effect leads the excited compound (known as the fluor) to emit photons of light. The fluorescence can be detected and quantified. The process is known as scintillation and when the light is detected by a photomultiplier, forms the basis of scintillation counting. The electric pulse that results from the conversion of light energy to electrical energy in the photomultiplier is directly proportional to the energy of the original radioactive event. This is a considerable asset of scintillation counting, since it means that two or even more isotopes can be separately detected and measured in the same sample, provided they have sufficiently different emission energy spectra. Scintillation counting provides information of two kinds:

(i) **Quantitative:-** the number of scintillations is proportional to the rate of decay of the sample, i.e. the amount of radioactivity.

(ii) **Qualitative:-** the intensity of light given out and therefore signal from the photomultiplier is proportional to the energy of radiation.

12.6.3.1 Types of scintillation counting

There are two types of scintillation counting, which are illustrated diagrammatically in Fig. 12.8a & b. In solid scintillation counting the sample is placed adjacent to a crystal of fluorescent

material. The crystal that is normally used for γ-isotopes is sodium iodide, whereas for α-emitters zinc sulphide crystals are preferred and for β-emitters organic scintillators such as anthracene are used. The crystals themselves are placed near to a photomultiplier, which in turn is connected to a high voltage supply and a scaler (Fig. 12.8a). Solid scintillation counting is particularly useful for γ-emitting isotopes. These rays are electromagnetic radiation and collide only rarely with neighbouring atoms to cause ionisation or excitation. Clearly, in a crystal the atoms are densely packed, making collisions more likely. Conversely, solid scintillation counting is generally unsuitable for weak β-emitting isotopes such as ^{3}H and ^{14}C, because even the highest energy negatrons emitted by these isotopes would have hardly sufficient energy to penetrate the walls of the counting vials in which the samples are placed for counting. As many of the isotopes used in radioimmunoassay are γ-emitting isotopes, solid scintillation counting is frequently used in biological work.

In liquid scintillation counting (Fig. 12.8b), the sample is mixed with a scintillation cocktail containing a solvent and one or more fluors. This method is particularly useful in quantifying weak β-emitters such as ^{3}H, ^{14}C and ^{35}S, which are frequently used in biological work. For these isotopes, liquid scintillation counting is the usual counting method.

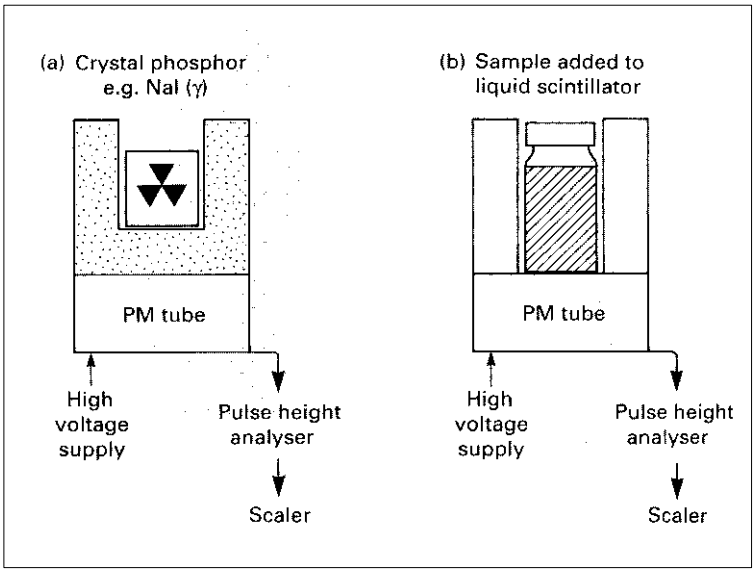
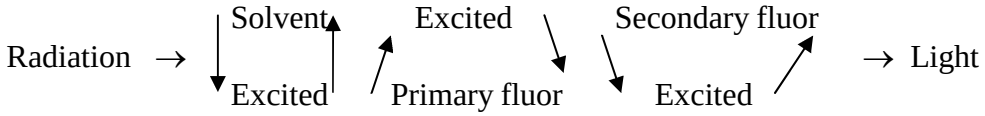


Fig. 12.8 Diagrammatic illustration of solid (a) and liquid (b) scintillation counting methods

12.6.3.2 Energy transfer in liquid scintillation counting

A small number of organic solvents fluoresce when bombarded with radioactivity. The light emitted is of very short wavelength and is not efficiently detected by most photomultipliers. However, if a compound is dissolved that can accept the energy from the solvent and itself fluoresce at a longer wavelength, then the light can be more efficiently detected. Such a compound is known as a primary fluor and the most frequently used example is 2,5-diphenyloxazole (PPO). Unfortunately the light emitted by PPO is not always detected with very high efficiency (depending

on the photomultiplier detector) but this can be overcome by including a secondary fluor or wavelength shifter such as 1,4-bis(5-phenyloxazol-2-yl) benzene (POPOP). Thus, the energy transfer process becomes



The question obviously arises as to why a primary fluor and a secondary fluor are necessary when it is the latter that emits light at the best wavelength for detection. The answer is simply that the solvent cannot transfer its energy directly to the secondary fluor.

PPO and POPOP were among the original fluors used in liquid scintillation counting and remain a favourite choice. However compounds such as 2-(4'-t-butylphhenyl)-5-(4"-biphenylyl)-1,3,4-oxadiazole (BUTYL-PBD) is a better primary fluor but is quite expensive and is affected by extremes of pH.

Most laboratories now buy their scintillation cocktails already prepared and there are many different makes and recipes on the market. Competition and an increasing awareness of health and safety mean that scintillation cocktails are gradually becoming less toxic and have a lower fire hazard. Some cocktails are designed for aqueous samples and others for organic sample; it is important that the appropriate formulation is used.

12.6.3.3 Advantages of scintillation counting

The very fact that scintillation counting is widely used in biological work indicates that it has several advantages over gas ionisation counting. These advantages are:

(i) The rapidity of fluorescence decay (10^{-9}s), which, when compared to dead time in a Geiger-Müller tube (10^{-4}s), means much higher count rates are possible.

(ii) Much higher counting efficiencies particularly for low energy β-emitters; over 50% efficiency is routine in scintillation counting and efficiency can rise to over 90% for high energy emitters. This is partly due to the fact that the negatrons do not have to travel through air or pass through an end-window of a Geiger- Müller tube (thereby dissipating much of the energy before causing ionisation) but interact directly with the fluor; energy loss before the event that is counted is therefore minimal.

(iii) The ability to accommodate samples of any type including liquids, solids, suspensions and gels.

(iv) The general ease of sample preparation.

(v) The ability to count separately different isotopes in the same sample, which means dual labelling experiments can be carried out.

(vi) Scintillation counters are highly automated, hundreds of samples can be counted automatically and built-in computer facilities carry out many forms of data analysis, such as efficiency correction, graph plotting, radioimmunoassay calculations, etc.

12.6.3.4 Disadvantages of scintillation counting

These disadvantages of scintillation counting include the following:

(i) The cost per sample of scintillation counting is not insignificant; however, other factors including versatility, sensitivity, ease and accuracy outweigh this factor for most applications.

(ii) At the high voltages applied to the photomultiplier, electronic events occur in the system that are independent of radioactivity but contribute to a high background count. This is referred to as photomultiplier noise and can be partially reduced by cooling the photomultipliers.

(iii) The greater disadvantage of scintillation counting is quenching.

Most of the inherent disadvantages have been overcome by improvement in instrument design.

12.7 Applications of Radioisotopes in the Biological Science

Radioisotopes are used in a variety of studies encompassing basic research and applications. Important areas where radioisotopes widely used are agriculture, health care, industry and physical sciences.

12.7.1 Agriculture Applications

In agriculture, radioisotopes are used to improve the quality and productivity of agricultural products as well as for optimum utilization f fertilizers, insecticides and pesticides without harmful effects to plants and mankind. They are also used for insect, pest and disease management and, for preservation of foods and agricultural products. These agricultural applications are based on tracer techniques and utilization of radiation energy.

(a) Genetic improvement of crop plant: In 1930, an American Scientst L.J. Stadler first used the X-rays in the development of genetic variation in plants. Radiation energy utilising technique used for inducing mutations in plants cells is widely used to obtain a desired variation in the quality of the product. Plants genetically modified to produce recombinant proteins such as peptides,

vaccines, to improve fruit quality, to insect increase disease resistance and to improve shelf life of fruits and vegetables. High yielding varieties of tur, green gram, black gram, groundnut, jute and rice were developed by using radioisotopes at BARC. Different types of radiation can be used to induce genetic variation. However, ^{60}Co is the most commonly used radiation source.

(b) Insect, Pest and disease management: Pest control by a technique called "Sterile (male) insect technique (SIT)" involves the release large number of male flies rendered sterile by γ-radiation into the field. SIT has been effectively utilised in the case of the Mexican fruit fly along the US - Medican border to prevent flies from entering California.

(c) Food Preservation: Conventional food preservation methods like sun drying, salting and picking are known since the existence of mankind. Cold storage canning, pasteurising, vacuum drying and preservation by chemical additives are some of the modern techniques used for food preservation. Each of these techniques has its own a merits and demerits. Further development in the area of food preservation is radiation processing of food with ionising radiation like γ-rays from ^{60}CO. When food is exposed to radiation, its interaction with DNA causes the death of micro-organisms and insects. Additionally, in food items like onions and potatoes, garlic and ginger, the ionising radiation impairs the sprouting processes resulting in longer storage periods. Food preservation by exposing to X-rays was first patented in France is 1930. On the basis of radiation dose deployed, applications are classified into 3 categories:

(i) Low dose applications (upto 1 kGy)

1. Inhibition of sprouting of potatoes, onions, garlic and ginger during storage.
2. Prevents the insect induced damage to stored grains, pulses, dry fruits, nuts and dry fish.
3. Destruction of parasites found in meat and meat products, e.g., entomoeba hystolytica and toxoplasma gondi.

(ii) Medium dose applications (1-10 kGy)

1. Shelf-life extension of mushrooms.
2. Prevention of food poisoning caused by Salmonella, Vibrio etc., present in fresh as well as frozen meat, poultry and sea foods.

(iii) High dose applications (10-45 kGy)

Destruction of spoilage organisms including bacterial spores to obtain shelf stable sterile products without refrigeration.

12.7.2 Applications in Health care

The applications of radioisotopes in the field of health care are very extensive. Radio isotope techniques are used in diagnosis and prognosis in a variety of diseases as well as providing relief by both curative and palliative treatment. The diagnostic techniques are performed in-vitro

using radioimmunoassay (RIA) reagents and in-vivo using radiopharmaceuticals. The therapeutic procedures could be performed using implanted or externally located sealed sources of radioisotopes or by systematic administration of appropriate radiopharmaceuticals.

(a) Diagnosis: Radioisotopes are very widely used for diagnostic tests. More than 100 different radio isotopes have been used for diagnosis. Among the most important ones are, ^{51}Cr, $^{57/58}CO$, $^{52/59}Fe$, ^{67}Ga, ^{81}Kr, ^{82}Rb, ^{99}Tc, $^{123/125/131}I$, ^{133}Xe, ^{169}Yb, $^{195/198}Au$, ^{201}Tu, and ^{131}I. Sodium iodide is used for the diagnosis of some thyroid diseases. ^{131}I-iodohippuric acid is used for kidney function tests. ^{133}Xe is used for lung function tests. ^{51}Cr is used for blood volume determination and investigations into certain kinds of anemia. ^{201}Thallium isotope mimics potassium ion behaviour in its uptake from the blood stream into the normal cells of heart. ^{131}I-MIBG used in neuro-endocrine tumour imaging.

Organ scintigraphy provides valuable information on the size, shape and location of organs and lesions. Radiocolloids are widely used in liver scanning, bone scanning lung imaging and imaging of the blood flow to myocardium and brain. Radio Iodine is used for diagnosis of metastatic sites of thyroid cancerous tumour.

(b) Therapy

(i) Radionuclide therapy: Radionuclide provided ammunition to the physicians to tackle some diseases since early times, especially cancer. Selective irradiation of the unwanted cells, e.g., cancer cells, is the main aim in these procedures. Radionuclides widely used in therapy are α-amitters, Auger/conversion electron emitters, hard beta emitters and soft beta emitters. ^{131}I radioiodine is used for efficacious treatment of thyroid disorders are thyrotoxicosis (benign) and thyroid cancer (malignant). ^{32}P as phosphate, $^{89}SrCl_2$ and ^{186}Re- phosphonate complexes are the important radionuclide products in treating the metastatic bone pain. Radiation synovectomy is, an alternative to surgery, suitable for treatment of rheumatoid arthritis. Radioimmunotherapy (RIT) and radiopeptidetherapys (RPI) are the major recent advances in combating caner.

(ii) Radiotherapy: Radiotherapy deals with treatment of cancer using radiations emitted from radioisotopes or X-ray machines or using high energy electrons. Radiotherapy is the only method for treatment of cervical cancer and also used as palliative treatment in patients with advanced cancer.

(iii) Teletherapy: In this therapy, a high intensity beam (~ 10,000 curies) from a tiny ^{60}CO source is collimated on to the tumour area.

(iv) 'Gamma knife': A recent advance in external radiation therapy is the development of 'Gamma Knife' for stereotactic radiosurgery of tumours and arteriovenous malformations in the brain. In Gamma knife treatment radiaton source is ^{60}CO radioisotope.

(v) Brachytherapy: Application of small sealed radiation sources in close proximity to the tumour for treatment is called brachy therapy. This therapy used for treatment of cancer of eye, skin cancer,

breast cancer and prostrate cancer. Isotopes like ^{192}Ir, ^{60}CO, ^{198}Au and ^{137}Cs are radiation sources for brachy therapy.

12.7.3 Applications in Biological research

(i) Molecular Biology: A radiotracer is generated by suitably labelling a biomolecule of interest with the radioisotope of choice. Radiolabels provide a very high degree of sensitivity. It is possible to detect concentrations as low as attomolar (10^{-18}). Nucleic acid and peptide probes thus generated, by using radioisotopes, are used for a variety of applications such as insitu hybridization, molecular cloning and screening, preparation of genomic libraries, structural and functional genetics, identification of gene mutations, identification of genetic disorders, molecular diagnosis of diseases and forensic applications such as DNA finger printing. Radioisotopes widely used in molecular biology are ^{35}S, ^{32}P, ^{33}P, ^{131}I, ^{3}H, ^{14}C, and ^{45}Ca.

(ii) Pharmacological studies: Another field where radioisotopes are widely used in the development of new drugs The central dogma of drug development is the principle of ADME (Adsorption, Distribution, Metabolism, Excretion). These four factors characterize the function of a drug in vivo. Thus, radioisotopes are useful at each and every stage of the ADME principle and have proved to be a great asset in drug development. In addition to ^{14}C and ^{3}H labeled compounds, there is a huge application of Positron Emission topography (PET) in this area.

iii) Ecological Studies: In particular, migratory patterns and behaviour patterns of many animals can be monitored using radiotracers. Another ecological application is in the examination of food chains where the primary producers can be made radioactive and the path of radioactivity followed throughout the resulting food chain.

(iv) Metabolic Studies: Isotopes have been employed in the study of almost every phase of metabolism viz., Amino acid metabolism, protein biosynthesis, photosynthesis (carbon flow), the EMP pathway, Krebs cycle, PPP pathway, nucleic acid synthesis, heme biosynthesis, fatty acid degradation and biosynthesis, and cholesterol steroid metabolism etc. Isolation and identification of metabolites of major metabolic pathways has been made possible by use of isotopes. Moreover, minor metabolic pathways which have a potential to become a major pathways in disease have been elucidated by mainly by the use of isotopes. Where several pathways exist, it has become possible to calculate the relative proportion traversing each route. Precursor product relationship has been established by means of radioactivity. By using the isotopes the whole metabolic pathway can be established.

(v) Metabolic turn over time determination: Radioisotopes provide a convenient method of ascertaining turnover times for particular compounds. On the basis of radioisotopic experiments, it could be found that the proteins of the liver cell have a half-life of about 5-6 days. On the other hand, proteins of muscle tissue turn over quite sluggishly showing a half-life of about 30 days.

(vi). Studies of absorption, accumulation and translocation: Radioisotopes have been very widely used in the study of the mechanism and rates of absorption, accumulation and translocation

of organic and inorganic compounds by both plants and animals. **Eg.** ^{45}Ca, ^{24}Na and ^{42}K have been used to study the fluxes of these elements through the plasma membrane of various cells. studies with ^{32}P have shown that in the liver, kidney and in the erythrocyte, the formation ATP on the cell surface in the principal mechanism for the entry of phosphate in to the cell interior. Thymidine permeability in bacteria was tested using radioactive isotopes ^{32}P and ^{3}H. The exchange reactions that take place in the bone with respect to calcium and phosphorous have been cleaned from the studies made using ^{45}Cow ^{32}P.

(vii) Fate of the cells and sub-cellular particles: Isotopes have provided much information about the behaviour, movement, activities and life span of cells and other functioning particles in the body. Isotope ^{51}Cr in the chromate state used in the measurement of the in vivo life span of erythrocytes.

(viii) Isotope incorporation and Isotope exchange studies: Isotope incorporation studies provides information concerning the position of the bond breakage and formation during a reaction. As an example consider the cleavage of glucose-1-phosphate as catalyzed by the alkaline phosphatase. This reaction carried out in the presence of H_2 ^{18}O enriched water. Isotope exchange studies yield information about the existence of reaction intermediates. Sucrose phosphorylase provides an example of the isotope exchange studies.

(ix) Enzyme and ligand binding studies: Generally any enzyme reaction can be assayed using radiotracer methods. The use of radioisotopes in enzyme assays is restricted to applications where it is possible to separate easily the radiolabelled forms of substrate and product. The assay of glutamate decarboxylase could be based on the evolution of $^{14}CO_2$.

$HOOC\ CH_2\ CH_2\ CH\ (NH_2)\ ^{14}COOH$ Glutamate $^{14}CO_2 + HOOCCH_2\ CH_2\ CH_2\ NH_2$

glumatic acid — decarboxylase → γ-amino butyric acid

Radiotracer-based enzyme assays are more expensive than other methods, but frequently have the advantage of a higher degree of sensitivity. Radioisotopes have also been used in the study of the mechanism of enzyme action and in ligand binding studies. ^{3}H, ^{14}C, ^{32}P, ^{35}S and ^{125}I are the most common radionuclides used in ligand binding studies.

(x) Radiodating: Aquite different analytical use for radioisotopes is in the dating (i.e. determining the age) of fossils, sediments and rocks. For long term dating, isotopes with long half lives are necessary, such as ^{235}U, ^{238}U and ^{40}K, whereas for shorter term dating ^{14}C is widely used. Palaeontologists and anthropologists widely use this technique.

(xi) Isotope dilution analysis: There are many compounds present in living organisms that cannot be accurately assayed by conventional means because they are present in such low amounts and in mixtures of similar compounds. Isotope dilution analysis offers a convenient and accurate way of overcoming this problem and avoids the necessity of quantitative isolation. For example, the amount of iron in protein preparation is determined by using ^{59}Fe radioisotope.

12.8 Summary

Radioactivity results from the spontaneous nuclear disintegration of unstable isotopes. Those isotopes exhibit radioactivity are called Radioisotopes. Radioactivity decay is a spontaneous phenomenon of emission of particles or electromagnetic radiation from an atom (nucleus). Radioactive decay is of three main types are α, β and γ decay. The basic unit of radioactivity is the curie, Ci. One curie is the amount of radioactive material that emits particles at a rate of 3.7×10^{10} disintegrations per second (dps), or 2.2×10^{12} min^{-1} (dpm). The detection and quantification of radioactivity is accomplished by three methods. These are Geiger Muller counting, liquid-scintillation on counting and photographic emulsion methods. The measurement of radioactivity is now a tool used in all areas of experimental biochemistry, including enzyme assays – biochemical pathways of synthesis and degradation, analysis of biomolecules, measurement of antibodies, binding and transport studies and many others. Autoradiography is very sensitive method and is widely used to locate the distribution of radioactivity in biological specimens of different types, but it is not useful in quantifying the radioactivity.

12.9 Model Questions

1. Define the following terms related to radioactivity units: (a) Becquerel, and (b) Millicurie.
2. Radioactive decay.
3. Describe the detection methods based upon gas ionisation.
4. Describe the autoradiography.
5. Applications of radioisotopes in the biology.

12.10 Reference Books

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5. Sood, D.D., Reddy, A.V.R. and N. Ramamoorthy (2004). Fundamentals of Radiochemistry, 2nd Edition, IANCAS Publishers, Mumbai, pp.1-455.

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