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## S.Y.B.Sc. SEM-4

## Subject: Biochemistry

## Paper-401: BIOPHYSICAL & BIOCHEMICAL TECHNIQUES

## Unit -5

# Electrophoresis

## Unit - 5 ELECTROPHORESIS

- Electrophoresis is the migration of charged particles or molecules in a medium under the influence of an applied electric field.

- It is an important method for separation and characterization of biological molecules because it usually does not affect the native structure of biopolymers and secondly it is highly sensitive to small differences in both charge and mass.

- The modern day scientists however use it for diverse specialized purpose like determination of molecular weight of protein, DNA sequencing, hybridization of nucleic acid etc.

### PRINCIPLE OF ELECTROPHORESIS

- Upon suspension in aqueous solvent, almost all particles (e.g. Red Blood Cell, Bacteria etc.) and many important biomolecules (e.g. Nucleotides, nucleic acids, amino acids, peptides, proteins etc.) acquire either positive or negative charge.

- Large number of ionizable amino and carboxyl group on protein surfaces determine net charge density of the protein molecules which makes it move in an electrical field in a direction and at velocity depend upon the sign and the quantity of this net charge density.

- Protein molecules with net negative charge migrate towards anode and those with net positive charge migrate towards cathode.

- Electrophoresis can also be applied for the separation of uncharged biomolecules such as carbohydrates. Carbohydrates can be made to possess weak charge through derivatization as borates and phosphates.

- Even if two molecules same charge quality and quantity they might not migrate together because if there is difference in their molecular weights, they will have different charge/mass ratio.
- Taken together, these factors govern the migration of charged compounds under the influence of electrical field and this is the basis of electrophoresis.
- Consider a situation where spherical molecule of net charge q is placed in an electrical field.
- The force F acting upon this molecule will depend upon
  **(i) The net charge density of the molecule**
  (ii) **The strength of the field in which it is placed**.
  The above relationship may be mathematically describe as

$$\mathbf{F} = \frac{\Delta E}{d} X\, q$$

- Where ΔE/d is the field strength is applied (**ΔE is the potential difference** between the two electrodes, **d being distance** between them.)

- Since the particle has been suspended in a solution, we have to consider the friction occurring between the accelerating molecules and the solution, to arrive at valid relationship of the electrophoretic migration.

- The extent of friction will depend upon:
- The size and shape of molecules
- The viscosity of the medium through which the molecule will migrate.

- Thus frictional force F can be expressed by the formula:

$$\mathbf{F=6\pi r\ \eta\ v}$$

- Where F is the friction exerted on the spherical molecule, **r is the radius** of the molecule, **η is the viscosity of the solution**, and **v is the velocity** at which the molecule is migrating. This frictional force will oppose the accelerating force generated by the electrical field

$$\frac{\Delta E}{d} X\ q = \mathbf{6\ \pi\ r\ \eta\ v}$$

$$\mathbf{Therefore\ v} = \frac{\mathbf{\Delta Eq}}{[\mathbf{d6\pi r\eta}]}$$

Thus it can be seen that the velocity (v) of migration of the molecules is directly proportional to:
(i) Field strength (ΔE/d) and
(ii) charge q and the molecules and inversely proportional to
(iii) particle size (r) and
(iv) viscosity of the solution (η).

## FACTORS AFFECTING ELECTROPHORETIC MOBILITY:

Factors that affect the electrophoretic mobility of particles or macromolecules include: the sample itself, electrical field, supporting medium, the buffer, etc.

### The Sample:

Charge/mass ratio of the sample dictates its electrophoretic mobility. The mass consists of not only the size (molecular weight) but also the shape of the molecule.

- **Charge:** Protein molecules with net negative charge migrate towards anode and those with net positive charge migrates towards cathode.
- Two protein molecules having net negative charge but differing in quantity of the charge will migrate in the same direction (towards anode) but the velocity would be different.
- The protein with less negative charge would migrate at lower speed then that of the protein having high negative charge.
- **Size:** The bigger the molecule, greater are the frictional and electrostatic force exerted upon it by the medium of suspension. Consequently, large particles /molecules have lesser electrophoretic mobility compare to the smaller particles /molecules.
- **Shape:** Rounded contours elicit lesser frictional and electrostatic retardation compared to sharp contours. As an example consider the case of globular and fibrous proteins of the same size (molecular weight) and charge. The globular protein will migrate faster than the fibrous protein.

### Electrical field:

- **Potential:** Force acting upon a molecule of charge q is ΔEq/d. The rate of migration depends on the potential difference and distance between the two electrodes.
- An increase in the potential difference increases the rate of migration. While increase in the distance between two electrodes decreases the rate of migration.
- **Current Charge:** The current (total charge carried per second to the electrode) in the solution placed between two electrodes is carried by the buffer ions, only a small proportion is carried by sample ions.

- An increase in the potential difference therefore increases the current.
- **Temperature:** During any electrophoretic run some power dissipates with consequent generation of heat. The temperature within the medium rises resulting into decreased viscosity of the solution.
- Consequently, the ions present can move faster more charge is carried per second to the electrode i.e. the current increases while resistance decreases as a result of increasing the buffer ion concentration.
- This results in more charge being carried by the buffer ions move faster, while the sample ions move slowly and separation slows down.
  Ideally one should maintain a constant current in high voltage electrophoresis; even with constant voltage heat will be generated and could slow down the migration of sample molecules.
- Therefore the electrophoresis is carried out on a constant current mode and not on the constant voltage mode.

## Supporting medium:

An inert supporting medium is chosen for electrophoresis but even this insert medium can influence the rate of migration by exerting adsorption and/or molecular sieving effects on the molecules.

- **Adsorption:** Retention of a component on surface of supporting medium is adsorption. Such components have two forces acting upon it; the applied electrical field and adsorption. Thus component is not resolved as sharp band but as a diffused band.
- **Molecular sieving:** Supporting media such as polyacrylamide, agar, starch and sephadex have cross linked structures giving rise to pores within the gel beads.
- When molecules are larger than pore size they will be excluded from entering the gel and those molecules migrate faster in sephadex gel.
- In case of polyacrylamide, starch and agarose, the larger molecules are also made to squeeze through the pores.
- The smaller molecules here pass through the pores easily but the larger molecules are retarded. Molecular sieving effects of the supporting medium thus modify the electrophoretic mobility.

## The buffers

Apart from maintaining the pH of the supporting medium, the buffer can affect the electrophoretic mobility of sample in various other ways.

- **Composition:** Commonly used buffers are formate, citrate, phosphate, acetate, tris etc. The choice of buffer depends on the types of sample being separated by electrophoresis.
  The buffer can effect electrophoretic mobility if is able to blind to components of the sample being separated.
- **Ionic strength:** Increased ionic strength of the buffer means large share of the current being carried by the buffer ions and very small proportion carried by the sample components.
- A decrease in the ionic strength would mean larger share of the current being carried by the sample molecules leading to faster separation.

  However, in low ionic strength buffers diffusion (especially of small molecules) tends to be high with concomitant loss of resolution. The chosen ionic strength of the buffer is therefore between 0.005-0.01 M.

- **pH:** pH determines the degree of ionization of organic compounds; it can also effect the rate of migration. Increased pH increases ionization of organic acids and decreased pH increases ionization of organic bases.

  Therefore, the direction and extent of the migration of charged particles/macromolecule is dependent on pH.

***One word Question***

| *Sr. No.* | Question | Answer |
|---|---|---|
| *1* | Which factors are affecting electrophoresis mobilty ? | Molecular size & Shape |
| *2* | Which technique separates charged particles using electric field? | Electrophoresis |
| *3* | Which type of buffer are used in electrophoresis | Ionic Buffer |
| *4* | The _____depends on the potential difference and distance between the two electrodes | Rate of migration |
| *5* | Electrophoresis is applied for the sample contain | Charge |

**GEL**

**ELECTROPHORESIS:**

The separation of molecules by the gel electrophoresis depends on charge and mass (size) of the molecule.

## Types of gel:

Various types of gels are available for electrophoresis and few are discussed below.

### A.) Starch gel:

Starch gel is used as a stabilizing medium for zone electrophoresis. Starch when heated and cooled in an appropriate buffer sets a gel. High porosity starch gels are obtained by using 2% (w/v) starch and low porosity gels are obtained by adding 10-15% starch to buffer. The resolving power of starch gel is good and hence used for analysis of isoenzyme patterns. But because of several disadvantages starch gels are not widely used.

- ♣ The pore size in starch gel is not uniform and this is the biggest drawback of these gels.
- ♣ Starch gels are not satisfactory for separation of basic proteins.
- ♣ It is difficult to prevent contamination of starch gels by microorganisms.
- ♣ Upon staining with coloring reagents, starch gel becomes opaque hence direct photoelectric determination of separation on starch gel is not possible.

### B.) Agarose gel:

- ♣ Agarose is galactose-based polymer; this solubilizes in aqueous buffers above 40°C and sets to form a gel at about 38°C.
- ♣ This can be used as a stabilizing medium for electrophoresis. Low quantity of agarose in buffer gives a larger pore size and almost no molecular sieving action. Therefore these gels have low diffusion resistance and consequently used in immuno electrophoresis and immuno diffusion assays to detect antigenic proteins.
- ♣ Agarose gel is being used to separate high molecular weight macromolecules like proteins and nucleic acids.

♣

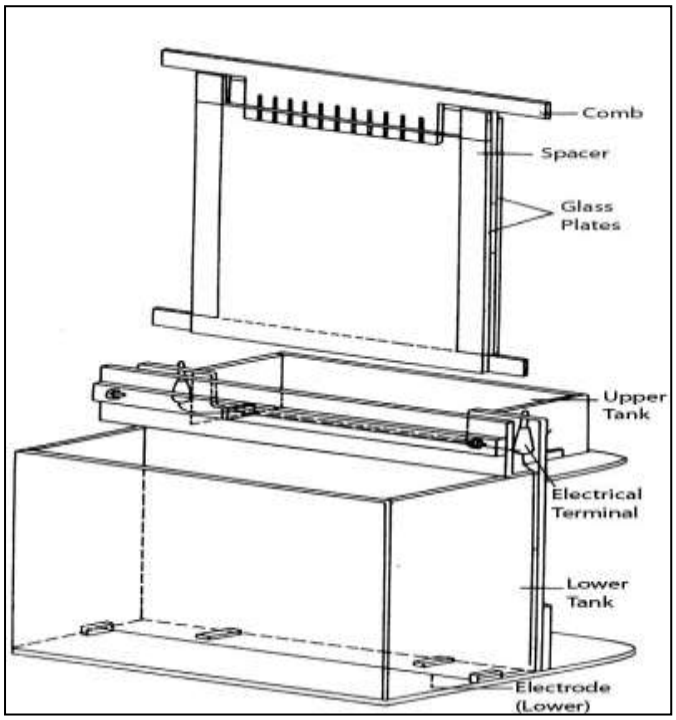
## C). Polyacrylamide gel:

- ♣ **Acrylamide monomer** and N, N'-methylene bis acrylamide (bis) are components of the matrix of polyacrylamide gel.
- ♣ **Ammonium per sulfate** when dissolved in aqueous medium gives free radicals ($SO_4$). These free radicals can activate acrylamide monomers inducing them to react other acrylamide molecules forming long chains become cross-linked to give rise the pores if the reaction is carried out in presence of N, N' - methylene bis acrylamide.
- ♣ **Tetramethylene diamine (TEMED)** acts as catalyst in gel formation because of its ability to exist in free radical form Riboflavin can also be used in place of ammonium presulfate and TEMED in mixture of acrylamide and N, N'-methylene bis acrylamide.
- ♣ In presence of oxygen when mixture is subjected to UV light, riboflavin undergoes photodecomposition to give rise to free radicals which acts in same way to polymerize the gel described above.
- ♣ The pore size of polyacrylamide gel depends on the amount (proportion) of acrylamide and N, N'- methylene bis acrylamide used. Higher amount of N, N'- methylene bis acrylamide will cause higher degree of cross-linking and smaller pore size and vice a versa.
- ♣ These polyacrylamide gels are widely used for sharper resolution of nucleic and or protein mixtures because of low adsorption and lack of electro osmosis.

## Procedure of gel electrophoresis:

- ♣ The equipment consists of two components; an electrophoretic apparatus and a D.C. power supply.
- ♣ The electrophoretic apparatus consist of two buffer reservoirs that are connected by the gel. Platinum electrodes are positioned in each reservoir and are connected to terminal extending from top of the unit. A perspex shield covers the whole assembly.
- ♣ Two different types of apparatus are used for electrophoresis; vertical (**slab gel electrophoresis** and **horizontal type.**
- ♣ In slab gel electrophoresis the gel is set or polymerized into a thin slab between two glass plates. The thickness of the is adjusted by placing spacers between glass plates.
- ♣ Wells for loading the sample can be made at upper end by placing a comb into the gel before it polymerizes.
- ♣ After the gel has set the comb is removed leaving behind the sample wells ditched into the gel. Since a number of wells can be cast side by side, number of sample s can be loaded simultaneously and compared under identical conditions.
- ♣ This technique has become extremely popular in the field of molecular biology.
- ♣ The sample prepared in a high density components such as glycerol or ficoll to prevent its mixing with the upper reservoir buffer is on top of the gel inside the sample well .A tracking dye(usually bromo phenol blue)is often mixed with the sample.
- ♣ The extent of migration of dye gives an index of progress of the electrophoresis process .The dye migrates faster than all macromolecules. Thus if the electrophoresis is

stopped before or just as the dye comes of the bottom of the gel, one can be sure that all the macromolecules are still within the gel.



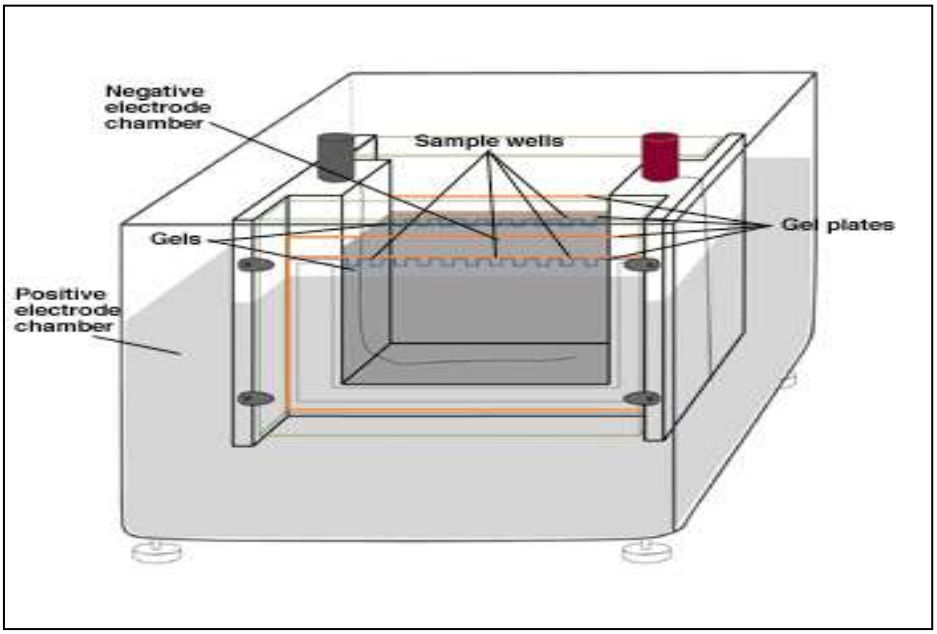




**Figure: Apparatus for slab gel electrophoresis.**

The pH of the buffer is usually fixed at 9 to impart net negative charge to all the macromolecules, which will migrates towards anode under the influence of applied electrical field.

## Detection and quantitative assay:

- ♣ To identify unknown components in the resolved mixture, the electrophoretogram may be compared with the standards.
- ♣ Individual compounds are usually detected and identified in situ by specific detection method. Some of the detection methods are described below.

- ♣ **UV absorption:** Proteins, peptides and nucleic acids absorb in the range of 260-280nm and this property can be exploited to detect these components on electrophoretogram.
- ♣ **Fluorescence detection:** Staining with ethidium bromide and subsequent visualization of electrophoretogram under UV light makes DNA and RNA fluoresce and thus facilitate their detection.
- ♣ Similarly fluorescamine staining is utilized for detecting amino acids, amino acid derivatives, peptides and proteins. Dansyl chloride may also be used in place of fluorescamine.
- ♣ **Staining with specific coloring reagents:** Different dyes are used for detection of different compounds. Many times during staining, the zones of resolved components spread. As a safeguard measure, a fixative may be applied before staining. Excess stain is removed after staining procedure is completed.
- ♣ Proteins can be stained by using bromo phenol blue, Commassie brilliant blue dye or by silver staining procedure and detected on electrophoretogram.
- ♣ Nucleic acid can be visualized after staining with pyronine, methylene blue, toluidine blue or methyl green-pyronine.
- ♣ Lipoproteins are stained with Sudan black. Glycoprotein are subjected to periodate oxidation followed by treatment with Schiff's reagent to give colored complex. For detection of polysaccharide iodine can be used.

♣ **Detection of enzyme in situ:** If the component to be separated is an enzyme, special staining techniques may be used (activity staining) to detect it.

♣ **Autoradiography:** If the components separated are radio labeled, they can be detected by autoradiography.

♣ **Quantitative estimation:** Color intensity of the separated components can be compared with that of the standards to have rough idea about the quantity of each component.

♣ However for more accurate estimation, the compound is eluted from electrophoretogram and the color density is measured colorimetrically and when compared with known standard the concentration (amount) in the sample can be found out.

## APPLICATION OF GEL ELECTROPHORESIS:

♣ Apart from separation of a large number of proteins, nucleic acids and other molecules this technique has several analytical applications in molecular biology and protein chemistry. Some of them are listed below:

♣ Determination of nucleotide sequence of DNA (DNA sequencing).

♣ Nucleic acid hybridizations: Southern (DNA-DNA) Blotting and Northern (DNA-RNA) blotting.

♣ Restriction mapping of DNA.

♣ DNA foot printing (to find out regions of DNA which interact with proteins).

♣ DNA finger printing has wider applications in the field of medicine, law and forensic science laboratories.

- ♣ Restriction Fragment Length Polymorphism (RFLP) is used to detect mutations in various genes in carcinogenesis and other diseases.
- ♣ To check conformational change in nucleic acids and proteins.
- ♣ Determination of subunit stoichiometry of oligomeric proteins or enzymes.
- ♣ Determination of molecular weight of proteins by SDS-PAGE (sodium dodecyl sulfate – polyacrylamide gel electrophoresis).

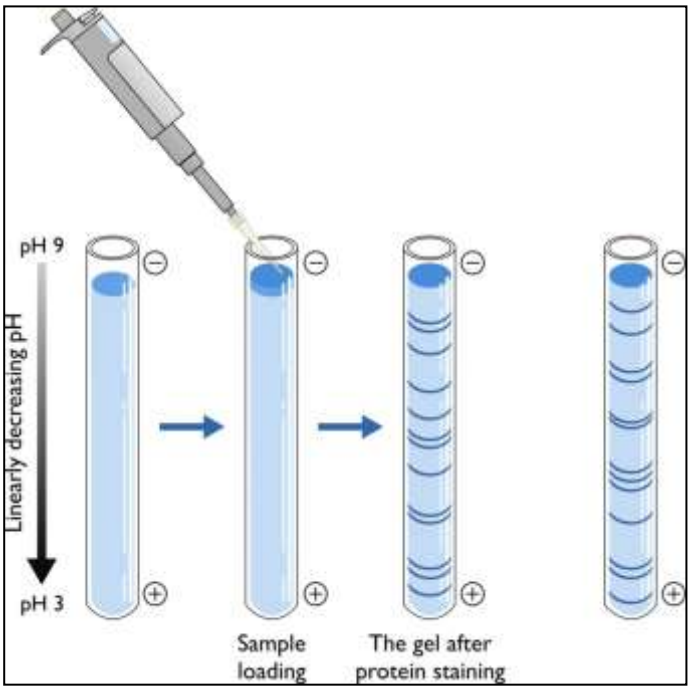
***One word Question***

| *Sr. No.* | Question | Answer |
|---|---|---|
| *1* | What does the electrophoresis apparatus consist of? | Power pack and electrophoresis unit |
| *2* | Agarose gel Electrophoresis is used to separation of | Nucleic acid |
| *3* | What is Ethidium Bromide? | Dye |
| *4* | Agarose gel made up of | Agarose powder |
| *5* | Staining dye use in agarose electrophoresis | Commissive Brilliant blue |
| *6* | Agarose gel is type of _____? | Horizontal electrophoresis |

## ❖ SDS POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE):

- ♣ The mobility of the proteins in acrylamide gels depends on both net charge and size of protein. Hypothetically, two proteins of different molecular weights may migrate towards the anode at the same rate if their size differences are balanced by compensating charge differences.
- ♣ **Sodium dodecyl sulfate (SDS)** is an anionic detergent. When mixture of protein is separated in presence of SDS, the SDS birds to the hydrophobic regions of protein and separates most of them into component subunit, if any disulfide bond are present to hold the protein subunits together then they can be broken in presence of SDS and β-mercaptoethanol.
- ♣ On an average one molecule of SDS binds for every two amino acid residues of proteins, SDS binding also imparts a large negative charge to the denatured, randomly coiled polypeptides.
- ♣ This charge masks the original charge of the protein and all the proteins become uniformly negatively charged. Now the separation of the protein will therefore exclusively depends on their mass (molecular size).
- ♣ The mobility of the proteins is a linear function of their molecular weights. Therefore this technique of SDS PAGE is widely used for studying subunit stoichiometry of oligomeric proteins and for determination of molecular weight of the proteins.

## ISOELECTRIC FOCUSING (IEF):



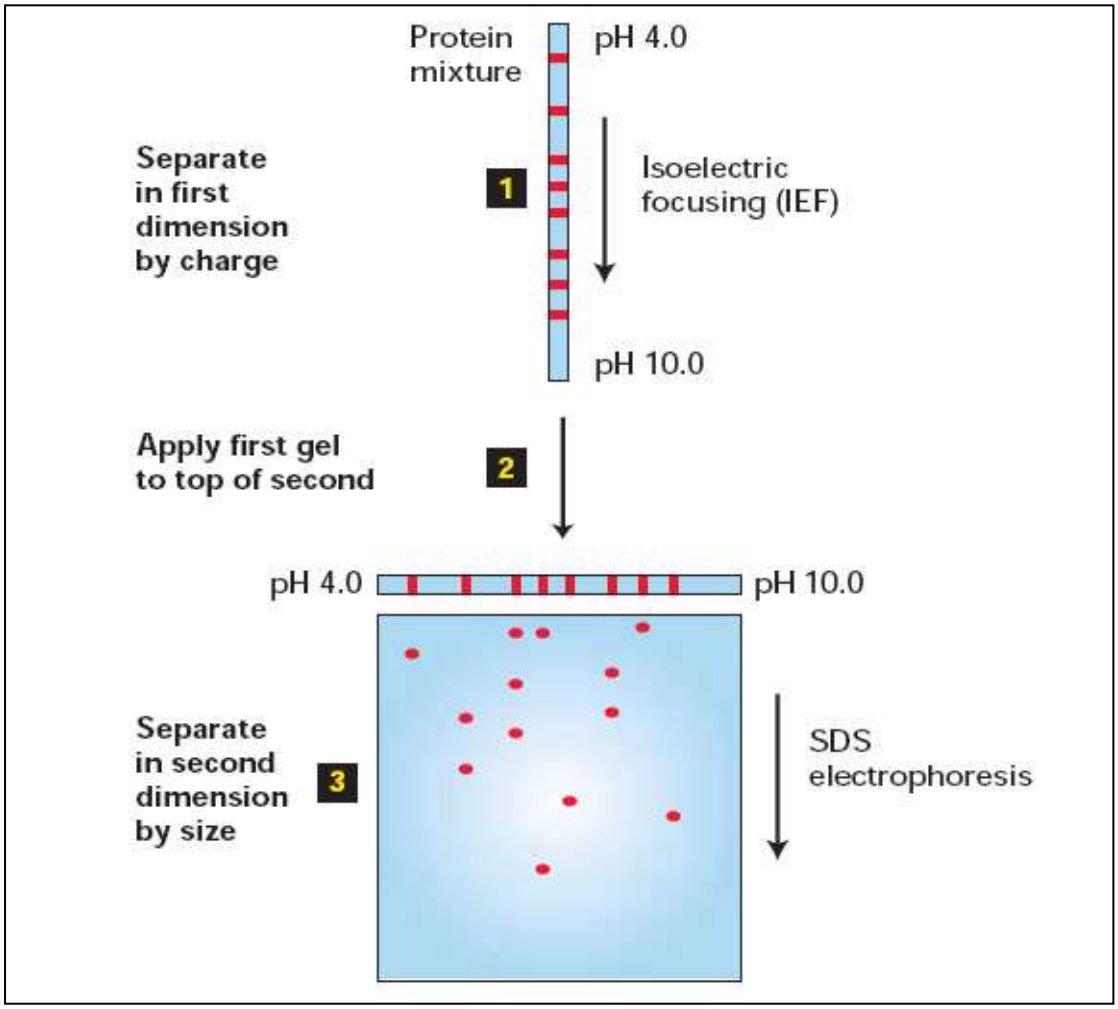

- This method is ideal for the separation of amphoteric substance such as proteins because it is based on the separation of molecules according to their different isoelectric points.
- The method has high resolution and being able to separate the proteins that differing in their isoelectric point by as little as 0.01 of a ph unit due to minute charge differences.
- IEF is carried out much in the same way as electrophoresis with the exception that ampholytes (complex mixture of poly amino ploy carboxylic acid in different ph ranges) are electrophoresed through the gel prior to sample addition in order to establish a pH gradient within it.
- As a protein migrates along the ph gradient, when the pH of the surrounding is same as the isoelectric point of the protein, the net charge on protein will be zero and therefore its movement will stop and it gets stabilized there and will not migrate any further under the influence of the applied electrical field.
- After the electrophoretic run, the gel is first washed with fixing solution (10% trichloro acetic acid) to precipitate the protein and to wash out the small ampholytes.
- Then finally the separated protein components are stained **Coomassie Brilliant Blue dye**.
- The isoelectric point (pI) of particular protein may be determined conveniently by running a mixture of protein of known isoelectric point on the same gel.
- This method is very useful to detect minute charge difference in protein isoelectric focusing is also use full for separation of isoenzymes (different forms of the same enzyme often differing only by one or two amino acid residues).

## TWO DIMENSIONAL GEL ELECTROPHORESIS:

- When the sample is containing mixture up to 5000 proteins then they can't be resolved properly using one dimensional electrophoresis.
- Two dimensional electrophoresis is a powerful technique to resolve such a high number of proteins in a sample.
- 2D electrophoresis involves first subjecting the sample mixture2 isoelectric focusing to separate proteins according to their isoelectric point and then carrying out the SDS polyacrylamide gel electrophoresis in another direction to separate the proteins according

to their molecular weight .since the isoelectric point and molecular weight of a protein are unrelated it is possible to obtain on even distribution of proteins using this parameters in two dimension.

- ♣ This method is capable of a routinely resolving between 1000 and 2000 proteins from a whole cell or tissue extract, and using giant 2D gels, sometime it is possible to resolve even up to 10000 proteins.
- ♣ This achievement had led to the introduction of the term proteome, which describes the complete set of proteins expressed by the genome in an organism. The proteins separated by 2D electrophoresis can be identified by partial sequence analysis followed by searching and matching of the database.
- ♣ The 2D protein profile can be compared between normal and diseased condition to understand the biochemical changes occurring during the particular condition. It is also useful to study tissue specific expression of specific proteins during development, effects of drugs treatment, effect of hormone or growth factor etc.




***One word Question***

| *Sr.* | Question | Answer |
|---|---|---|

| *No.* | | |
|---|---|---|
| *1* | In an SDS-PAGE used for separation of | Protein |
| *2* | SDS is a_____? | Anionic Detergent |
| *3* | Proteins can be visualized directly in gels by | CBB Dye |
| *4* | In isoelectric focusing, proteins are separated on the basis of their | Charge of sample |
| *5* | Proteins are separated in an SDS-PAGE experiment on the basis of their | Molecular weight |
| *6* | Isoelectric focusing method is ideal for the separation of | amphoteric substance |
| *7* | protein migrates along the ph gradient, when the pH of the surrounding is same as the isoelectric point of the protein, | Net charge zero |
| *8* | In 2D electrophoresis, DNA will migrate towards | Anode |
| *9* | The electrophoresis technique that used isoelectric focusing is | 2D electrophoresis |
| *10* | In isoelectric focusing, proteins are separated on the basis of their | Positive & Negative charge |