Electrophoresis

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Electrophoresis

- The term electrophoresis describes the migration of a charged particle under the influence of an electric field.
- Many important biological molecules, such as amino acids, peptides, proteins, nucleotides and nucleic acids, possess ionisable groups and, therefore, at any given pH, exist in solution as electrically charged species either as cations or anions .
- Under the influence of an electric field these charged particles will migrate either to the cathode or to the anode, depending on the nature of their net charge.
- The equipment required for electrophoresis consists basically of two items, a power pack and an electrophoresis unit.
- Electrophoresis units are available for running either vertical or horizontal gel systems. Vertical slab gel units are commercially available and routinely used to separate proteins in acrylamide gels

Definition-

Migration of charged particle in the medium under the influence of an applied electric field.

The rate of migration of an ion in electric field depend on factors-

- Net charge of the molecule
- Size and shape of particle
- Strength of electric field
- Properties of supporting medium
- Temperature

Electrophoretic mobility

- More commonly the term electrophoretic mobility (m) of an ion is used, which is the ratio of the velocity of the ion to field strength (v/E). When a potential difference is applied, therefore, molecules with different overall charges will begin to separate owing to their different electrophoretic mobilities. Even molecules with similar charges will begin to separate if they have different molecular sizes, since they will experience different frictional forces.

Apparatus of gel electrophoresis

Vertical gel apparatus: It is commonly used in sds PAGE for the separation of proteins.

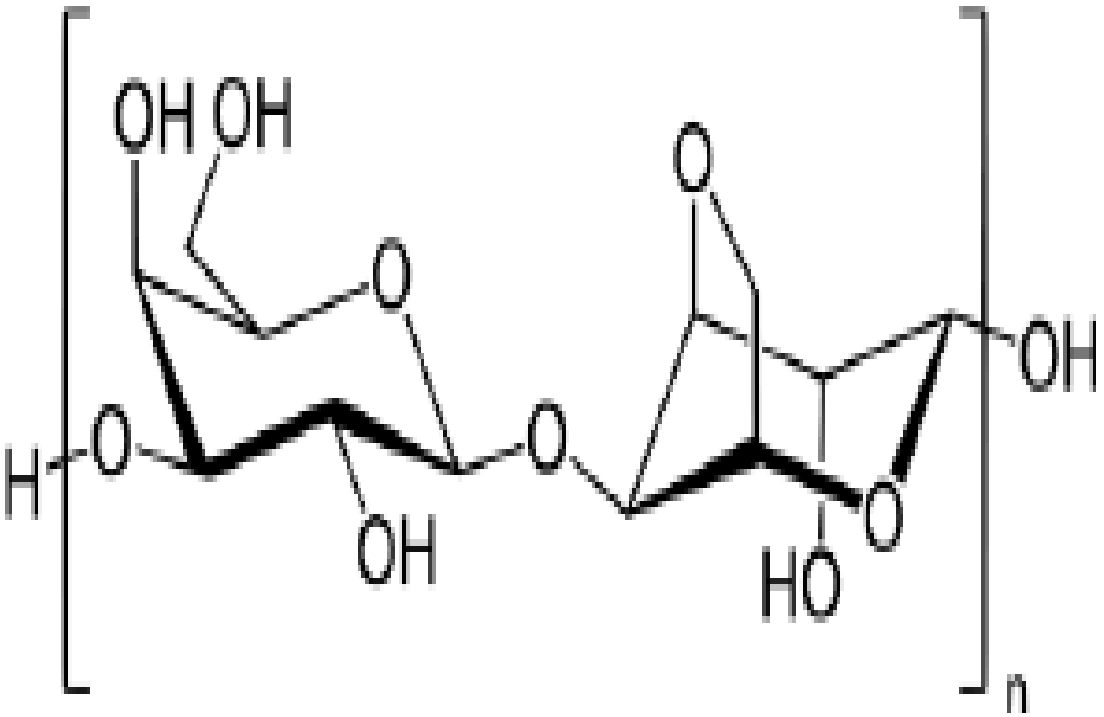
Horizontal gel apparatus: It is used for immune electrophoresis, iso-electric focusing and electrophoresis of DNA and RNA in the agarose gel.

Material required for gel Electrophoresis

- Buffer tank-to hold the buffer
- Buffer
- Electrodes- made of platinum or carbon
- Power supply
- Support media

Agarose gels

- Agarose is a linear polysaccharide (average relative molecular mass about 12 000) made up of the basic repeat unit agarobiose, which comprises alternating units of galactose and 3,6-anhydrogalactose .
- Agarose is one of the components of agar that is a mixture of polysaccharides isolated from certain seaweeds. Agarose is usually used at concentrations of between 1% and 3%.
- Agarose gels are formed by suspending dry agarose in aqueous buffer, then boiling the mixture until a clear solution forms. This is poured and allowed to cool to room temperature to form a rigid gel.
- The gelling properties are attributed to both inter- and intramolecular hydrogen bonding within and between the long agarose chains. This cross-linked structure gives the gel good anticonvectional properties. The pore size in the gel is controlled by the initial concentration of agarose;
- advantage of using agarose is the availability of low melting temperature agarose (62-65 C). As the name suggests, these gels can be reliquified by heating to 65 C and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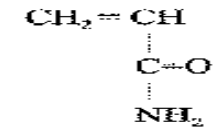
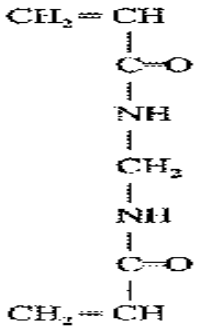
Polyacrylamide gels

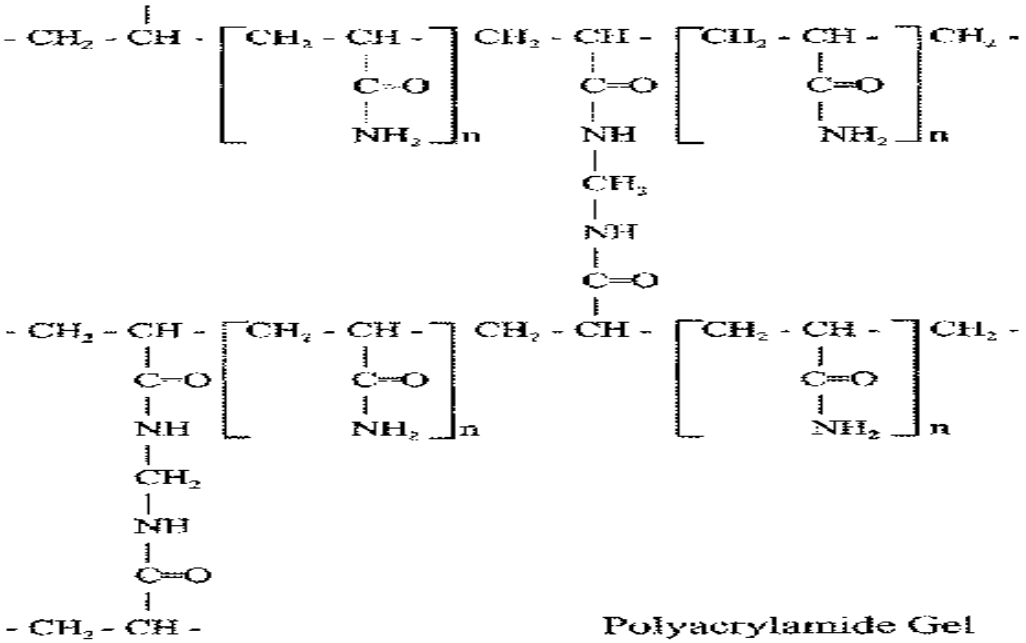
- Electrophoresis in acrylamide gels is frequently referred to as PAGE, Cross-linked polyacrylamide gels are formed from the polymerisation of acrylamide monomer in the presence of smaller amounts of N,N0-methylene-bisacrylamide (normally referred to as 'bis'-acrylamide) . bisacrylamide is essentially two acrylamide molecules linked by a methylene group, and is used as a cross-linking agent.
- The polymerisation of acrylamide is an example of free-radical catalysis, and is initiated by the addition of ammonium persulphate and the base N,N,N0,N0-tetramethylenediamine (TEMED). TEMED catalyses the decomposition of the persulphate ion to give a free radical
- Photopolymerisation is an alternative method that can be used to polymerise acrylamide gels. The ammonium persulphate and TEMED are replaced by riboflavin and when the gel is poured it is placed in front of a bright light for 23 h. Photodecomposition of riboflavin generates a free radical that initiates polymerisation



Acrylamide



N,N'-methylene-bis-acrylamide



Polyacrylamide Gel

Electrophoresis of Nucleic acid

Agarose gel Electrophoresis of DNA

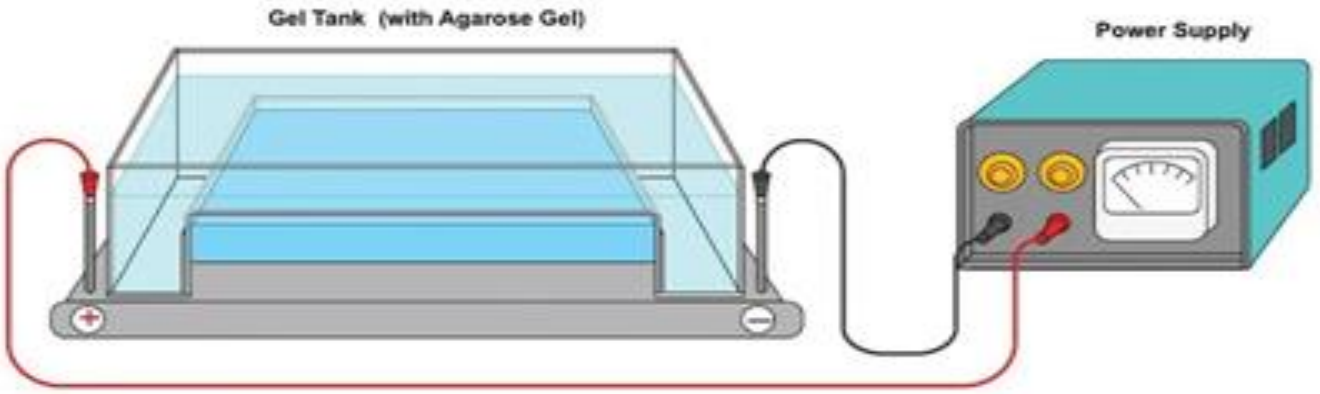
- **Principle** For the majority of DNA samples, electrophoretic separation is carried out in agarose gels. DNA size was referred to in terms of base-pairs (bp) or kilobase-pairs (kbp), when referring to doublestranded DNA. When talking about single-stranded DNA it is common to referto size in terms of nucleotides (nt). Since the charge per unit length (owing to the phosphate groups) in any given fragment of DNA is the same, all DNA samples should move towards the anode with the same mobility under an applied electrical field.
- separation in agarose gels is achieved because of resistance to their movement caused by the gel matrix. The largest molecules will have the most difficulty passing through the gel pores (very large molecules may even be blocked completely), whereas the smallest molecules will be relatively unhindered. Consequently the mobility of DNA molecules during gel electrophoresis will depend on size, the smallest molecules moving fastest.

Electrophoresis Apparatus

- An **electrophoresis chamber** and **power supply**
- **Gel casting trays**, which are available in a variety of sizes and composed of UVtransparent plastic. The open ends of the trays are closed with tape while the gel is being cast, then removed prior to electrophoresis.
- **Sample comb** around which molten medium is poured to form sample wells in the gel.
- **Electrophoresis buffer**, usually Tris-acetate-EDTA (TAE) or Tris-borate-EDTA (TBE).
- **Loading buffer**, contains something dense (e.g. glycerol) to allow the sample to "fall" into the sample wells, and Nucleic acid is before loading on to a gel is first mixed with the gel loading buffer, which usually consists of:-
- Salts: It creates environment with favorable ionic strength and pH of the sample, e.g., Tris-HCl

- Metal chelator: It prevents nucleases to degrade the nucleic acid such as EDTA.
- Loading dyes: It provides color for tracking and easy monitoring of sample. Such as, bromophenol blue, xylene cyanol., which migrate in the gel and allow visual monitoring or how far the electrophoresis has proceeded.
- **Staining:** DNA molecules are easily visualized under an ultraviolet lamp when electrphoresed in the presence of the extrinsic fluor ethidium bromide.
- **Transilluminator** (an ultraviolet light box), which is used to visualize stained DNA in gels.

Agarose Gel Electrophoresis



Procedure

- Agarose, dissolved in gel buffer by boiling, is poured onto a glass or plastic plate, surrounded by a wall of adhesive tape or a plastic frame to provide a gel.
- Loading wells are formed by placing a plastic well-forming template or comb in the poured gel solution, and removing this comb once the gel has set.
- The gel is placed in the electrophoresis tank, covered with buffer, and samples loaded by directly injecting the sample into the wells.
- Samples are prepared by dissolving them in a buffer solution that contains **sucrose, glycerol or Ficoll**, which makes the solution dense and allows it to sink to the bottom of the well. A dye such as **bromophenol blue** is also included in the sample solvent; it makes it easier to see the sample that is being loaded and also acts as a marker of the electrophoresis front.
- All the molecules in the well pile up against the gel within a few minutes of the current being turned on, forming a tight band at the start of the run.

- Once the system has been run, the DNA in the gel needs to be stained and visualised. The reagent most widely used is the fluorescent dye ethidium bromide.
- The gel is rinsed gently in a solution of ethidium bromide and then viewed under ultraviolet light. Ethidium bromide is a cyclic planar molecule that binds between the stacked base-pairs of DNA (i.e. it intercalates) The ethidium bromide concentration therefore builds up at the site of the DNA bands and under ultraviolet light the DNA bands fluoresce orange-red.

Applications of Agarose Gel Electrophoresis

- Agarose gel electrophoresis is a routinely used method for separating DNA or RNA.
- Estimation of the size of DNA molecules
- Analysis of PCR products, e.g. in molecular genetic diagnosis or genetic fingerprinting
- Separation of restricted genomic DNA prior to Southern analysis, or of RNA prior to Northern analysis.
- The agarose gel electrophoresis is widely employed to estimate the size of DNA fragments after digesting with restriction enzymes, e.g. in restriction mapping of cloned DNA.
- Agarose gel electrophoresis is commonly used to resolve circular DNA with different supercoiling topology, and to resolve fragments that differ due to DNA synthesis.
- In addition to providing an excellent medium for fragment size analyses, agarose gels allow purification of DNA fragments. Since purification of DNA fragments size separated in an agarose gel is necessary for a number molecular techniques such as cloning, it is vital to be able to purify fragments of interest from the gel.

- **Electrophoresis of RNA**

Electrophoresis of RNA is usually carried out in agarose gels, and the principle of the separation, based on size, is similar to DNA

Procedure

- Integrity of RNA is checked by electrophoresis in a 2% agarose gel in about 1 h. Ribosomal RNAs (18 S and 28 S) are clearly resolved and any degradation (seen as a smear) or DNA contamination is seen easily. This can be achieved on a 2.5-5% acrylamide gradient gel with an overnight run. Native RNA run on gels can be stained and visualised with ethidium bromide.
- If the study objective is to determine RNA size by gel electrophoresis, then full denaturation of the RNA is needed to prevent hydrogen bond formation within or even between polynucleotides . There are three denaturing agents (formaldehyde, glyoxal and methylmercuric hydroxide) that are compatible with both RNA and agarose.
- After heat denaturation, each of these agents forms adducts with the amino groups of guanine and uracil, thereby preventing hydrogen bond reformation at room temperature during electrophoresis.
- Denatured RNA stains only very weakly with ethidium bromide, so acridine orange is commonly used to visualise RNA on denaturing gels.

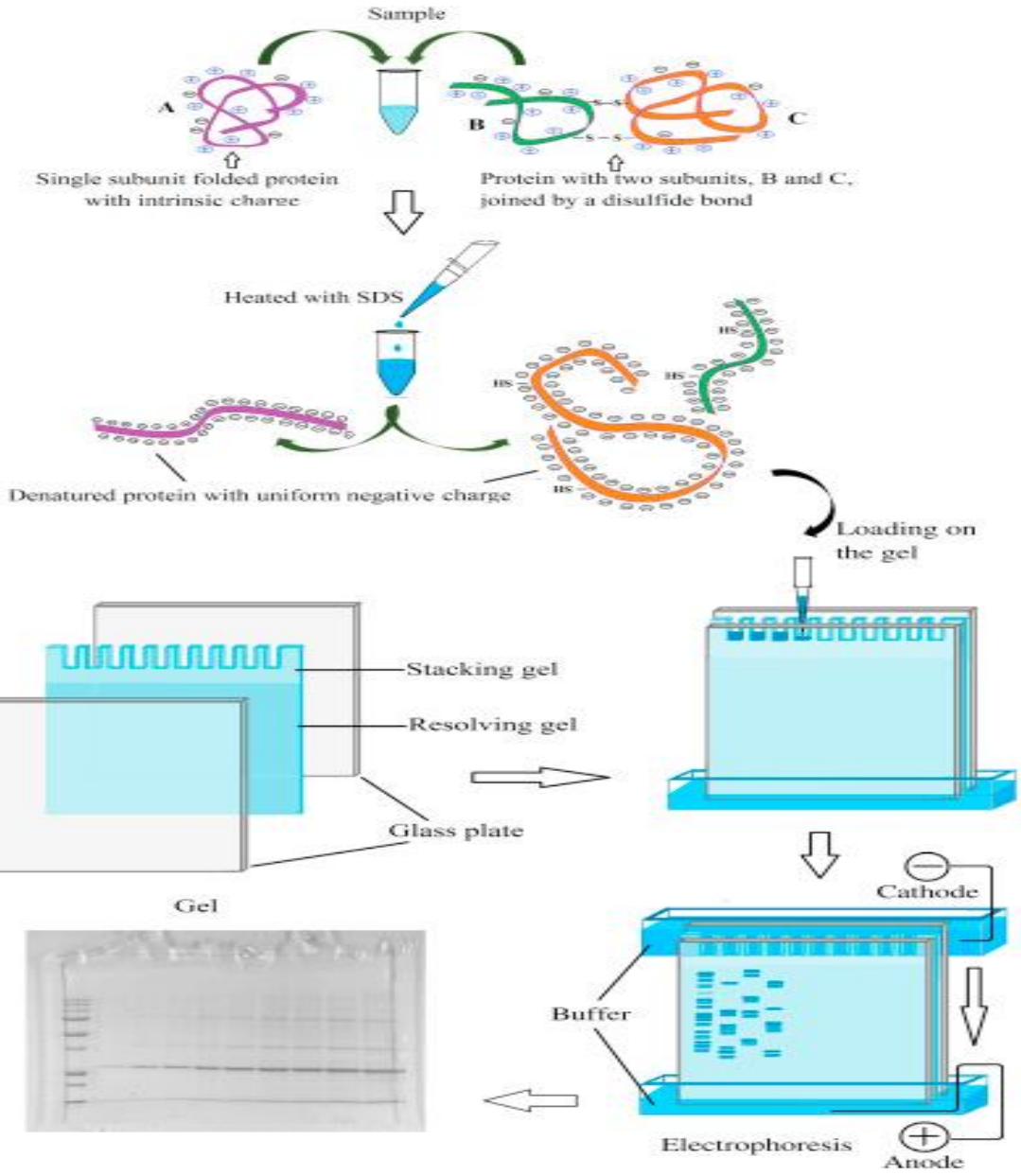
Electrophoresis of Protein

Sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis

- **Principle**-SDS–polyacrylamide gel electrophoresis (SDS–PAGE) is the most widely used method for analysing protein mixtures qualitatively. It is particularly useful for monitoring protein purification and, because the method is based on the separation of proteins according to size, it can also be used to determine the relative molecular mass of proteins.
- **Procedure-**
- **Sample preparation-**Samples to be run on **SDS–PAGE** are firstly boiled for 5 min in sample buffer containing **b-mercaptoethanol**
- **SDS** is an anionic detergent.
- The **mercaptoethanol** reduces any disulphide bridges present that are holding together the protein tertiary structure, and the SDS binds strongly to, and denatures, the protein
- Each protein in the mixture is therefore fully denatured by this treatment and opens up into a rod-shaped structure with a series of negatively charged SDS molecules along the polypeptide chain. On average, one SDS molecule binds for every two amino acid residues.

- The original native charge on the molecule is therefore completely swamped by the negatively charged SDS molecules.
- The sample buffer also contains an ionisable tracking dye, usually **bromophenol blue**, that allows the electrophoretic run to be monitored, and **sucrose or glycerol**, which gives the sample solution density thus allowing the sample to settle easily through the electrophoresis buffer to the bottom when injected into the loading well
- Once the samples are all loaded, a current is passed through the gel. The samples to be separated are not in fact loaded directly into the main **separating gel.** When the main separating gel (normally about 5 cm long) has been poured between the glass plates and allowed to set, a shorter (approximately 0.8 cm) **stacking gel** is poured on top of the separating gel and it is into this gel that the wells are formed and the proteins loaded. The purpose of this stacking gel is to concentrate the protein sample into a sharp band before it enters the main separating gel.

The negatively charged protein–SDS complexes now continue to move towards the anode, the smaller the protein the more easily it can pass through the pores of the gel, whereas large proteins are successively retarded by frictional resistance due to the sieving effect of the gels. Being a small molecule, the bromophenol blue dye is totally unretarded and therefore indicates the electrophoresis front. When the dye reaches the bottom of the gel, the current is turned off, and the gel is removed from between the glass plates and shaken in an appropriate stain solution usually Coomassie Brilliant Blue, and then washed in destain solution. The destain solution removes unbound background dye from the gel, leaving stained proteins visible as blue bands on a clear background.


Sample
A
B
C
Single subunit folded protein with intrinsic charge
Protein with two subunits, B and C, joined by a disulfide bond
Heated with SDS
Denatured protein with uniform negative charge
Loading on the gel
Stacking gel
Resolving gel
Glass plate
Gel
Cathode
Buffer
Electrophoresis
Anode

Visualization of Protein bands

Coomassie Brilliant Blue The most commonly used general protein stain for detecting protein on gels is the sulphated trimethylamine dye Coomassie Brilliant Blue R-250 (CBB). Staining is usually carried out using 0.1% (w/v) CBB in methanol:water:glacial acetic acid 45:45:10, by vol.). This acid–methanol mixture acts as a denaturant to precipitate or fix the protein in the gel, which prevents the protein from being washed out whilst it is being stained. The Coomassie stain is highly sensitive; a very weakly staining band on a polyacrylamide gel would correspond to about 0.1 mg (100 ng) of protein.

silver staining Silver stains are based on techniques developed for methods based on the photographic process. In either case, silver ions (Agþ) are reduced to metallic silver on the protein, where the silver is deposited to give a black or brown band. The silver stain is at least 100 times more sensitive than CBB, detecting proteins down to 1 ng amounts.

Glycoproteins have traditionally been detected on protein gels by use of the

periodic acid–Schiff (PAS) stain. PAS stain is not very sensitive and often gives

very weak, red-pink bands,

Isoelectric focussing gels

Principle-

- This method is ideal for the separation of amphoteric substances such as proteins because it is based on the separation of molecules according to their different isoelectric points . The method has high resolution, being able to separate proteins that differ in their isoelectric points by as little as 0.01 of a pH unit.
- Separation is achieved by applying a potential difference across a gel that contains a pH gradient. The pH gradient is formed by the introduction into the gel of compounds known as ampholytes, which are complex mixtures of synthetic polyaminopolycarboxylic acids
- Depending on which point on the pH gradient the sample has been loaded, proteins that are initially at a pH region below their isoelectric point will be positively charged and will initially migrate towards the cathode. As they proceed, however, the surrounding pH will be steadily increasing, and therefore the positive charge on the protein will decrease correspondingly until eventually the protein arrives at a point where the pH is equal to its isoelectric point

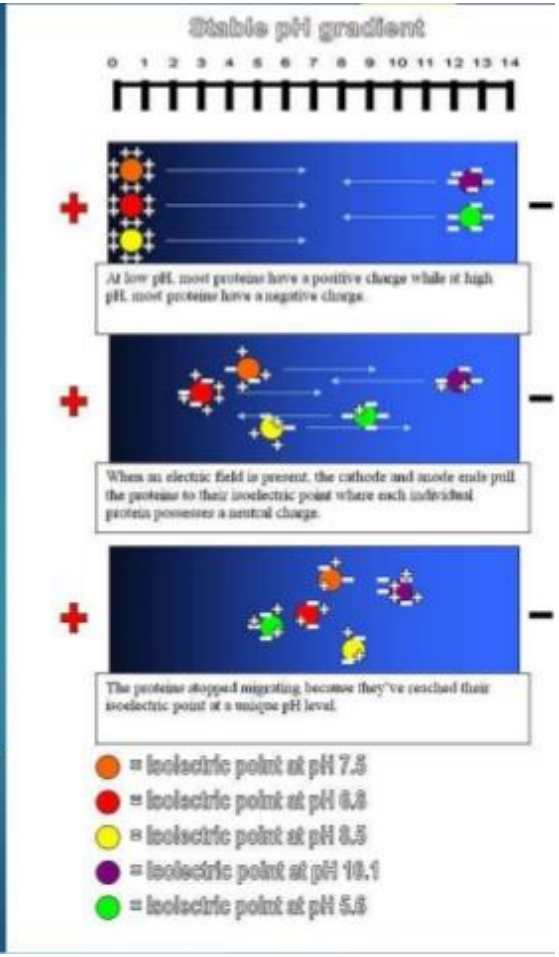
- The protein will now be in the zwitterion form with no net charge, so further movement will cease. Likewise, substances that are initially at pH regions above their isoelectric points will be negatively charged and will migrate towards the anode until they reach their isoelectric points and become stationary.

Procedure-

- To prepare a thin-layer IEF gel, carrier ampholytes, covering a suitable pH range ,polyacrylamide gel
- Once the gel has set, the glass plates are prised apart to reveal the gel stuck to one of the glass sheets. Electrode wicks, which are thick (3 mm) strips of wetted filter paper (the anode is phosphoric acid, the cathode sodium hydroxide) are laid along the long length of each side of the gel and a potential difference applied.
- Under the effect of this potential difference, the ampholytes form a pH gradient between the anode and cathode. The power is then turned off and samples applied by laying on the gel small squares of filter paper soaked in the sample.

Iso-electric Point (pI)

- The pH at which net charge on protein becomes zero.
  - Below pI = Positive Charge.
  - Above pI = Negative Charge.
- Proteins move toward the electrode with the opposite charge.
- During motion, proteins will either pick or loose protons.
- Different from conventional electrophoresis migrate to steady state.



- A voltage is again applied for about 30 min to allow the sample to electrophorese off the paper and into the gel, at which time the paper squares can be removed from the gel.
- Following electrophoresis, the gel must be stained to detect the proteins. However, this cannot be done directly, because the ampholytes will stain too, giving a totally blue gel. The gel is therefore first washed with fixing solution (e.g. 10% (v/v) trichloroacetic acid). This precipitates the proteins in the gel and allows the much smaller ampholytes to be washed out. The gel is stained with Coomassie Brilliant Blue and then destained

Applications

- IEF is a highly sensitive analytical technique and is particularly useful for studying microheterogeneity in a protein.
- The method is particularly useful for separating isoenzymes which are different forms of the same enzyme often differing by only one or two amino acid residues.

2D gel electrophoresis

- Analysis of sample by one-dimensional electrophoresis is the most common form of protein gel electrophoresis.
- For separation and analysis of hundreds to thousands of proteins in one gel, a powerful electrophoretic method called two-dimensional gel electrophoresis is used.
- 2D gel electrophoresis separates a mixture of proteins according to two properties, one in each dimension.
- The first dimensions involve the separation based on native isoelectric point (pI), using form of electrophoresis called isoelectric focusing (IEF).
- Second dimensions separate mass using SDS-PAGE.
- This technique provides highest resolution for the protein analysis.