ELECTROPHORESIS

Introduction

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

Father of Electrophoresis

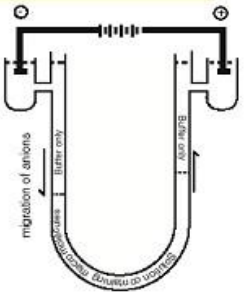
Arne Tiselius
(Sweden, 1902-1971)

The Nobel Prize in Chemistry 1948

"for his research on electrophoresis and adsorption analysis, especially for his discoveries concerning the complex nature of the serum proteins"

This type of cell is essentially a bent glass tube with electrolyte reservoirs containing the cathode and anode, and a buffer containing the macromolecules that need electrophoresed.

He tested horse serum in the apparatus and found 4 distinct bands consisting of albumin and 3 globulin components, which he named "α," "β," and "γ."



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Electrophoresis

- I t is a separation technique used for the separation of colloidal substances carrying positive or negative charge.
- It is Simple, rapid and highly sensitive.

It is used for separation of

- Proteins in body fluids: serum, urine, CSF
- Proteins in erythrocytes: hemoglobin
- Nucleic acids: DNA, RNA

Clinical applications of Electrophoresis

- Serum Protein Electrophoresis
- Lipoprotein Analysis
- Diagnosis of Haemoglobinopathies and Haemoglobin A1c
- Determination of Serum Protein Phenotypes and Micro heterogeneities eg. α1- antitrypsin deficiency, MM
- Genotyping of Proteins eg. ApoE analysis for Alzheimer's disease (polymorphic protein)
- Small Molecules (Drugs, Steroids) Monitoring
- Cerebrospinal Fluid Analysis
- Urine Analysis (determination of GNs)

Principle :

- Any charged ion or molecule migrates when placed in an electric field.
- The rate of migration depends upon
    1. Net charge of molecule
    2. Size and shape of particle
    3. Strength of electrical field
    4. Properties of Supporting medium
    5. Temperature of operation

It can be represented by equation:

$$v = E * q / r$$

v = velocity of migration of the molecule.

E = electric field in volts per cm

q = net electric charge on the molecule

f = frictional coefficient

The movement of charged particle in an electric field is expressed in terms of electrophoretic mobility, denoted by μ.

where, $\mu = v/E$ OR $\mu = q/f$

For molecules with similar conformation f varies with size but not with shape. Thus electrophoretic mobility (μ) of a molecule is directly proportional to charge density (charge\mass ratio).

Factors affecting Electrophoresis

Electrophoretic velocity depends on:

Inherent Factors

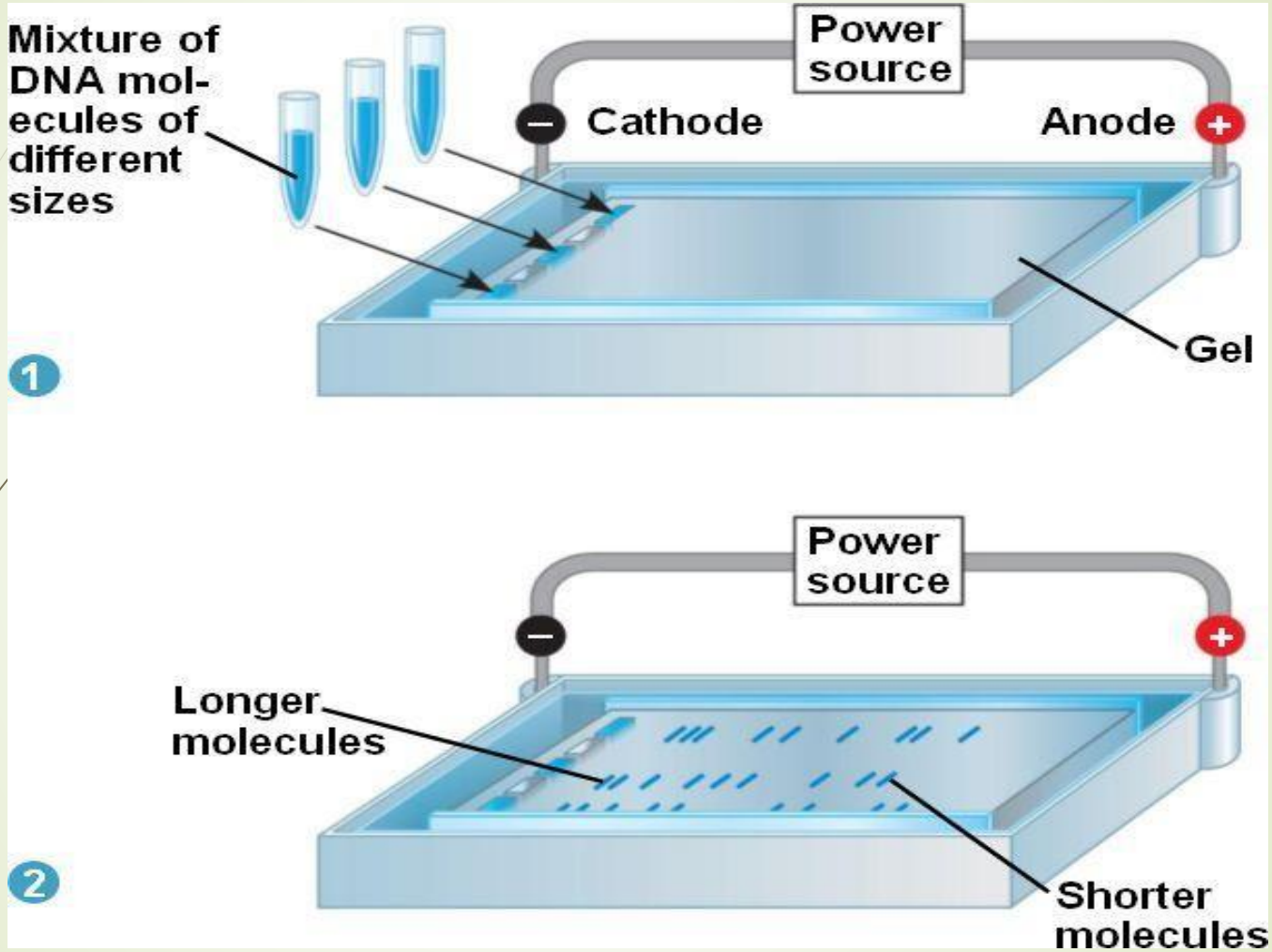
- Magnitude of its charge
- Charge density
- Molecular weight
- Tertiary or quaternary structure (i.e., its shape).

External Environment

- Solution pH
- Electric field
- Solution viscosity
- Temperature

Mobility

- Under the electrical field, the mobility of the particle is determined by two factors:
  - Its charge, – higher the charge greater the electrophoretic mobility.
  - Frictional coefficient
- Size and shape of the particle decide the velocity with which the particle will migrate under the given electrical field and the medium. Bigger the molecule greater are the frictional and electrostatic forces exerted on it by the medium. Consequently, larger particles have smaller electrophoretic mobility compared to smaller particles rounded contours elicit lesser frictional and electrostatic retardation compared to sharp contours. Therefore globular protein move faster than fibrous protein.

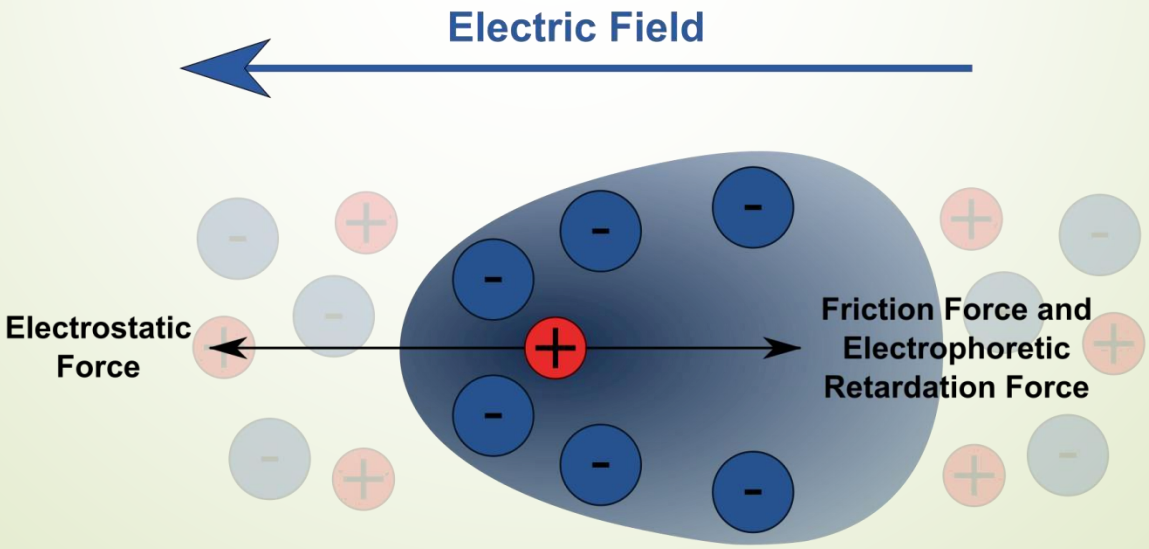

Mixture of DNA mol-ecules of different sizes
Power source
Cathode
Anode
Gel
1
Power source
Longer molecules
Shorter molecules
2

Strength of electrical field

- It determined by the force exerted on the particle, and the charge the particle carrying.

F=QV

when force is exerted on the particle it start moving, however the moment is restricted by the experience of the frictional force because of the viscosity.



Effect of pH on Mobility

- As the molecule exist as amphoteric , they will carry the charges based on the solvent pH.
- Their overall net charge is NEUTRAL when it is at zwitter ion state. And hence the mobility is retarded to zero.
- Mobility is directly proportional to the magnitude of the charge, which is functional of the pH of solvent.
- The pH is maintained by the use of Buffers of different pH.

Power supply

- Drives the moment of ionic species in the medium and allow the adjustment and control of the current or voltage.
- Constant delivery is required.
- Pulsed power can also be applied.

Buffer

- The buffer in electrophoresis has two fold purpose:
  - Carry applied electrical current
  - They set the pH as which electrophoresis is carried out.
- Thus they determine;
  - Type of charge on solute.
  - Extent of ionization of solute
  - Electrode towards which the solute will migrate.
- The buffer ionic strength will determine the thickness of the ionic cloud.

Commonly buffers used;

Buffer	pH value
Phosphate buffer	around 7.0
Tris-Borate-EDTA buffer (TBE)	around 8.0
Tris-Acetate EDTA buffer (TAE)	above 8.0
Tris Glycine buffer (TG)	more than 8.5
Tris -Citrate-EDTA buffer (TCE)	around 7.0
Tris -EDTA buffer (TE)	around 8.0
Tris -Maleic acid -EDTA buffer (TME)	around 7.5
Lithium Borate - buffer (LB)	around 8.6

Supporting medium

- Supporting medium is an matrix in which the protein separation takes place.
- Various type has been used for the separation either on slab or capillary form.
- Separation is based on to the charge to mass ratio of protein depending on the pore size of the medium, possibly the molecular size.

Properties:

Chemical nature	inert
Availability	easy
Electrical conductivity	high
Adsorptivity	low
Sieving effect	desirable
Porosity	controlled
Transparency	high
Electro-endosmosis (EEO)	low
Rigidity	moderate to high
Preservation	feasible
Toxicity	low
Preparation	easy

Types of Electrophoresis

1) Zone Electrophoresis

a) Paper Electrophoresis

b) Gel Electrophoresis

c) Thin Layer Electrophoresis

d) Cellulose acetate Electrophoresis

2) Moving Boundary Electrophoresis

a) Capillary Electrophoresis

b) Isotachophoresis

c) Isoelectric Focussing

d) Immuno Electrophoresis

ZONE Electrophoresis-

Paper Electrophoresis

- It is the form of electrophoresis that is carried out on filter paper. This technique is useful for separation of small charged molecules such as amino acids and small proteins.
- Filter paper : It is the stabilizing medium. We can use whatman filter paper, cellulose acetate filter paper or chromatography paper.
- Apparatus : Power pack, electrophoretic cell that contains electrodes, buffer reservoirs, support for paper, transparent insulating cover.

Paper Electrophoresis

- Sample application :The sample may be applied as a spot(about 0.5 cm in diameter) or as a uniform streak.
- Electrophoretic run: The current is switched on after the sample has been applied to the paper and the paper has been equilibrated with the buffer. The types of buffer used depends upon the type of separation. Once removed, the paper is dried in vaccum oven.
- Detection and quantitative assay: To identify unknown components in the resolved mixture. The electrophoretogram may be compared with another electrophoretogram on which standard components have been electrophoresced under identical conditions. Physical properties like fluorescence, ultraviolet absorption or radioactivity are exploited for detection.

Gel Electrophoresis

It is a technique used for the separation of Deoxyribonucleic acid, Ribonucleic acid or protein molecules according to their size and electrical charge using an electric current applied to a gel matrix.

- What is a gel?

Gel is a cross linked polymer whose composition and porosity is chosen based on the specific weight and porosity of the target molecules.

Types of Gel:

- Agarose gel.
- Polyacrylamide gel.

Agarose Gel Electrophoresis

- A highly purified uncharged polysaccharide derived from agar.
- Used to separate macromolecules such as nucleic acids, large proteins and protein complexes.
- It is prepared by dissolving 0.5% agarose in boiling water and allowing it to cool to 40°C.
- It is fragile because of the formation of weak hydrogen bonds and hydrophobic bonds.

ADVANTAGES:

- Easy to prepare and small concentration of agar is required.
- Resolution is superior to that of filter paper.
- Large quantities of proteins can be separated and recovered.
- Adsorption of negatively charged protein molecule is negligible.
- It adsorbs proteins relatively less when compared to other medium.
- Sharp zones are obtained due to less adsorption.
- Recovery of protein is good, good method for preparative purpose.

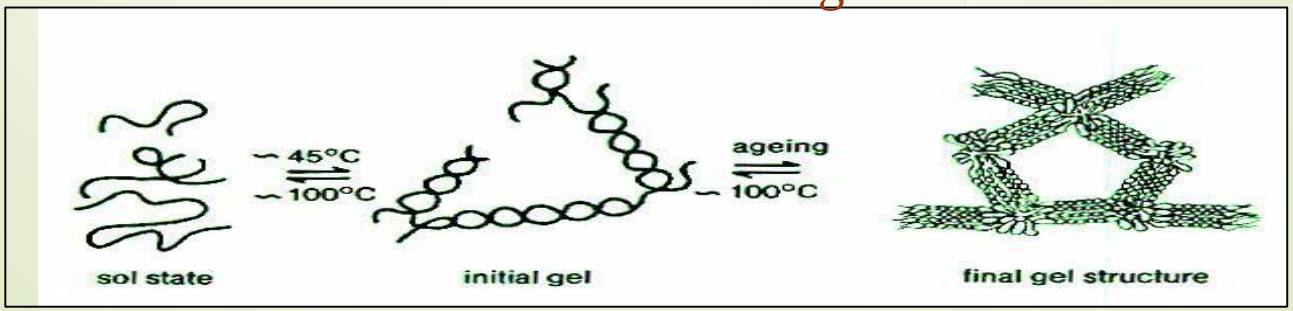
DISADVANTAGES:

- Electro osmosis is high.
- Resolution is less compared to polyacrylamide gels.
- Different sources and batches of agar tend to give different results and purification is often necessary.

APPLICATION:

- Widely used in immuno electrophoresis

Gel Structure of Agarose:



Polyacrylamide gel Electrophoresis

- Frequently referred to as PAGE.
- Cross-linked polyacrylamide gel are formed from the polymerization of the monomer in presence of small amount of N,N''-methylene- bisacrylamide.
- Bisacrylamide – two acrylamide linked by the methylene group.
- The polymerization of the acrylamide is an example for free radical catalysis.
- They are defined in terms of total percentage of acrylamide present, and pore size vary with conc.

Polyacrylamide gel Electrophoresis

- Commonly used components: Acrylamide monomers, Ammonium persulphate, Tetramethylenediamine (TEMED), N,N'-methylenebisacrylamide.
- These free radicals activate acrylamide monomers inducing them to react with other acrylamide monomers forming long chains.
- Used to separate most proteins and small oligonucleotides because of the presence of small pores.

Polyacrylamide gel Electrophoresis

Made in conc. between 3-30% acrylamide.
Thus low % has large pore size and vice versa.

Proteins are separated on the basis of charge to mass ratio and molecular size, a phenomenon called Molecular sieving.

ADVANTAGES:

- Gels are stable over wide range of pH and temperature.
- Gels of different pore size can be formed.
- Simple and separation speed is good comparatively.

Cellulose acetate Electrophoresis

- Thermoplastic resin made by treating cellulose with acetic anhydride to acetylate the hydroxyl group.
- When dry, membrane contain about 80% air space within fibers and brittle film.
- As the film is soak in buffer, the space are filled.
- Because of their opacity, the film has to be made transparent by soaking in 95:5 methanol:glacial acetic acid.
- It can be stored for longer duration.

Moving Boundary Electrophoresis

Isoelectric focusing electrophoresis

- IEF separates amphoteric compounds, such as proteins, with increased resolution in a medium possessing a stable pH gradient

Isotachophoresis

- Completely separates smaller ionic substances into adjacent zones tat contact one another with no overlap &all migrate at the same rate.

Capillary Electrophoresis

- Capillary electrophoresis is an analytical technique that separates ions based on their electrophoretic mobility with the use of an applied voltage.
- The electrophoretic mobility is dependent upon the charge of the molecule, the viscosity, and the atom's radius. The rate at which the particle moves is directly proportional to the applied electric field--the greater the field strength, the faster the mobility. Neutral species are not affected, only ions move with the electric field. If two ions are the same size, the one with greater charge will move the fastest. For ions of the same charge, the smaller particle has less friction and overall faster migration rate.
- Capillary electrophoresis is used most predominately because it gives faster results and provides high resolution separation. It is a useful technique because there is a large range of detection methods available.

Capillary Electrophoresis

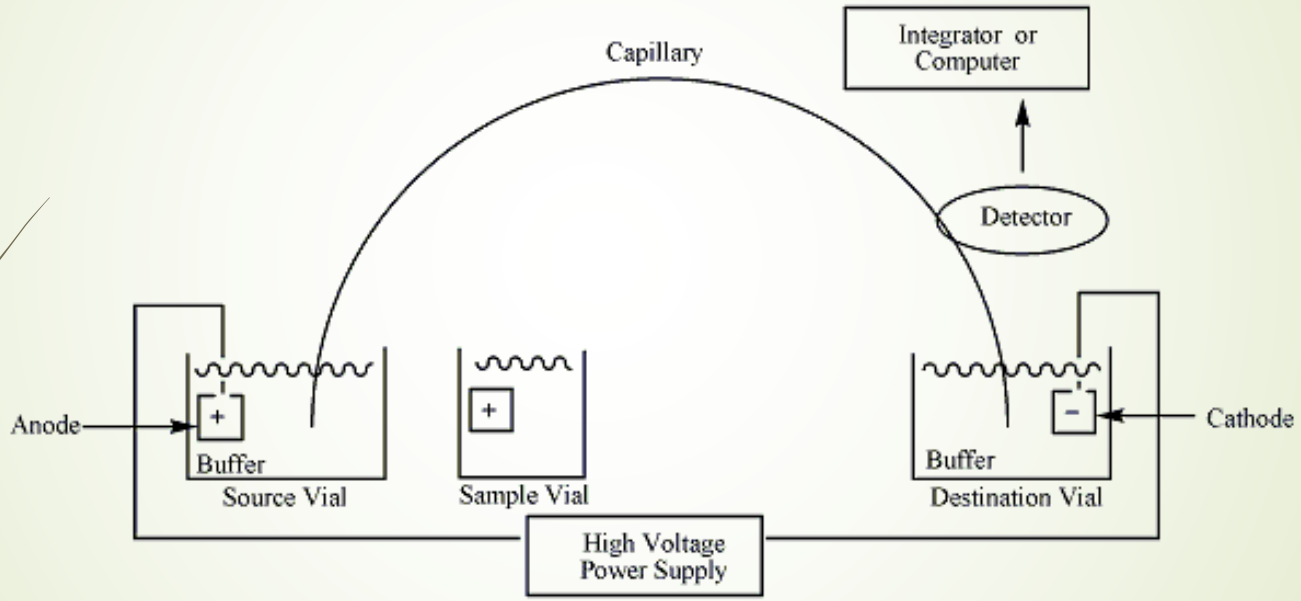
- Endeavors in capillary electrophoresis (CE) began as early as the late 1800's. Experiments began with the use of glass U tubes and trials of both gel and free solutions.
- In 1930, Arnes Tiselius first showed the capability of electrophoresis in an experiment that showed the separation of proteins in free solutions. His work had gone unnoticed until Hjerten introduced the use of capillaries in the 1960's. However, their establishments were not widely recognized until Jorgenson and Lukacs published papers showing the ability of capillary electrophoresis to perform separations that seemed unachievable.
- Employing a capillary in electrophoresis had solved some common problems in traditional electrophoresis. For example, the thin dimensions of the capillaries greatly increased the surface to volume ratio, which eliminated overheating by high voltages. The increased efficiency and the amazing separating capabilities of capillary electrophoresis spurred a growing interest among the scientific society to execute further developments in the technique.

Capillary Electrophoresis

Instrumental Setup

- A typical capillary electrophoresis system consists of a high-voltage power supply, a sample introduction system, a capillary tube, a detector and an output device.
- Some instruments include a temperature control device to ensure reproducible results. This is because the separation of the sample depends on the electrophoretic mobility and the viscosity of the solutions decreases as the column temperature rises. Each side of the high voltage power supply is connected to an electrode. These electrodes help to induce an electric field to initiate the migration of the sample from the anode to the cathode through the capillary tube. The capillary is made of fused silica and is sometimes coated with polyimide.
- Each side of the capillary tube is dipped in a vial containing the electrode and an electrolytic solution, or aqueous buffer. Before the sample is introduced to the column, the capillary must be flushed with the desired buffer solution. There is usually a small window near the cathodic end of the capillary which allows UV-VIS light to pass through the analyte and measure the absorbance. A photomultiplier tube is also connected at the cathodic end of the capillary, which enables the construction of a mass spectrum, providing information about the mass to charge ratio of the ionic species.

Capillary Electrophoresis



Capillary Electrophoresis

Types

There are six types of capillary electroseparation available:

1-Capillary zone electrophoresis (CZE)

2-Capillary gel electrophoresis (CGE)

3-Micellar electrokinetic capillary chromatography (MEKC)

4-Capillary electrochromatography (CEC)

5-Capillary isoelectric focusing (CIEF)

6-Capillary isotachophoresis (CITP).

Capillary Electrophoresis

Applications-

- May be used for the simultaneous determination of the ions $NH_4^{+,}$, Na^+, K^+, Mg^{2+} and Ca^{2+} in saliva.
- One of the main applications of CE in forensic science is the development of methods for amplification and detection of DNA fragments using polymerase chain reaction.
- Capillary electrophoresis (CE) has become an important, cost-effective approach to do DNA sequencing.
- A specialized type of CE, affinity capillary electrophoresis (ACE), utilizes intermolecular binding interactions to understand protein-ligand interactions.
- A major use of CE by forensic biologists is typing of STR from biological samples to generate a profile from highly polymorphic genetic markers which differ between individuals.

Continuous and discontinuous buffer systems

- A continuous buffer system has only a single separating gel and uses the same buffer in the tanks and the gel.
- In a discontinuous system a non-restrictive large pore gel, called a stacking gel, is layered on top of a separating gel.E.g. SDS PAGE Electrophoresis.
- The resolution obtainable in a discontinuous system is much greater than that obtainable in a continuous one. However the continuous system is easier to set up.

ENHANCED-RESOLUTION TECHNIQUES:

- Isotachophoresis
- Disk electrophoresis
- Isoelectric focusing

Isoelectric focusing

- Electrophoretic method that separates proteins according to the iso-electric points.
- Is ideal for separation of amphoteric substances.
- Separation is achieved by applying a potential difference across a gel that contain a pH gradient.
- Isoelectric focusing requires solid support such as agarose geI and poly acrylamide gel.
- Isooelectric focusing gels contain synthetic buffers called ampholytes that smooth the pH gradients.
- Ampholytes are complex mixtures of synthetic polyamino-polycarboxylic acids
- Commercially available ampholytes are-

  BIO-LYTE, PHARMALYTE

Isoelectric focusing

- It gives good separation with a high resolution compared to any other method
- Resolution depends on

1. The pH gradient,
2. The thickness of the gel
3. Time of electrophoresis
4. The applied voltage
5. Diffusion of the protein into the gel

Preparation of IEF gel

Carrier ampholytes (suitable pH) and riboflavin mixed with acrylamide solution

↓

Mixture is poured over a glass plate which contain spacer

↓

Second glass plate is placed on first

↓

Gel is polymerised

Preparation of IEF gel

This takes 2-3 hr

After the gel has set glass plates are prised apart

Electrode wicks are laid along the long length of each side of the gel

Potential difference is applied

Ampholytes form a pH gradient between anode and cathode

Preparation of IEF gel

The power is then turned off

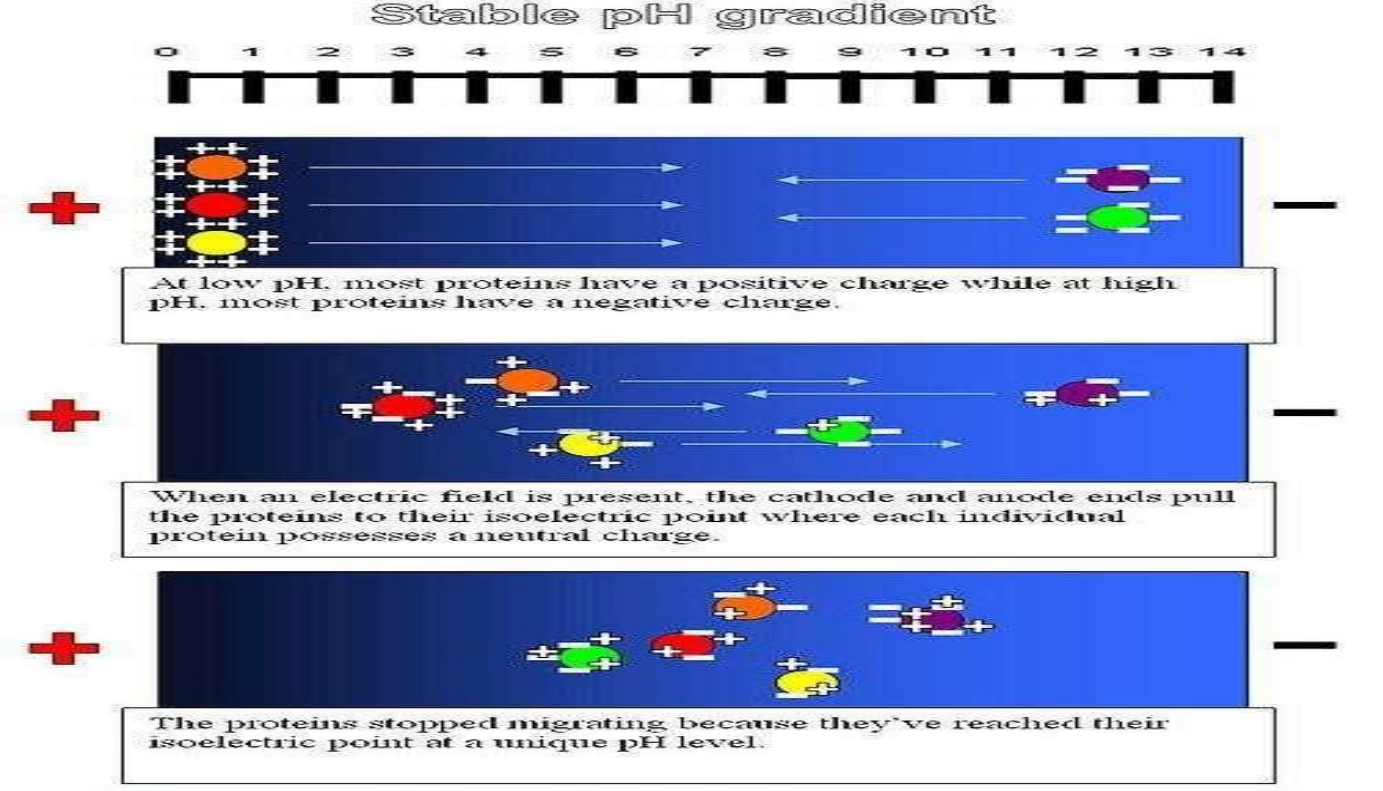
Samples applied by laying on gel filter paper soaked in the sample

Voltage is again applied for 30 min

Proteins having positive charge will migrates towards the cathode. negatively charged protein will migrates towards anode

Become stationary when they reaches isoelectric point

Preparation of IEF gel



Preparation of IEF gel

The gel is washed with trichloroacetic acid

↓

This precipitaes the proteins and allows smaller ampholytes to be washed out

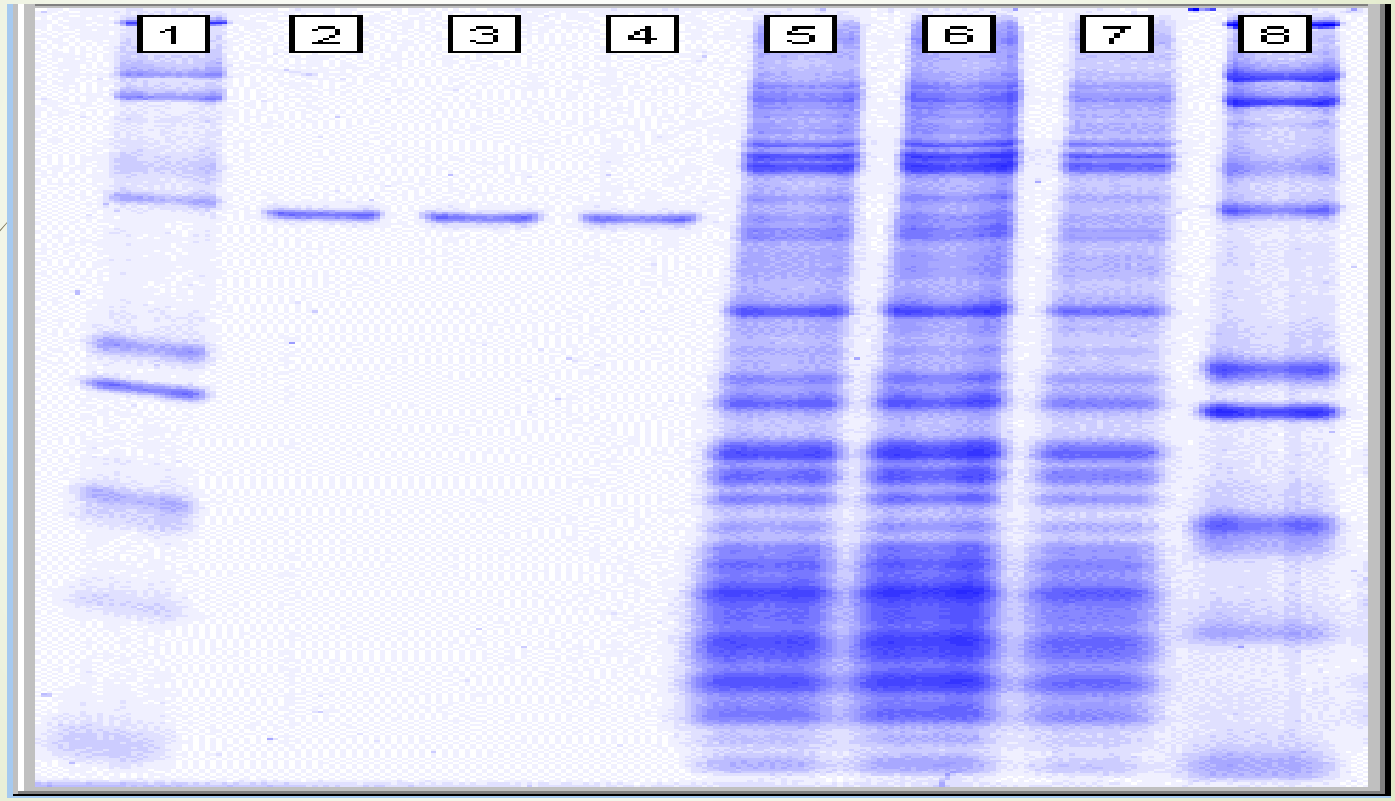
↓

Gel is stained with Coomasie Brilliant Blue

↓

Destained

A typical isoelectric focusing gel



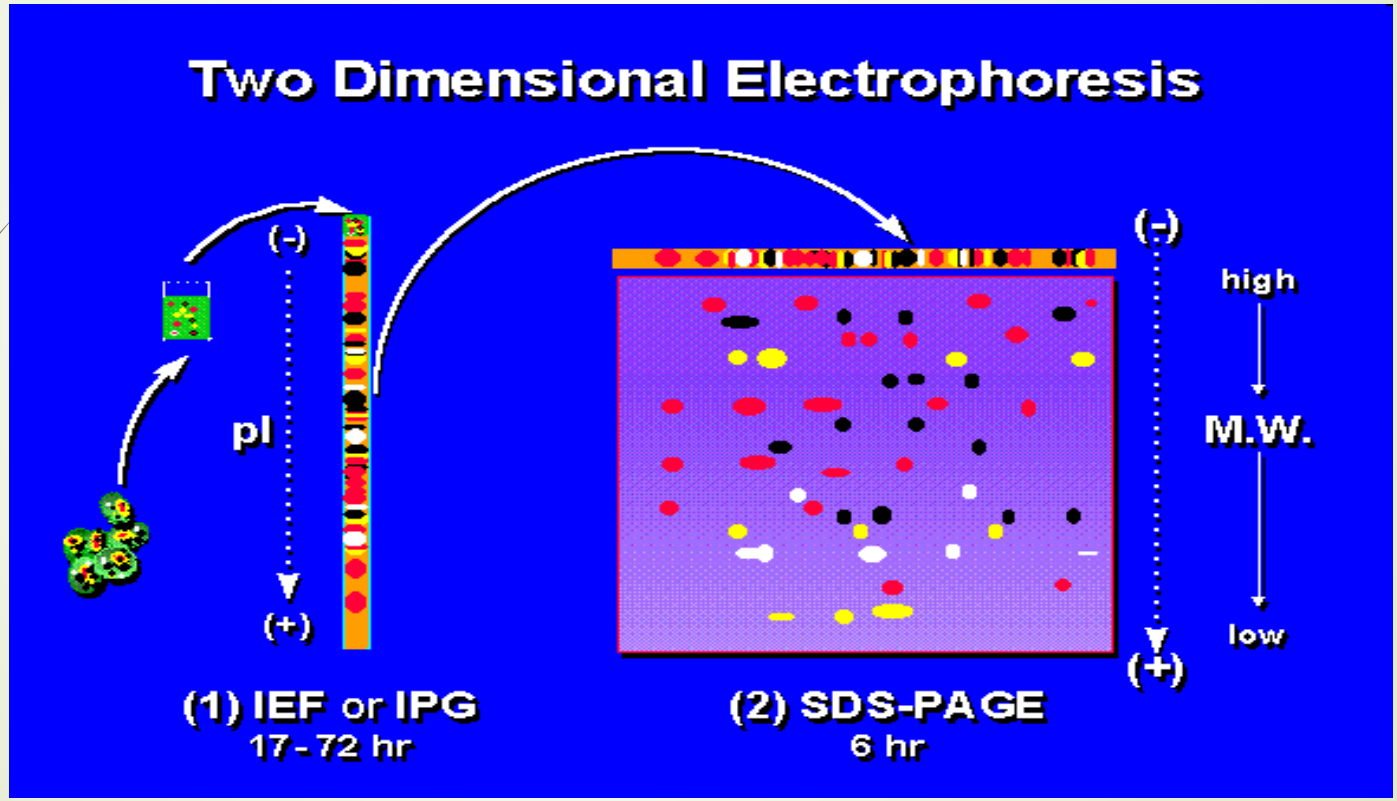
Two-dimensional Electrophoresis

- This technique combines the technique IEF (first dimension), which separates proteins in a mixture according to charge (PI), with the size separation technique of SDS- PAGE (second dimension).
- The combination of these two technique to give two-dimension(2-D) PAGE provides a highly sophisticated analytical method for analysing protein mixtures.
- To maximise separation, most workers use large format 2-D gels(20cm x 20cm).

Two-dimensional Electrophoresis

- Although the mini gel system can be used to provide useful separation in some cases.
- For large-format gels the first dimension(isoelectric focusing) is carried out in an acrylamide gel that has been cast on a plastic strip(18cm x3mm wide).
- The gel contains ampholytes (for forming pH gradient) together with 8M urea and a non-ionic detergent, both of which denature and maintain the solubility of the proteins being analysed.

Two-dimensional Electrophoresis



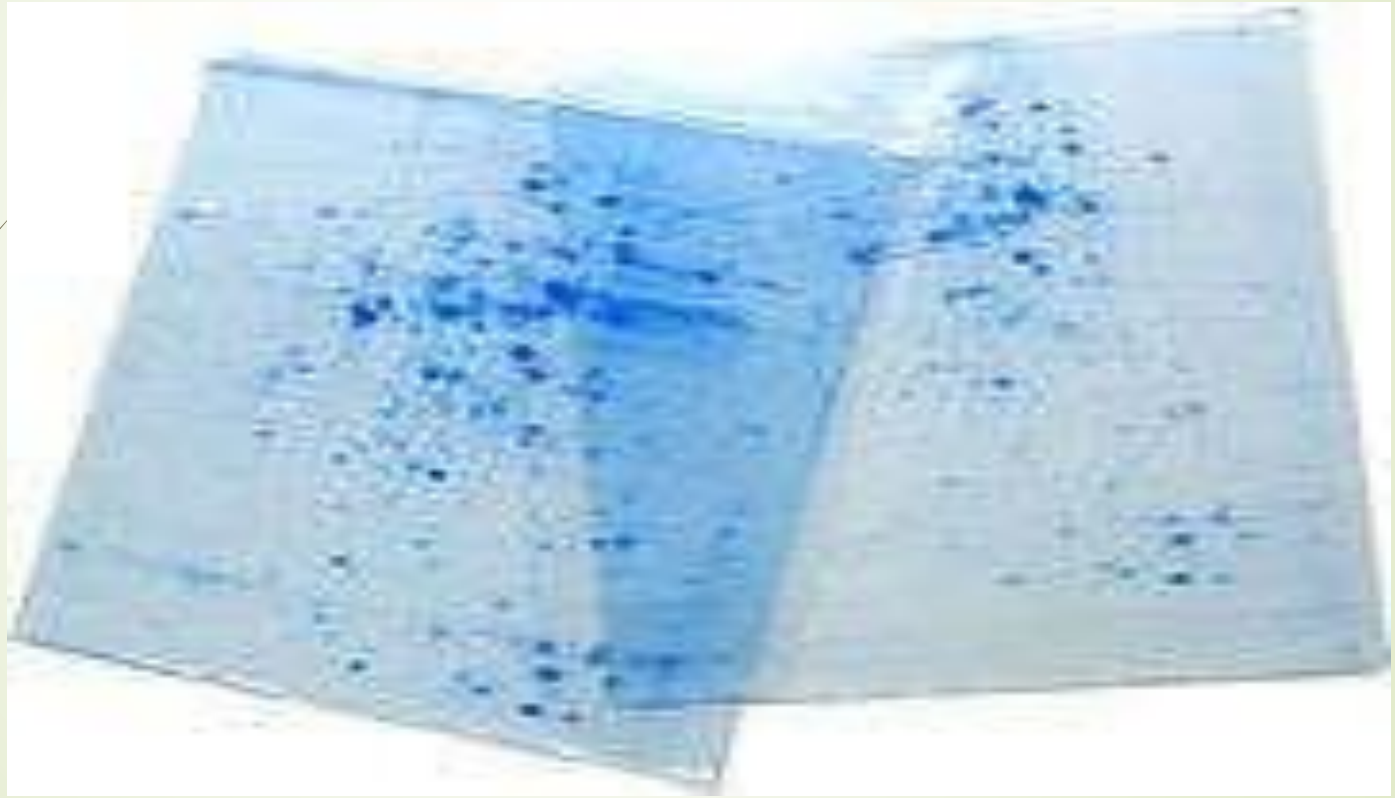
Two-dimensional Electrophoresis

- The denatured proteins therefore separate in this gel according to their isoelectric points.
- The IEF strip then incubated in a sample buffer containing SDS (thus binding SDS to the denatured proteins) and then placed between the glass plates of a previously prepared 10% SDS-PAGE gel.
- Electrophoresis is commenced and the SDS-bound proteins run into the gel and separate according to size.
- The IEF gels are provided as dried strips and need rehydrating overnight.
- The first dimension IEF run then takes 6-8h, the equilibration step with SDS sample buffer takes about 15 min, and then the SDS-PAGE step takes about 5h.

Two-dimensional Electrophoresis

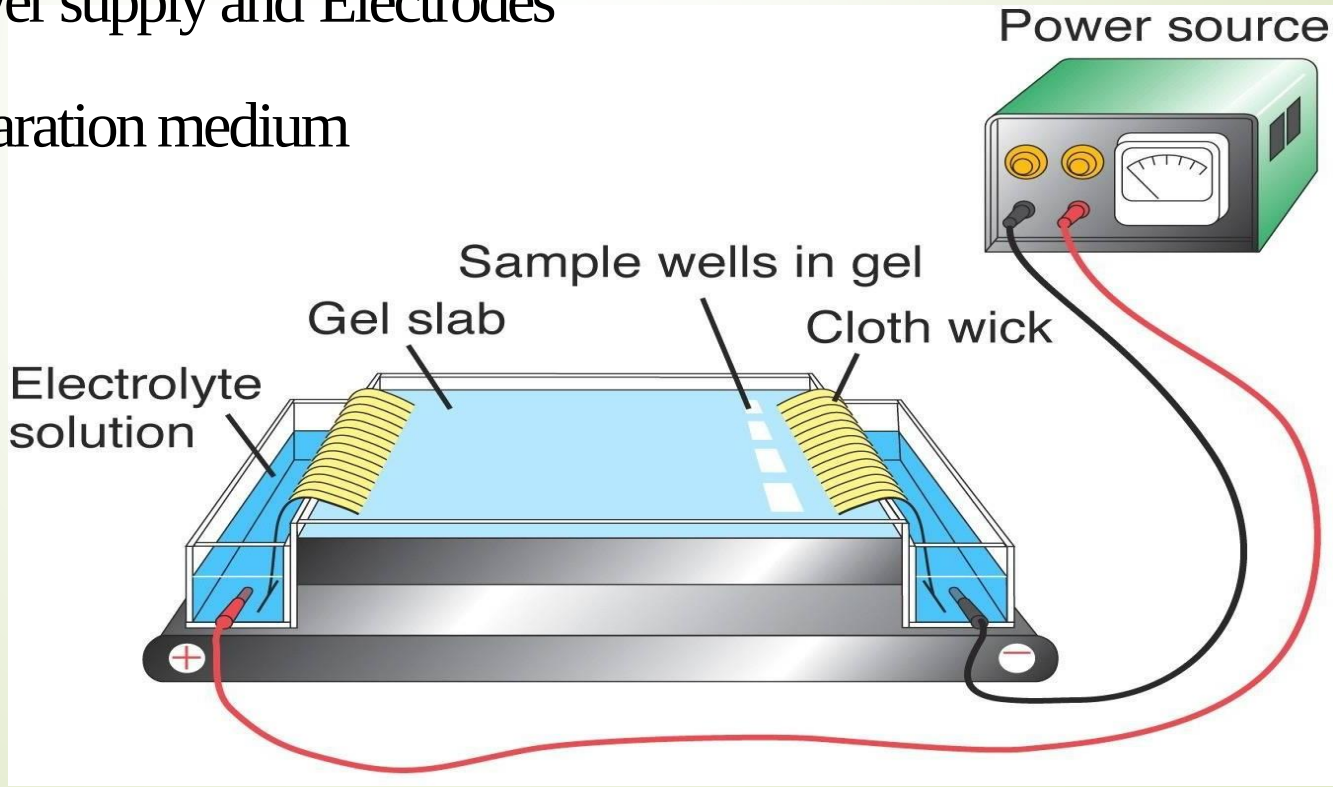
- Using this method one can routinely resolve between 1000 and 3000 proteins from a cell or tissue extract and in some cases workers have reported the separation of between 5000 and 10000 proteins.
- The result of this is a gel with proteins spread out on its surface. These proteins can then be detected by a variety of means, but the most commonly used stains are silver and coomasie staining.

2D-gel (coomassie stained)



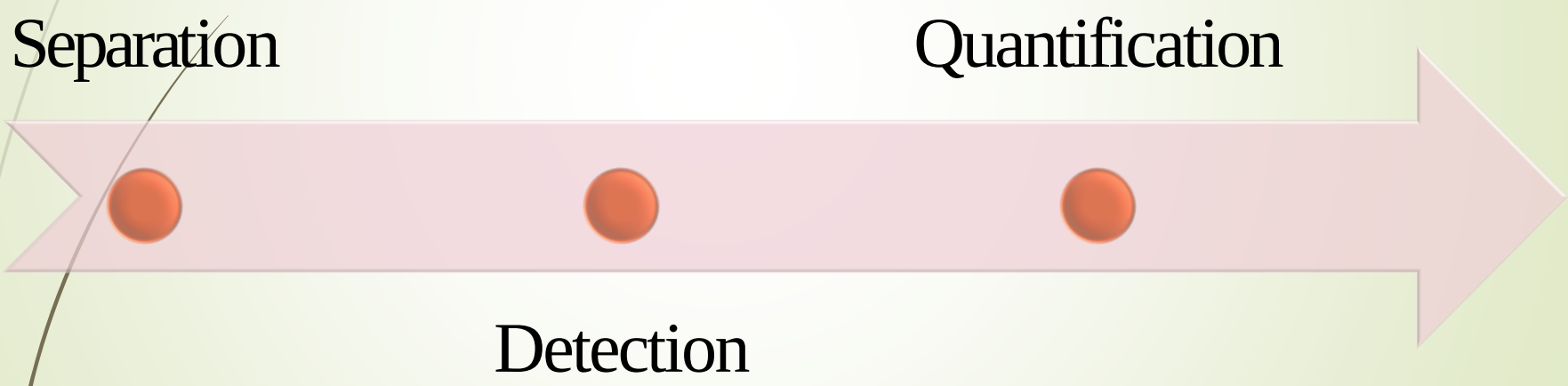
Conventional electrophoresis

- Instrumentation :
  - Two reservoir for the buffer
  - Power supply and Electrodes
  - Separation medium



General Operation of Electrophoresis

The general operation of the conventional electrophoresis include;

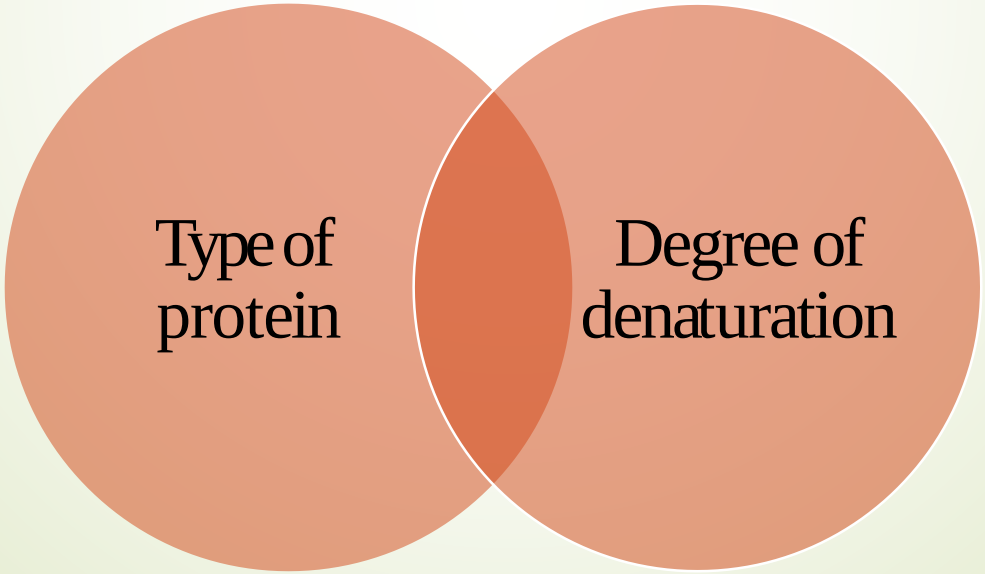


a. Electrophoresis Separation

- When performed on precast or agarose gel, following steps are followed;
  - Excess buffer removed
  - 5-7 µL sample
  - Placed in electrode chamber
  - Current application
  - Gel is rinsed, fixed and dried
  - Stained
  - Scanned under densitometry

b. Staining

- Protein is ppt in gel by using acetic acid or methanol (this will prevent diffusion of protein out of the gel when submerged in stain solution)
- Amount of dye taken by sample is affected by many factors,



Different stains of Electrophoresis

- Plasma Proteins
  - Amido black
  - Coomassie Brilliant Blue
  - Bromophenol Blue
- Hemoglobins
  - Amido black
  - Coomassie Brilliant Blue
  - Ponceau Red
- Lipoproteins
  - Sudan Black
- DNA (Fluorescent dyes)
  - Ethidium Bromide
  - Sybr Green, Sybr Gold

Staining Systems

Proteins

General – Coomassie brilliant blue R, Kenacid blue, Amido black.

Specific – Oil red O, PAS, Rubeanic acid, Transferrin-specific & for calcium binding proteins

Steps
* fixing
* staining
* destaining

Allozymes - Histochemical staining

DNA -EtBr, SyBR green, Propidium iodide and silver staining

C. Detection and Quantification

- Once separated, protein maybe detected by staining followed by the quantification using the densitometer or by direct measuring using an optical detection system under set at 210nm.

Separation type	Wavelength
Serum protein	520-640nm
Isoenzymes	570nm
Lipoproteins	540-600nm
DNA fragments	254-590nm
CSF protein	----

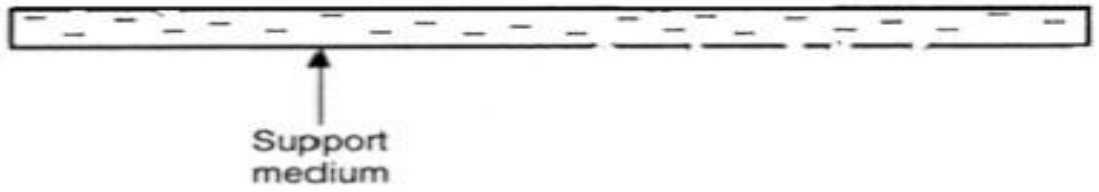
The selection of the wavelength is the property o type of stain used for the identification of separation.

Few technical considerations

- Buffers
  - Barbital → bacterial growth → pH change
  - Barbital, pH 8.6 most often used
  - Discard after each run

- Sample
  - Optimal amount of sample applied to gel
  - Avoid 'overloading': dilute serum prior to application (0.050 ml serum + 0.2 ml buffer)

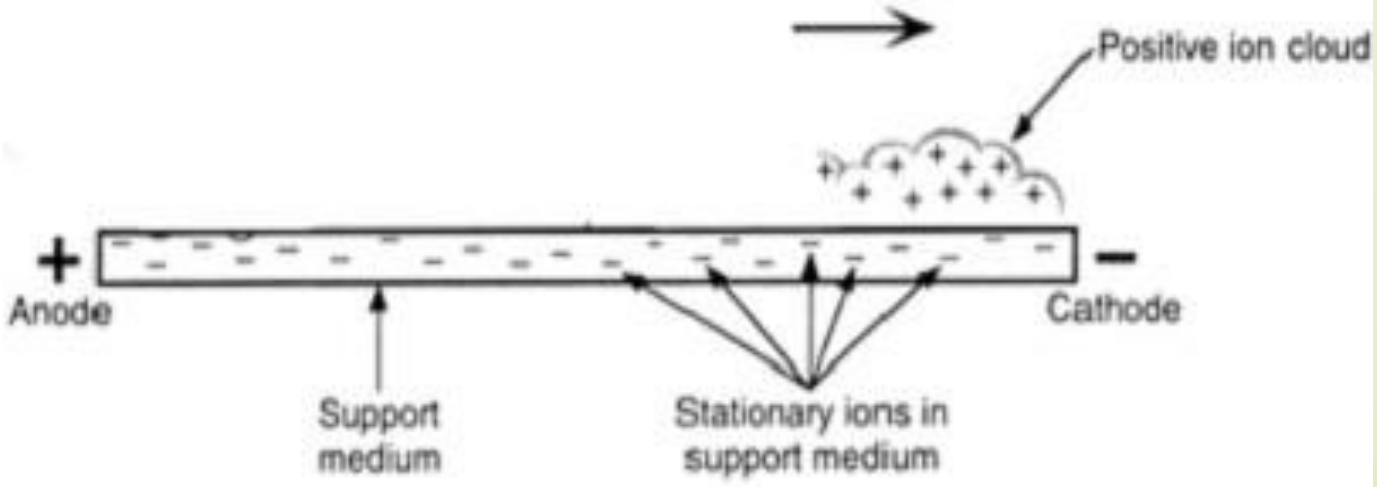
- Evaporation and wick flow



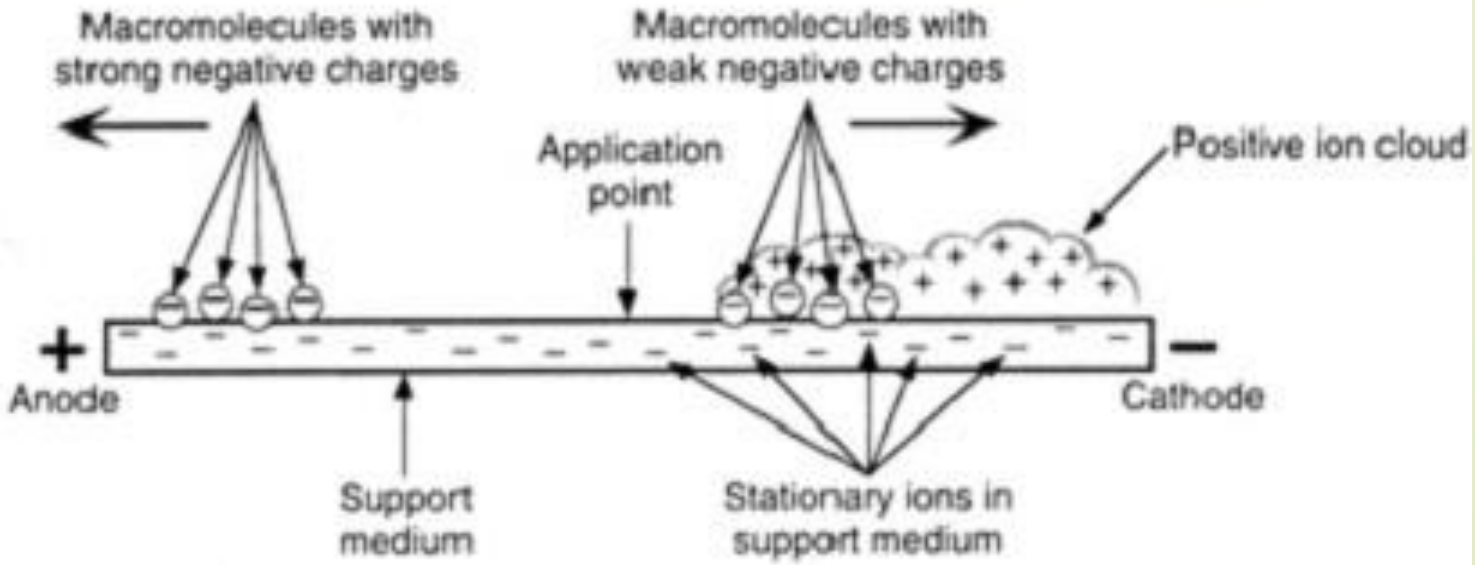
- Electroendosmosis

What is EEO &why low???

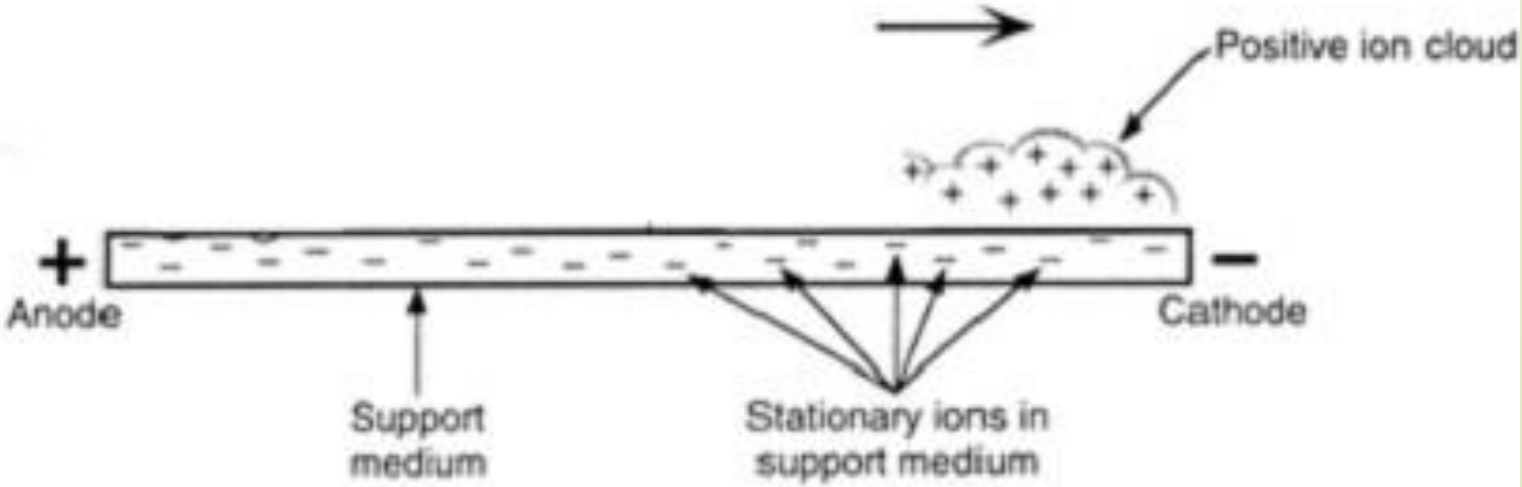
- Ionic cloud is mobile
- Electrical current causes positive ionic cloud to move toward the cathode



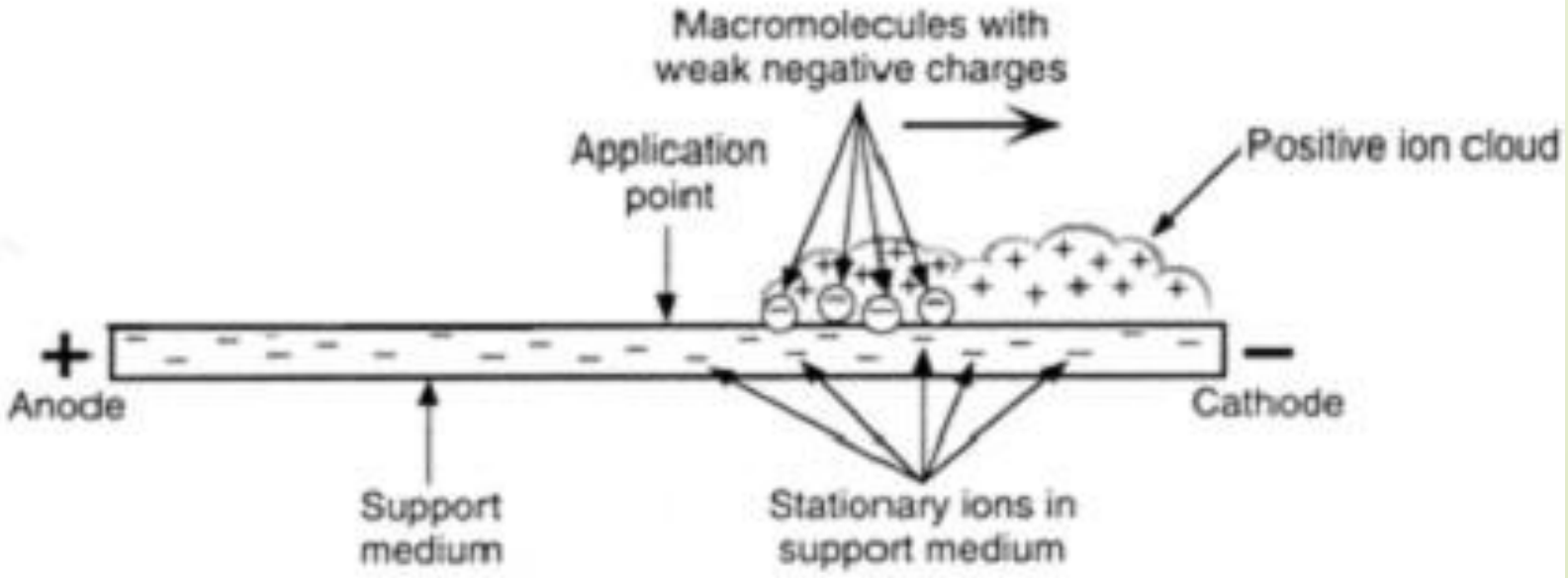
- Macromolecules (proteins) that have a sufficiently strong enough charge are able to oppose the flow of the positive ion cloud and move in the opposite direction towards the electrode of opposite polarity



- Surface of gel is negatively charged
- Surface gel ions are immobile
- Positive buffer ions (pH 8.6) orient with negative surface ions = positive ionic cloud



- Molecules on surface of gel that hold a weak negative charge are 'pushed' toward the cathode despite migration direction toward the anode



Common effect of variables on separation

pH	Changes charge of analyte, effective mobility; structure of analyte- denaturing or dissociating a protein.
Ionic strength	Changes in voltage; increased ionic strength reduces migration velocity and increase heating.
Ions present	Change migration speed; cause tailing of bands.
Current	Too high current cause overheating.
Temperature	Overheating cause denature protein; lower temp reduce diffusion but also migration; there is no effect on resolution.
Time	Separation of bands increases linearly with time, but dilution of bands increase with square root of time.
Medium	Major factors are endosmosis and pore size effect, which effect migration velocities.

Support media in separation

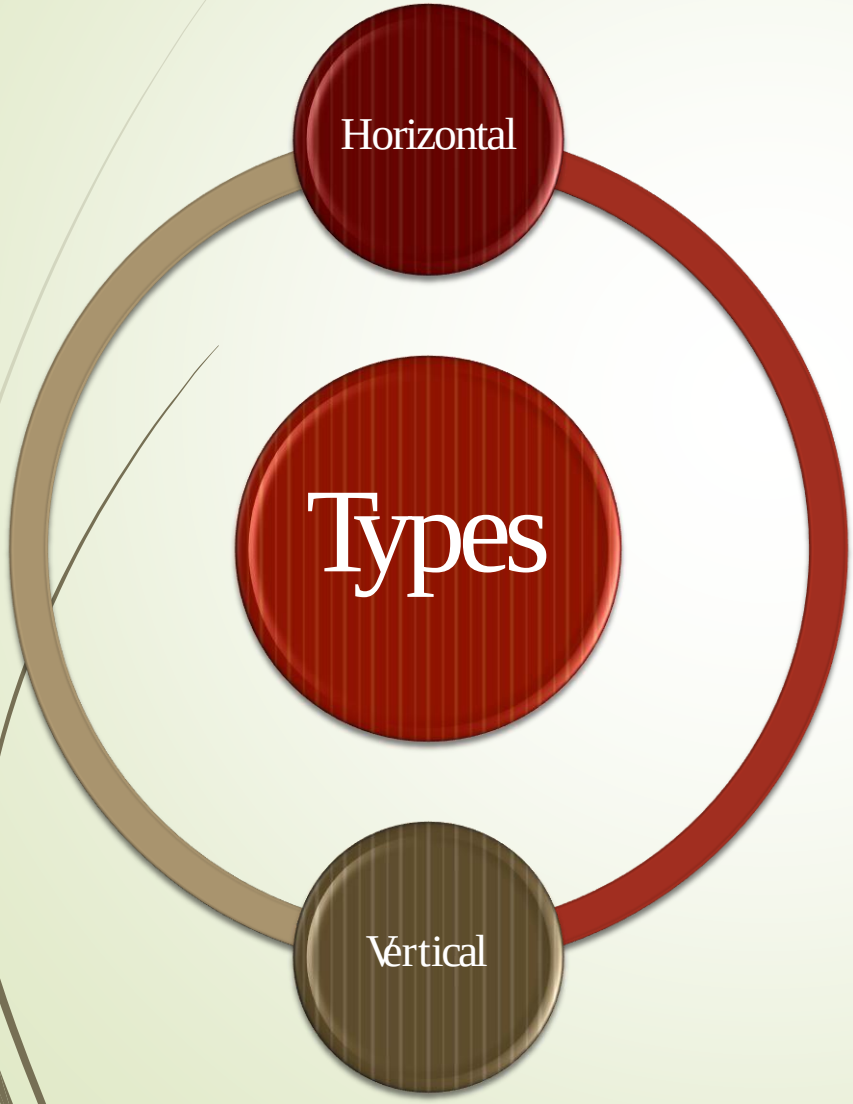
Molecular size

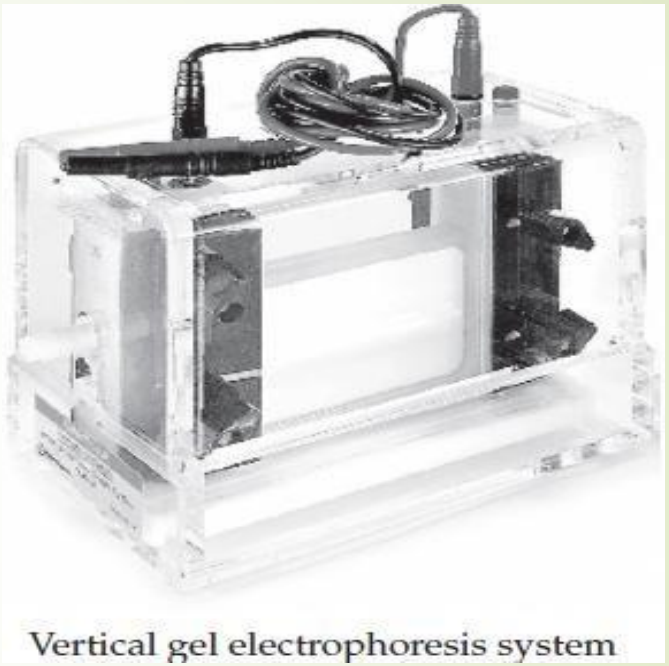
- Gradient gels
- Gels containing denaturants

Molecular size & Charge

- Gel electrophoresis
- Immunoelectrophoresis
- 2D electrophoresis

System classification





Vertical gel electrophoresis system

Horizontal gel electrophoresis system

Electrophoresis of proteins

- The most commonly used technique for the separation of proteins is Sodium dodecyl sulphate-polyacrylamide gel electrophoresis(SDS PAGE).

Procedure-

- Protein sample is first boiled for 5 mins in a buffer solution containing SDS and β-mercaptoethanol.
- Protein gets denatured and opens up into rod-shaped structure.
- Sample buffer contains bromophenol blue which is used as a tracking dye, and sucrose or glycerol.
- Before the sample is loaded into the main separating gel a stacking gel is poured on top of the separating gel.

Electrophoresis of proteins

- Current is switched on.
- The negatively charged protein-SDS complexes now continue to move towards the anode.
- As they pass through the separating gel, the proteins separate, owing to the molecular sieving properties of the gel.
- When the dye reaches the bottom of the gel, the current is turned off.
- Gel is removed from between the glass plates and shaken in an appropriate stain solution.
- Blue colored bands are observed under UV rays.


Thank
you !!!
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