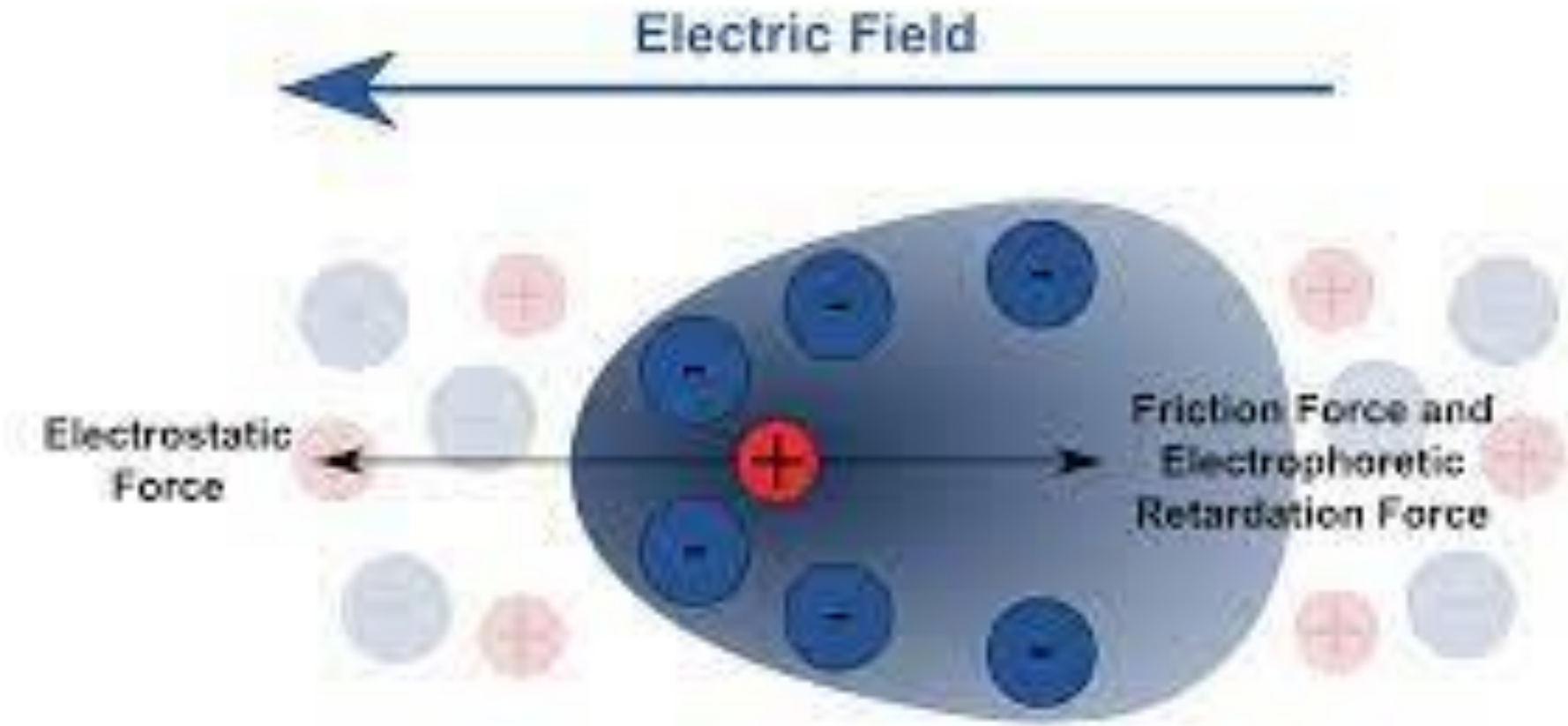
Definition

- ***Electro*** means *Electricity*
- ***Phoresis*** means *Separation*
- Separation of serum proteins by the effect of an electric current.
- Electrophoresis is a physical method of analysis which involves separation of the compounds that are capable of acquiring electric change in conducting electrodes.
- Electrophoresis may be defined as the migration of the charged particle through a solution under the influence of an external electrical field.
- Ions that are suspended between two electrodes tends to travel towards the electrodes that bears opposite charges


Electric Field
Electrostatic Force
Friction Force and Electrophoretic Retardation Force

- Macromolecules can be characterized by the rate of movement in an electric field.

- This property is used to determine protein molecular weights, to distinguish molecules by virtue of their net charge or their shape and to separate different molecular species quantitatively.

- Rate of movement of macromolecules in an electric field is useful parameter to know any change in amino acid regarding its charge.

- Electrophoresis is similar to chromatography.
- Electric field is used as a dragging force.
- Technique is simple, very effective and clean.
- Large number of samples can be separated, identified and quantitatively measured.

- As movement of ions or their mobility depends upon the frictional coefficient, which in turn depends on the function of some of the physical properties of the molecules such as weight, molecular shape, size etc.

  The law of electrostatics states:

$$F_{electric} = qE$$

- where $F_{electric}$ is electrical force on anion, q is the charge on the ion and E is the electric field strength.

- The resulting electrophoretic migration of the ion through the solution is opposed by a frictional force

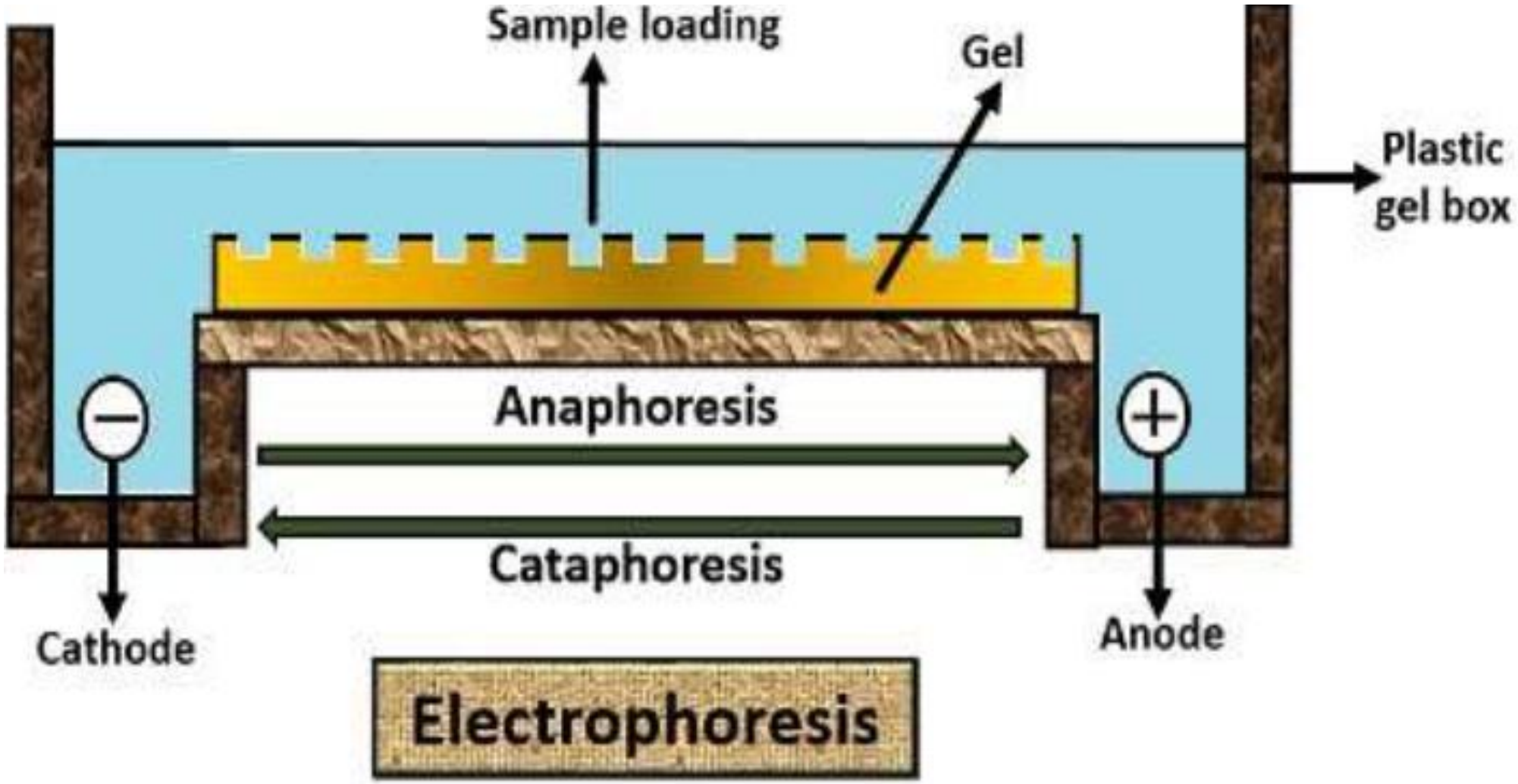
$$F_{friction} = V f$$

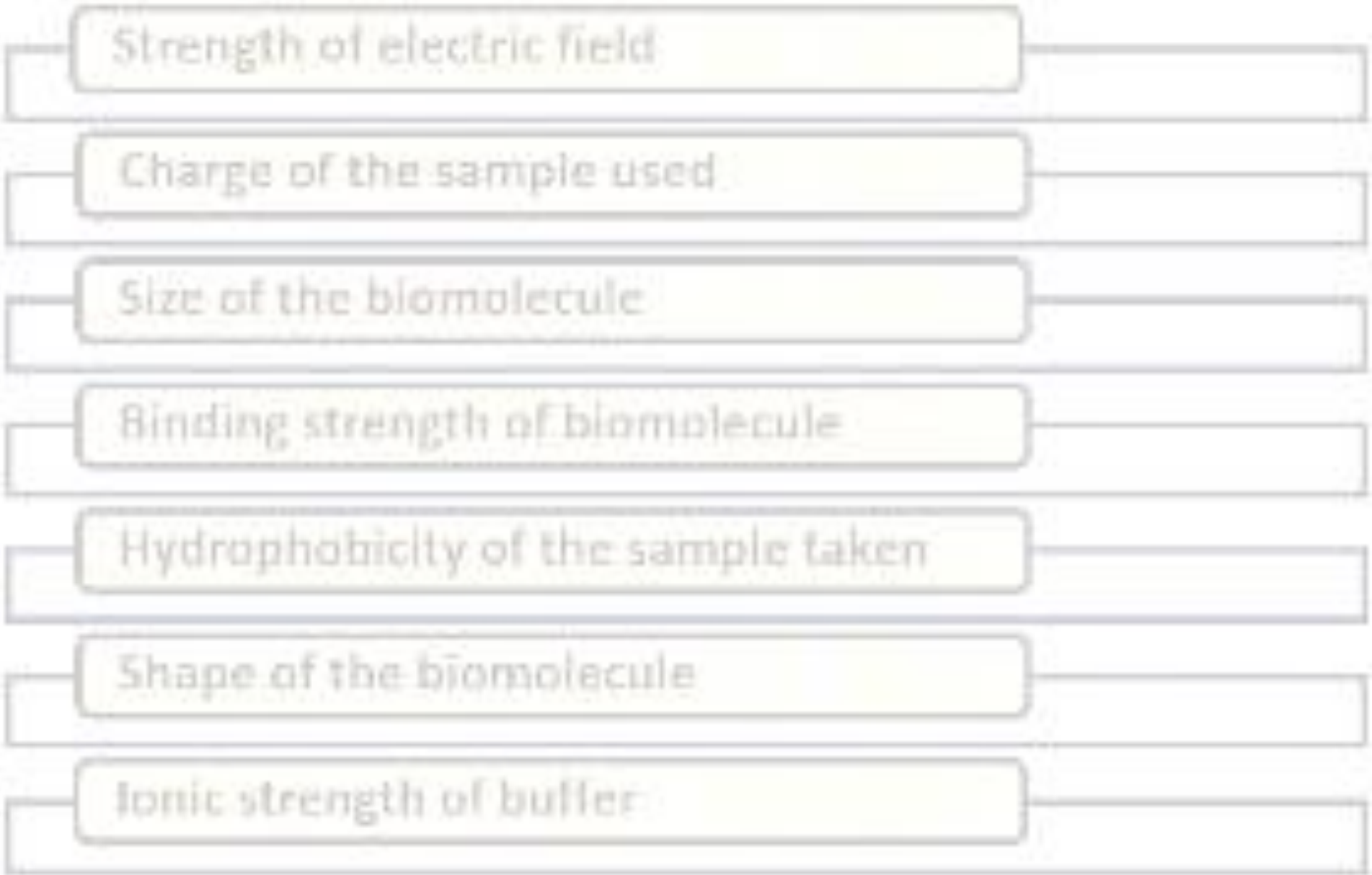
- where V is velocity (rate of migration) of the ion and f is its 'frictional coefficient'.
- The frictional coefficient is a measure of the drag that the solution exerts on the moving ion and is dependent on the size, shape and state of the ion as well as on the viscosity of the solution.

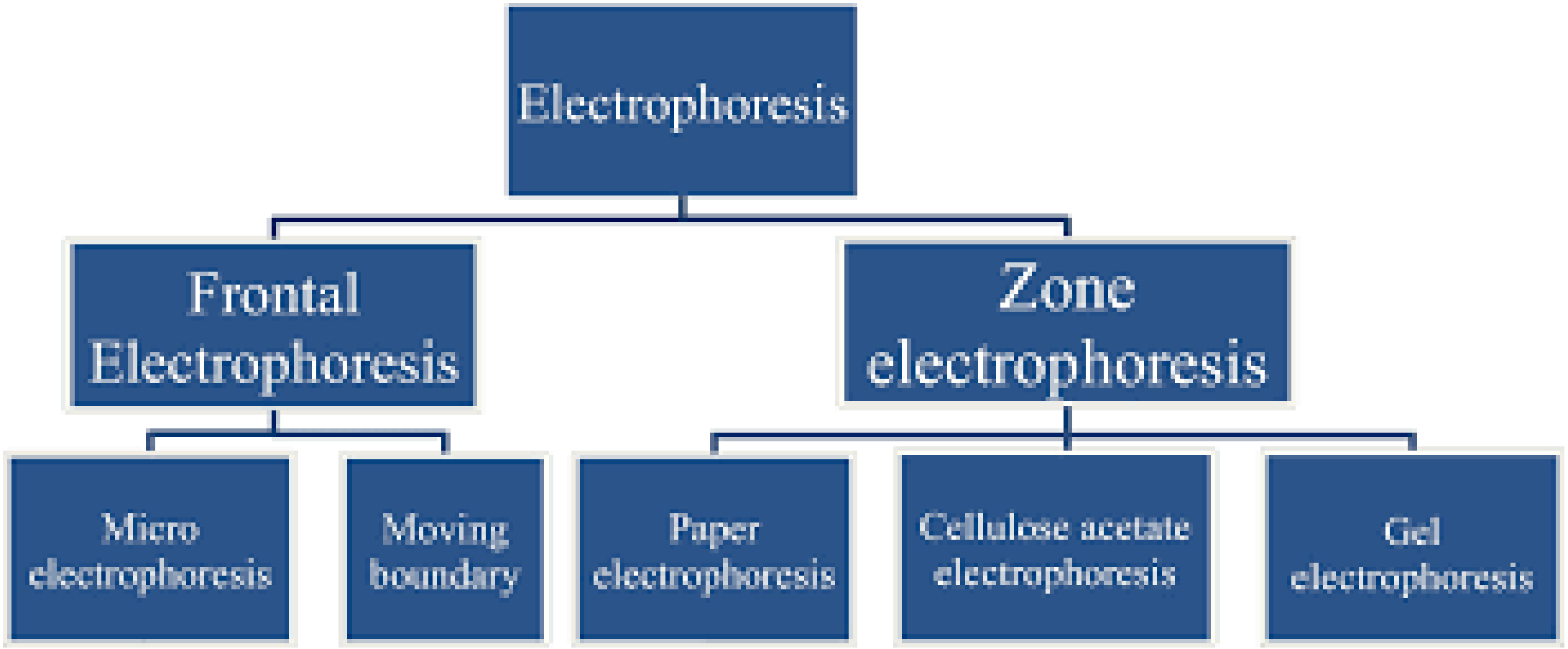
- The working principle of electrophoresis is that it causes the separation of the molecules, ions or colloidal particles that suspends in the matrix under the force of an electric field.

- The electric field allows the migration of the positively charged molecule towards the anode and the migration of negatively charged molecule towards the cathode.

- Therefore, electrophoresis is the **electrokinetic phenomenon** where the motion of molecules occurs under an electric field.


Sample loading
Gel
Plastic
gel box
Anaphoresis
Cataphoresis
Cathode
Anode
Electrophoresis

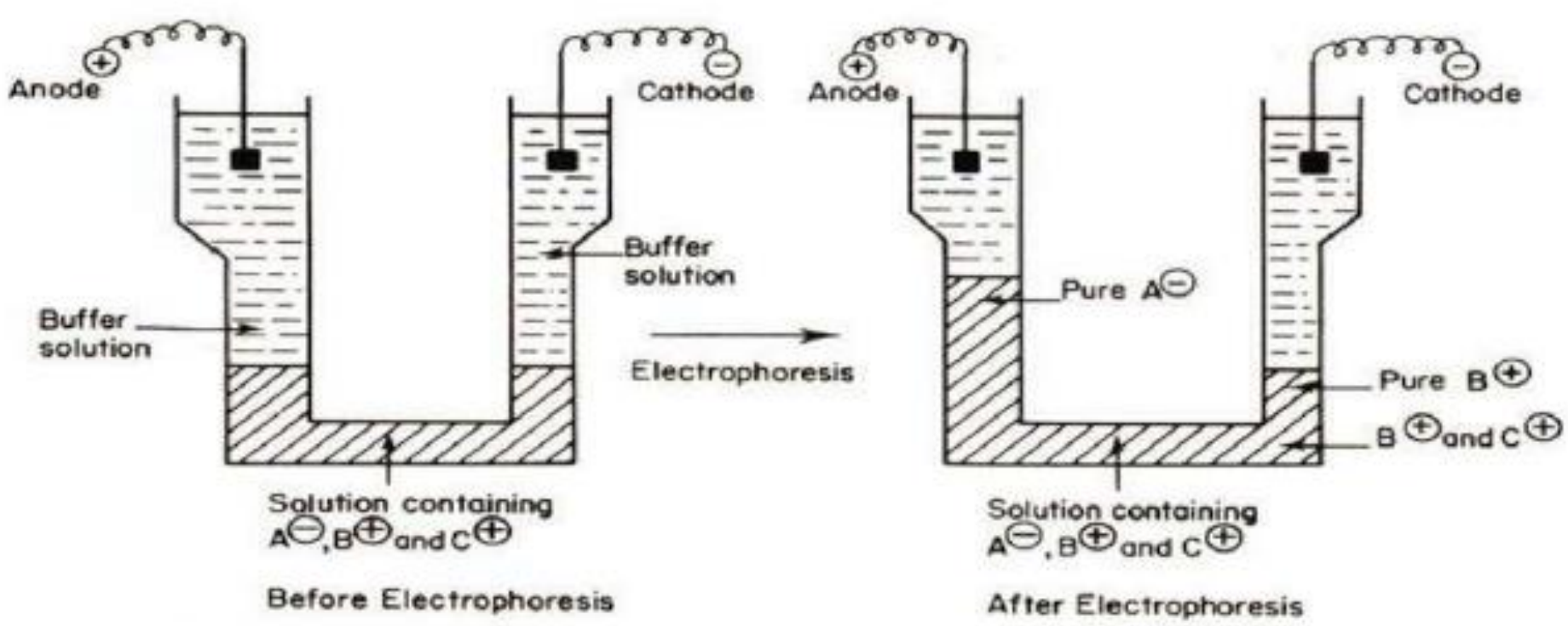

Strength of electric field
Charge of the sample used
Size of the biomolecule
Binding strength of biomolecule
Hydrophobicity of the sample taken
Shape of the biomolecule
Ionic strength of buffer


Electrophoresis
Frontal Electrophoresis
Zone electrophoresis
Micro electrophoresis
Moving boundary
Paper electrophoresis
Cellulose acetate electrophoresis
Gel electrophoresis

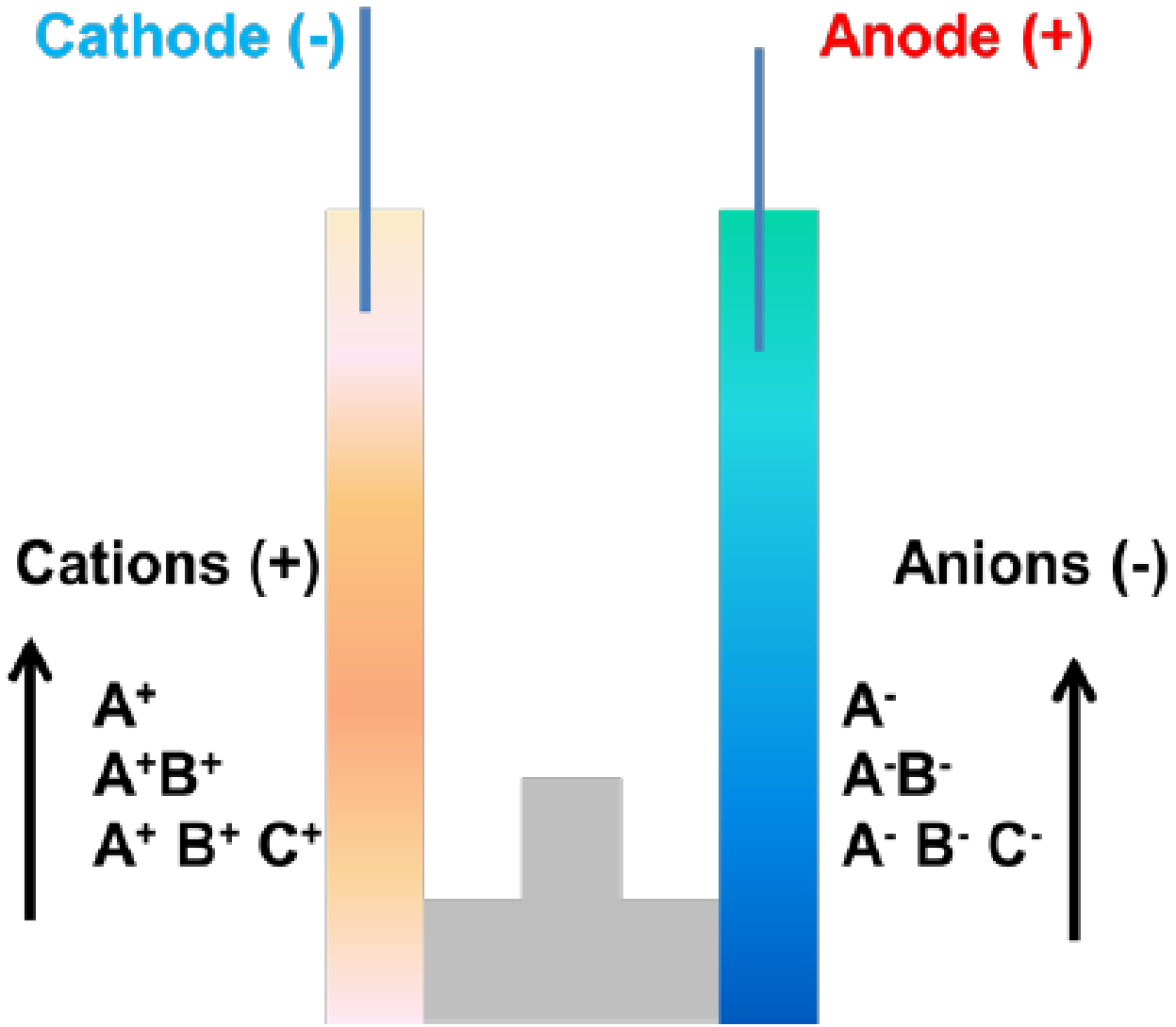
Moving boundary electrophoresis

- First used by Swedish biochemist Arne Tisellus, to separate proteins in 1937.
- In this method, the electrophoresis is carried in solution, without a supporting media.
- The sample is dissolved the buffer and molecules move to the irrespective counter charge electrodes.
- Moving boundary electrophoresis is carried out in a U shape tube with platinum electrodes attached to the end of both arms

- At the respective ends, tube has refractometer to measure the change in refractive index of the buffer during electrophoresis due to presence of molecule.
- Sample is loaded in the middle of the U tube and then the apparatus is connected to the external power supply.
- Charged molecule moves to the opposite electrode as they passes through the refractometer, a change can be measured.
- As the desirable molecule passes, sample can be taken out from the apparatus along with the buffer.



Moving boundary electrophoresis.


Cathode (-)
Anode (+)
Cations (+)
Anions (-)
A+
A+B+
A+ B+ C+
A-
A-B-
A- B- C-

Disadvantages of Moving Boundary electrophoresis-

- The resolution of the technique is very low due to the mixing of the sample as well as over-lapping of the sample components.

- The electrophoresis technique is not good to separate and analyse the complex biological sample instead it can be used to study the behaviour of the molecule in an electric field.

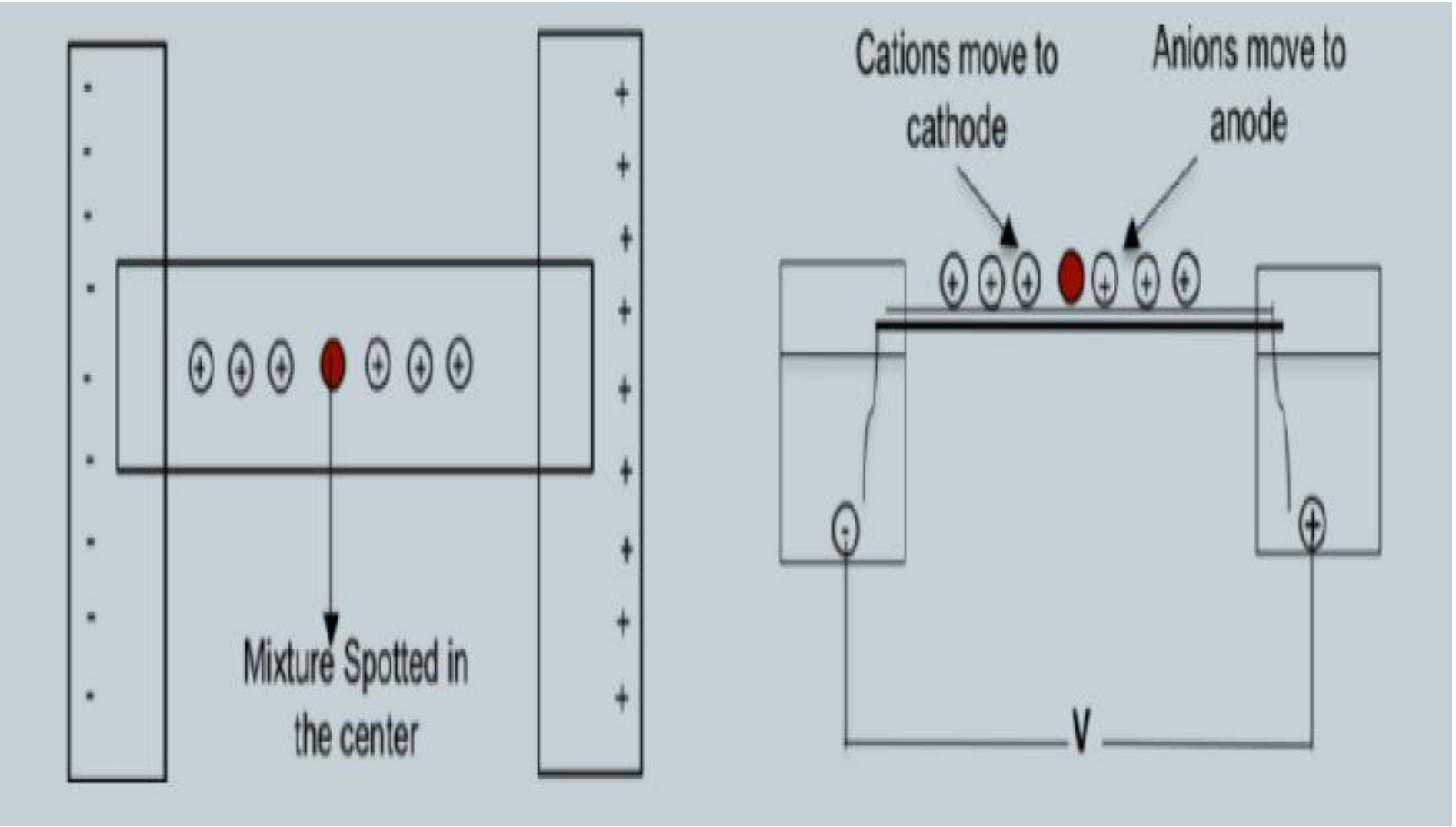
Zone electrophoresis

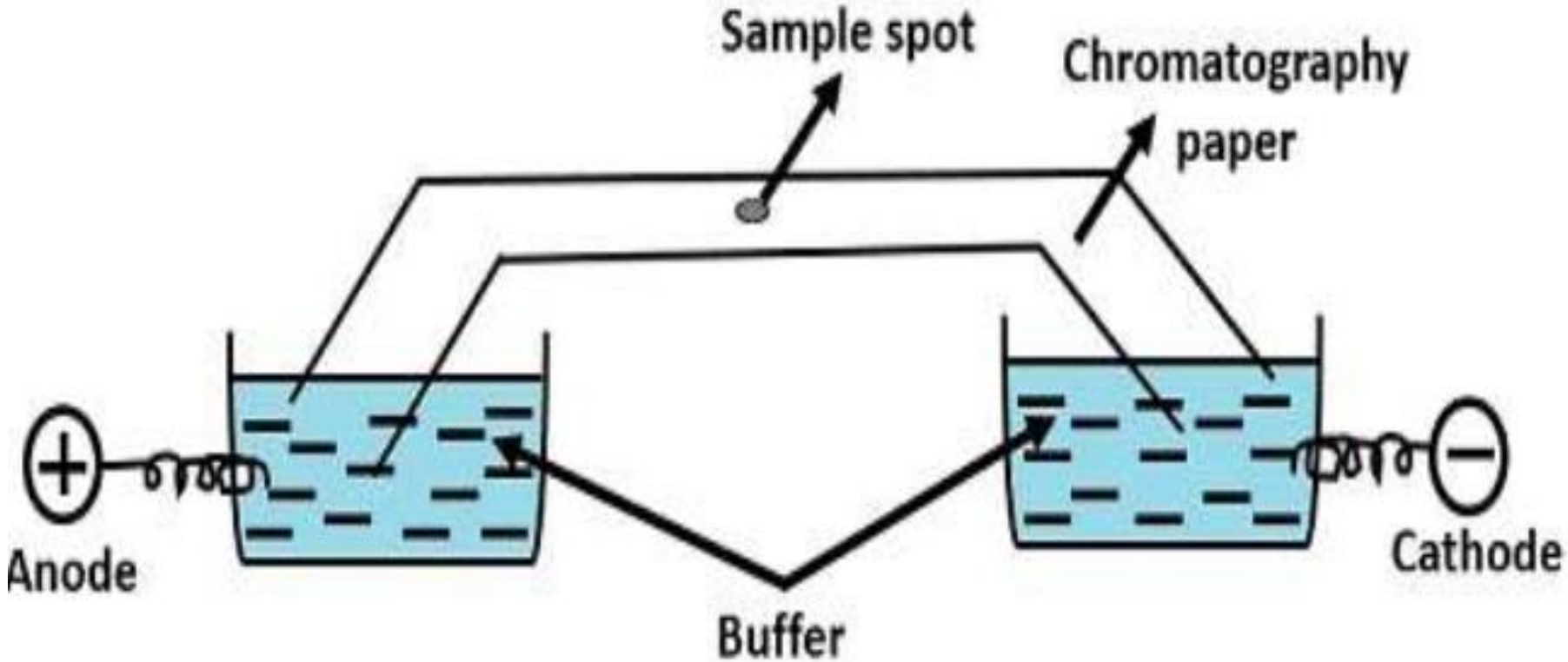
- In this method, an inert polymeric supporting media is used between the electrodes to separate and analyze the sample.
- The supporting media used in zone electrophoresis are absorbent paper, gel of starch, agar and polyacrylamide.
- The major advantage of presence of supporting media is that it minimizes mixing of the sample and immobilization of the molecule after electrophoresis.
- It makes the analysis and purification of the molecule from the gel much easier than the moving boundary electrophoresis.
- The gel electrophoresis is the best example of zone electrophoresis.

Paper electrophoresis

- Paper electrophoresis (PE) is useful for these partition of small-charged molecules, such as amino acids and small proteins using a strip of paper (chromatography paper).
- In this technique, the motion of colloidal particle of solution occurs leading to subsequent separation along the paper strip.
- PE is easier in comparison to gel electrophoresis.
- It does not require matrix preparation and it does not contain charges that interfere with the separation of compounds.

- A strip of filter paper is moistened with buffer and the ends of the strip are immersed in to buffer reservoirs containing the electrodes.
- The samples are spotted in the center of the paper and high voltage is applied.
- Application of high voltage causes less diffusion of small molecules giving better resolution and it take less time to complete the process.
- Spots migrate according to their charges.
- After electrophoresis, separated component scan be detected by variety of staining techniques, depending upon their chemical composition.


Mixture Spotted in the center
Cations move to cathode
Anions move to anode
V



Paper Electrophoresis

Applications of Paper Electrophoresis

- A simple, inexpensive, and accurate laboratory procedure for various research and clinical studies.
- Easily available and easy to handle, allowing new methodologies to be tried and developed with convenience.
- Clinical applications of PE include study of sickle cell disease, hemoglobin C abnormalities, and separation of blood clotting factors and serum plasma proteins from blood sample.

Applications of Paper Electrophoresis

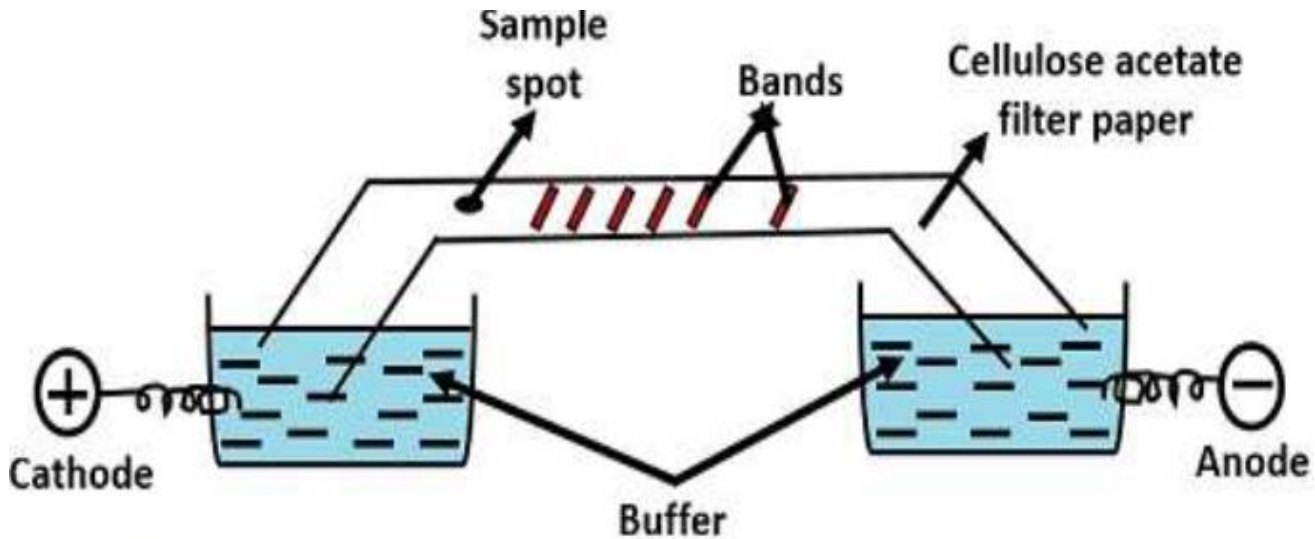
- Used in separation and identification of alkaloids.
- used for testing suitability of municipal water supplies, toxicity of water, and other environmental components.
- Drug-testing industry uses paper electrophoresis to determine presence of illegal or recreational drugs in job applicants and crime suspects.
- since 1950 it used by the investigators and in forensics to analyze inks used in currency to check the counterfeiters.
- Lack of sensitivity and reproducibility are limitations of PE

Cellulose acetate strip electrophoresis

- Many biological samples adsorb on cellulose, that is paper.
- The adsorption is because of hydroxyl groups present in cellulose.
- Adsorption reduces the movement and therefore causes tailing of spots/bands
- This spreading of spots reduces resolution.
- To solve this problem cellulose acetate membrane is used where most of the hydroxyls have been converted acetate groups.
- Cellulose acetate is preferred because of its simplicity and high resolution at low applied voltage.

Cellulose acetate strip electrophoresis

- It contains 2-3acetyl groups per glucose unit and its adsorption capacity is less than that of paper.
- It gives sharper bands.
- Provides a good background or staining glycoprotein
- Application:
- Widely used in analysis of clinical and biological protein sample (albumin and globulins).
- Alternative to paper electrophoresis.



Cellulose acetate Electrophoresis

Gel electrophoresis

- It makes the use of gel as a support matrix.
- Most popular and commonly used method.
- Used for both analytical and preparative processes.
- It is the most common method to carry out the process of electrophoresis.

Principle:

- In this porous gel matrix is used which consist of the cross-linked polymer network.
- Through this network, molecules of different size, charge and shape pass through.
- This relies upon the fact that negatively charged molecule will attract towards the positive end and viceversa.
- After the migration, bands will appear on the gel matrix at different levels those which lag behind will be the heavy molecules and those which moves faster are lighter molecules through the pores of the gel matrix.

Types of Gel electrophoresis

- Horizontal (Agarose gel electrophoresis)

- Vertical (SDS-PAGE)

Electrophoresis is defined as movement of charged particles in an electric field leading to their separation. In other words, it is separation technique which is based on differences in mobility of charged particles in an electric field. It is essential to understand factors which determine mobility of particles and these are being explained with the help of following statements.

When voltage (potential difference) is applied across the electrodes, it results in generation of potential gradient (E) which is equal to applied voltage (V) divided by distance between the electrode (d). Thus,

$$\mathbf{E = V/d.}$$

It is required to be noted that ‘d’ will vary from equipment to equipment and thus for the same applied voltage, potential gradient will vary.

If charge on molecule is ‘q’ coulombs, then force exerted on particle is equal to ‘Eq’ newtons. This force causes the charged particles to move towards electrode. Particles carrying positive charge are referred as cations and will migrate towards cathode whereas particles carrying negative charge are called as anions and these will move towards anode. Thus, cathode is negatively charged and anode is positively charged.

It is also essential to be noted that particles under movement experiences frictional resistance which retards movement of charged particles. The frictional force is dependent on size, shape, and hydrodynamic volume of particle as well as on pore size of support matrix used for separation and viscosity of medium (buffer) used in electrophoresis. Thus, velocity 'v' is function of 'E' and frictional coefficient 'f' and is defined by following equation.

$$v = E / f$$

$$= Eq / f$$

Electrophoretic mobility ‘μ’ of an ion is defined as ratio of velocity to potential gradient and is expressed as

$$\mu = v / E = q / f$$

Molecules with identical or similar charges can be also separated if these molecules experiences different frictional force. Thus, separation of particles differing in charge as well as of similar charge is feasible in electrophoresis. In fact, separation of charged particles based on differences in frictional forces is routinely used in laboratories.

In electrophoresis, electric field must be switched off before ions reach respective electrode to allow their separation. Failure to do so will result in their neutralization at respective electrodes, a process called ‘electrolysis’.

During electrophoresis, heat is generated in supporting medium and is given by following equation

$$W = I^2R,$$

where

W is power generated in Watts,

I is current which increases with increase in voltage (V/I= R)

R is resistance.

Power generated is dissipated as heat.

Heating of medium (support) will cause following problems.

• Sample and buffer ions will diffuse resulting in broadening of bands

• Viscosity of buffer will decrease and thus, resistance of medium will also decrease. Thus, as per Ohm's law, it will result in increase in current at a given voltage. Heat can denature proteins and enzymes which may lose activity.

• If during electrophoresis, constant voltage is used, current will continue to slowly increase with time because of heat generated. More is the value of current, more is heat generated. Thus, this increased amount of heat will further increase current. As such amount of heat will be continuously increasing with time. Some workers prefer to run electrophoresis at constant current. Thus it should be clear that higher is the current, higher is heat generated and higher is velocity.

In order to minimize heat generation, low voltage could be an option. However, this will end in long separation time which ultimately will result in diffusion of separated bands. Affects of heat can be minimized if support is maintained at low temperature. This is achievable if electrophoresis is performed at low temperature or cold water is circulated through buffers in electrode chambers or support material is layered over surface with good heat conducting properties or through Peltier cooling. It is also repeated here that force exerted on charged particle is dependent on voltage, not on current. It is therefore advised to perform electrophoresis on constant voltage which when applied should not result in generation of excess heat and also should not lead to diffusion of separated bands.

Charge or Net Charge on Macromolecule

For the students of biochemistry or life-science subject, electrophoretic separation of protein is more frequently encountered and therefore this macromolecule is discussed here. Now we will explain how charge on protein is contributed. To make this clear, it is essential to understand concept of pH, buffer, dissociation constant, pKa and different amino acids which acts as monomeric unit in protein.

pH is minus logarithmic of hydrogen ion activity (concentration).

ie pH = -log [H+]

Water is weak electrolyte. It ionizes to small extent to give [H+] and [OH-]. Its dissociation and ionic product are summarized in following equations.

$$H_2O \rightleftharpoons [H^+] + [OH^-]$$

$$K = \text{Equilibrium constant} = \frac{[H^+]\,[OH^-]}{[H_2O]} = 1.8 \times 10^{-16}$$

$$Kw = \text{Ionic Product of Water} = K.\,[H_2O] = [H^+]\,[OH^-]$$

$$= 1.8 \times 10^{-16} \times [H_2O]$$

$$= 1.8 \times 10^{-16} \times 55.6 = 1 \times 10^{-14}\ mol^2\ dm^{-6}$$

Concentration of H+ and OH- ions from water is equal and is equal to 10^{-7} mol^2 dm^{-3}. Thus, pH of water is 7. For alkaline solution, pH is greater than 7 while for acidic solution, pH is lower than 7. Also, it may be noted that pH values for strong acids will be less than 0 and that of strong bases greater than 14. For weak acids such as acetic acid, extent of dissociation and concentration of acetic acid will determine pH of solution.

$$HA \rightleftharpoons [H^+] + [A^-]$$

$$Ka = \text{Dissociation constant} = \frac{[H^+]\,[A^-]}{[HA]}$$

$$[H^+] = \frac{Ka.\,[HA]}{[A^-]}$$

$$\text{Log}\,[H^+] = \text{Log}\,Ka + \text{Log}\,\frac{[HA]}{[A^-]}$$

$$-\text{Log}\,[H^+] = -\,\text{Log}\,Ka - \text{Log}\,\frac{[HA]}{[A^-]}$$

$$pH = pKa + \text{Log}\,\frac{[A^-]}{[HA]}$$

Above equation is referred as Henderson–Hasselbalch equation. This equation can be used for calculating extent of dissociation of group at a given pH.

pKa which is defined as negative logarithmic of dissociation constant is constant for a given group. Thus by changing pH, extent of dissociation can be changed and consequently the charge contribution.

Carboxylic group on dissociation will give one negative charge whereas un-dissociated will be charge-less. Contrary to it, amino group in un-dissociated state will carry one positive charge while dissociated state will be charge-less.

Proteins are polymers of amino acids which are linked together by peptide bond.

Amino acids contain amino group and carboxylic groups.

Carboxylic group of one amino acid is linked with amino group of other amino acid and thus the groups involved in peptide bond formation will not contribute to charge.

However, α-amino group of first amino acid and α- carboxylic group of last amino acid in protein or peptide will contribute to charge on molecule.

Different amino acids contain different side chains and some of them do contain groups which can contribute to charge on protein.

For example side chains of aspartic acids and glutamic acid have carboxylic group while that of lysine has amino group.

Similarly imidazole of histidine can contribute to charge.

Thus (i) pKa of α-amino, ε-amino, α-carboxylic, ß- carboxylic, γ- carboxylic and imidazole,

(ii) number of these groups in protein and (iii) pH will determine net charge on protein.

It is again emphasized that dissociation of carboxylic group will lead to one negative charge while dissociation of amino group will abolish one positive charge. pKa values of groups in protein is given table.