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Electrophoresis

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Separation of charged compounds of a mixture by means of difference in their migration rates in an electric field is one of the important methods of separation. As the migration depends upon sign and magnitude of charge (and also the size), compounds having different charges can be successfully separated. The method is particularly valuable for labile macromolecules as separation can be carried in aqueous media under mild conditions of temperature, pH and ionic strength.

Earlier electrophoretic separations were carried out in homogeneous solutions and were consequently restricted to frontal analysis. With the introduction of porous media to prevent convection of the solute boundary it became possible to carry out zonal separation.

Tiselius pointed out that electrophoresis, like most other separation processes, can be operated by frontal, zonal and displacement techniques and suggested the term zone electrophoresis for all electrical separations in which discrete solute zones are formed irrespective of the method used to stabilize these zones.

General Theory

Movement of charged particles in electric field

The factors governing movement of charged particles in an electric field can be considered to fall in the following classes :

(a) Those characteristics which relate to the ion itself, namely its charge, sign and magnitude, size, shape, tendency to dissociate and amphoteric behaviour, if any.
(b) Those factors which are related to environment in which the ion is present, such as electrolyte concentration, ionic strength, dielectric properties, chemical properties, pH, temperature, viscosity and presence of non-polar molecules which may influence viscosity or dielectric properties or which may interact with charged complexes.
(c) The characteristics of applied field, its intensity and distribution along the migration path.
(d) The secondary interactions between factors (a) and (b) either electrostatic or by van der Waals forces which may further influence the migration.

In a salt free very dilute system the force F exerted on a charged particle by an applied electric field is equal to the field strength X and net charge Q .

$$F = X Q \quad \ldots \ldots \ldots \qquad (1)$$

A particle moving in a constant field (constant force) in a viscous medium, however, attains a constant velocity because the viscous retarding force, F', opposing the movement increases linearly with acceleration. The viscous retarding force depends upon the geometry of the particle and for a sphere is given by Stoke's law :

$$\frac{F'}{V} = 6\pi rn \qquad \ldots \qquad \ldots \qquad (2)$$

V = electrophoretic velocity
n = viscosity of the medium
r = radius of the particle.

Thus for an isolated spherical particle obeying Stoke's law in the steady state, the two opposing forces are equal (F = F′) and we have from equations (1) and (2) :

$$X\ Q = 6\pi\, r\, \eta\, V$$

or $$\frac{X}{V} = \frac{6\pi\ r\ \eta}{Q}$$

The electrophoretic mobility, u, is the velocity V measured under standard conditions, i.e., per unit field, X, expressed as volts per c.m. or

$$u = \frac{V}{X} \text{ or } u = \frac{Q}{6\pi\, r\, n}$$

Experimentally one can not be restricted to ideal conditions, buffers or other salts are nearly always present and electrical interactions between charged ions can no longer be ignored as is permissible under the conditions of infinite dilution.

The electrical forces that are exerted between ions in aqueous solution e.g. Na^+ and Cl^- are of the same order as thermal forces and appreciably influence the distribution of other ions present. This results in a change in the net force that is applied by the electric field. This is the principal cause of decreased mobility with increasing ionic strength. The force applied remains XQ but a new retarding force is added, owing to the fact that ions of opposite charge, group about the migrating ions resulting in local ionic atmosphere of opposite charge. As a consequence, the fluid surrounding the charged particle moves in a direction opposite to that of migrating particle with an attendant decrease in mobility. The degree of effectiveness of various electrolytes in creating this retarding effect depends upon their concentration and the charge. It does not depend upon the specific chemical nature of the ion but primarily on its valence.

We have seen that the velocity, V, with which an ion migrates is proportional to electric force acting on per unit charge. This force is in turn, proportional to the field strength. Field strength, X, is expressed as potential in volts divided by the distance between the electrodes in c.m. In practice geometrical considerations make it impracticable to measure this distance directly. A simple Ohm's law transformation permits to measure field strength, X, at any cross section, s, between the electrodes to be calculated from the expression.

$$X_s = \frac{i}{q_s x k_s}$$

where i = current in amperes, q_s = area at cross section under consideration in square cm. k_s = specific conductivity in mhos/cm across cross section.

In order to carry out any type of electrophoretic separation, it is necessary that convection be controlled so that resulting mixing of the separated solutes does not take place and thus obscure or destroy the resolution. In the classical moving boundary method of Tiselius, convection control is effected by proper regulation of temperature gradient. In zone electrophoresis convection is prevented by the supporting medium, provided the medium is not overloaded with electrolyte.

It is obvious that greater the difference in the mobilities of any two ions A and B more easily and quickly they are separated; thus if u_a and u_b are the mobilities of components A and B

respectively which migrate distance d_a and d_b and X be the field strength and t be the time of separation. Then the difference in mobilities are represented by the equation :
$d_a - d_b = (u_a - u_b)$ x X x t

For weak electrolytes and amphoteric substances which are of considerable biological significance, the degree of separation depends also upon the degree of their dissociation.

During the course of electrophoresis, free diffusion occurs as a function of time, concentration and temperature. The diffusion, therefore, sometimes leads to limitation in the choice of field strength-time relationship chosen to effect a given separation. The rate of diffusion is roughly inversely proportional to the initial width of the sample section and imposes limits on the extent to which the width of sample section may be reduced in order to achieve equivalent separation of components with shorter migration distances. Thus to obtain maximum resolution in lesser time, applied field strength and shorter migration channel, the starting zone must be of infintisimally small size.

Secondary Effects of the Electric Field

Heating effect

When an electric current flows through a column of electrolyte of length L and cross sectional area A and specific conductance k, heat is produced according to the relation :

$$W(\text{watts/ml}) = \frac{Ei}{LA} = \frac{i^2}{kA^2}$$

where E = Electrical potential across the column and i is the current.

Electric heating provides the principal limitation on the use of high potential gradients for electrophoretic separation, since field strength is proportional to i and heat produced to i^2.

Excessive heating produces number of undesirable effects, notably (a) modification or destruction of labile solutes and inhomogeneities in the physical properties of separation system. The latter arises from the fact that heat is evenly produced across the migration channel, but is only removed at the periphery. Thus the central or inner portion tends to become warmer than the outer portion consequently a parabolic temperature gradient is established across the channel. Since in general, ionic mobility increases 2-3% per degree rise in temperature, ions near the centre of the channel will move faster than those at periphery, resulting in parabolic mobility of ions and curved zones will be obtained. The effect of electrical heating can be minimized either by changing the design of the apparatus or by changing the concentration of electrolyte.

Combining the equations $X = \frac{i}{AK}$ and $W = \frac{i^2}{KA^2}$ we get the equation :

$$\frac{X}{W} = \frac{A}{i}$$

Thus more favourable relation between field strength to heat produced can be achieved by reducing the current density in the channel. This can be achieved by increasing the cross sectional area A and/or by reducing the current i. To reduce the current is better and that can be

best afforded by using the buffer salts at low concentration and electrolyte conductance. Second method of reducing heating is by efficient transfer of heat from separation channel by increasing the ratio of perimeter length to cross sectional area. This can be best afforded by using rectangular tubes of maximum thermal conductivity.

Electroendo osmosis

When a potential is applied to a liquid contained in a non-conducting capillary system, bulk movement of liquid towards one of the electrodes takes place. This phenomenon is known as electroendo osmosis. The effect is of considerable significance in zone electrophoresis conducted in porous supporting media. Although it does not directly influence the course of separation , the bulk movement of liquid through medium will increase or decrease the effective length of migration channel accordingly as electro-osmotic flow occurs along or against the direction electrical migration of ions. The electro-osmotic flow must be considered in any attempt to calculate ionic mobility in porous system, as the observed boundary displacement is the algebraic sum of electro-osmotic and electric transport.

Boundary shape and electrophoretic migration

Under initial conditions of both front and zonal analysis the boundary between the solute mixture and the buffer solution forms a well defined plane surface at right angles to the direction of migration. As the migration proceeds, the initial sharp boundary undergoes a progressive deterioration in shape. The most important factor influencing this deterioration is diffusion. The initial state represents an extremely steep concentration gradient and the rate of diffusion is directly proportional to concentration gradient (Fick's first law of diffusion). Inhomogeneities in the migration channel such as variation in the temperature, electrolyte concentration, conductance and density will also produce deterioration. It has been shown that a sharp boundary of resolved solutes is favoured when the ratio of electrophoretic mobility to diffusion constant is high and which can be easily attained by increasing the field strength i.e. voltage across the electrodes. Lowering in temperature is also helpful.

Adsorption of solute on supporting gel also effects the sharpness of zones. If the solutes are irreversibly adsorbed there will not be any useful result, either there will not be any movement or the moving zone will be continuously depleted of the solute until they are evenly distributed along the migration channel. Reversible adsorption can affect the sharpness of zones. At the front of the boundary, those molecules which have moved ahead of the main zone due to diffusion or any other cause will be present in low concentration and will be strongly adsorbed. Since only charged molecules can migrate, the adsorbed molecules will be soon overtaken by main zone, where the molecules are present at higher concentration and are consequently less strongly adsorbed. Automatic sharpening of the front boundary will, therefore, occur. At the rear, a zone of more strongly adsorbed molecules (from dilute solution) would result in tailing.

Experimental Details

The electrophoresis can be subdivided into a number of techniques :

(a) Frontal methods usually carried out in free solution
(b) Zonal methods

1. Powdered or other porous solids as supporting media
2. Strip of paper or other fibrous material such as cellulose acetate
3. Gels: (i) agar-agar (ii) hydrolyzed starch, (iii) acrylamide and agarose

Frontal method

It is usually carried out in free solution and the method involves the movement of a boundary, separating a solute solution from a supernatant buffer. The movement of the boundary must take place in a vertical channel and the solution below any boundary must be denser than above it, otherwise gravitational convection will destroy the boundary system.

As shown in the figures 1a and b three solutes A, B and C are contained in a vertical u tube. After passage of electric current for suitable period of time, a series of boundaries would be set up depending upon relative mobilities of the solutes.

Boundary I separates pure component A from the supernatant buffer. Boundary II separates solution A from that of A + B, while boundary III separates latter from A+B+C. Similarly on descending side boundary VI separates a solution of slowest moving component C from the supernatant buffer, boundary VII separates C from B+C. The false boundary IV on the ascending side occupies the initial boundary and separates two solution in which every component is present on both the sides of the boundary and arises from different movement of various ions. Similarly, boundary V on the descending side, is also a stationary boundary. These conditions satisfy the gravitational stability, as each boundary separates from an underlying solution of greater density, owing to the presence of at least one additional component in each underlying solution. This would not, however, attain if boundary VII is allowed to migrate down in the horizontal section of u tube as the solution of components B and C would then be below the solution of component A, B and C. The consequent gravitational convection might pass into ascending limb of u tube and effect boundary I, II, III and IV. It can be seen that a boundary constitutes two frontal analyses, from which only fastest and slowest moving constituents, A and C, respectively, can be isolated in pure state. This is the basic disadvantage of the frontal analysis. Thus the method is of much historical significane and is of little advantage for preparative purposes and is of use for analytical purposes only.

Boundary method in free solution is wholly due to Tiselius, who introduced three major innovations:

1. Operation at $4^{o}C$, the temperature of maximum density of water, which allows greater potentials to be applied without marked thermal density changes and consequent convectional disturbances.
2. Use of a narrow rectangular migration channel which permits rapid thermal equilibrium.
3. Introduction of optical methods based on refractive index changes for localization of boundaries between colorless solutions and for accurate measurement of changes across the boundary. The subsequent introduction of interferometric method has improved the precision and convenience of these methods.

The migration channel is made in three parts. The upper part for the connection to electrode vessel, the migration channel proper and the lower bridge piece.

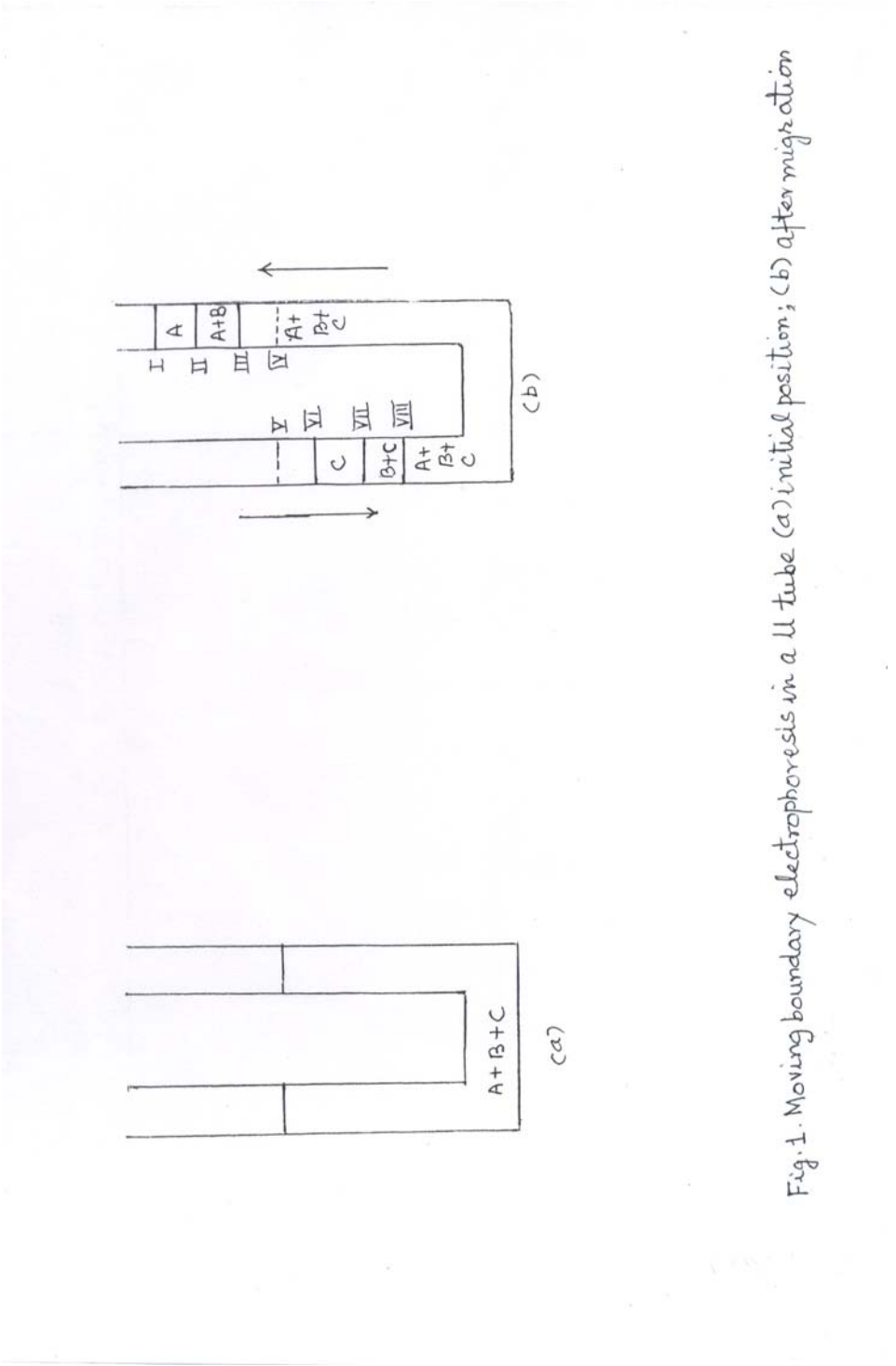


Fig. 1. Moving boundary electrophoresis in a U tube (a) initial position; (b) after migration

The left limb and section is filled with the solution to be examined while right limb and section is completely filled with appropriate buffer. At the end of experiment right limb is again displaced to isolate the separated boundary system for sampling or other examination.

Powdered or porous supporting media
Variety of finely divided powders such as potato starch, glass, various types of celluloses and polyvinyl chloride were tested as the anticonvectant supporting media. Other porous materials such as sponge, rubber and polyurethane have also been tested.

Electrophoresis has been conducted either horizontally or vertically, both the techniques have their own advantages and disadvantages depending upon the need the experiment.

Powdered anticonvectant medium is moistened with appropriate buffer and packed in rectangular open container. The container may be of glass or Perspex.

There are separate compartments to keep buffer and electrode to prevent migration of product of electrode reaction to the gel slab. Electrode compartment consists of normal saline. The two compartments are connected together with moist filter paper wads.

Powdered anticonvectant medium is formed in to slurry in suitable buffer and poured in the rectangular container. Excess buffer is removed by blotting it out.

Before electrophoresis proper electric current should be passed for some time to equilibrate the supporting medium and remove excess buffer. In order to minimize loss of buffer from porous medium, the surface of the medium after applying the sample is sealed either by covering it with glass plate or layering with-molten paraffin wax.

Application of sample:
(a) A narrow slot is cut in the porous medium and sample solution is introduced into it.
(b) To the sample small quantity of porous medium is added and the thick slurry, so obtained, is introduced into the slot cut in to the supporting medium.
(c) A filter paper strip is cut, soaked with the sample and introduced in to the narrow slit cut to the size of the paper strip in to the supporting medium.

The plate loaded with sample is subjected to electrophoresis. Electric current, usually 1-5 mA/cm^2 cross sectional area is applied at 0-4^oC for predetermined time. The voltage gradient varies from 10-15 V/cm. Higher voltage gradients can be applied for better results if there is provision for efficient cooling.

Detection of separated zones
After electrophoresis is complete, the cover is removed and solute zones are located by pressing a filter paper strip on the surface for some time so that the separated zones diffuse from supporting medium to the paper. The strip is then dried and stained with suitable developing reagent.

The other commonly employed method for detection of separated solutes is to cut small slices transversely and elute the solute by crushing the slice in suitable buffer.

Zone Electrophoresis on Solid Anticonvectant Media

Various solid supporting media including filter paper and cellulose acetate strips, porous gels made from agar-agar, partially hydrolyzed starch and polyacrylamide have been used to obtain discrete zones of solutes on the basis of differences in their electrophoretic mobility.

Paper and cellulose acetate strip

Filter papers of varying thickness have been widely used. The filter paper strip is soaked in appropriate buffer and connected to electrode compartments. A narrow streak or spot of sample is applied on the paper at an appropriate position and electric current is applied for predetermined time. Separated solute bands are visualized by appropriate staining. The paper electrophoresis has several disadvantages such as

(i) the technique, as such, can not be used for preparative purposes,
(ii) cellulose adsorbs many solutes, at times irreversibly, therefore, it takes longer time for separation to complete with attendant denaturation of labile molecules,
(iii) moreover adsorption of solutes of different ionic mobilities result in inferior separation and
(iv) separation here depends mainly on the basis of differences in the ionic charges of the solutes, resolution is therefore poor compared to porous gels where better resolution is obtainable because of molecular sieving action of the gels in addition to differences in the charge.

Better results are obtained by carrying electrophoresis on strips of cellulose-acetate instead of paper. Since cellulose acetate does not have any charged groups, separation takes much less time, separated bands are sharp and there is no tailing. After electrophoresis is over, separated solute zones may be visualized by staining with-suitable dyes (for proteins commonly employed dyes are bromophenol blue, amidoblack, Coomassie brilliant blue etc.)

Zonal electrophoresis on porous gels

Use of gels as supporting anticonvectant medium for zone electrophoresis has largely superseded other forms of electrophoresis because much improved resolutions are obtained and the technique is used for preparative work with the same efficacy as for analytical work. In general, gels are easier to handle, separation is improved due to the fact that electrophoretic separation of solutes is further facilitated by molecular sieving action of pores of gels on the solutes to be separated. Gels are semisolid, hydrophilic or semi-solid colloids. Most commonly employed gels are made from agar-agar, agarose, partially hydrolyzed starch and cross linked polyacrylamide.

Agar

Agar is a cheap, non-toxic, chemically ill defined complex powdery mixture of two galactose based polymers linear agarose chains and branched agaro-pectin. Agar dissolves in boiling aqueous buffers and sets to form a gel at about 38^oC. 1% w/v agar gel in buffer has high water content, good fibre structure and has, therefore, good anticonvectant property, large pore size and low frictional resistance, consequently, movement of ions is very rapid. Disadvantages in the use of agar include adsorption of solutes due to SO_4 ions, subsequent tailing and poor resolution and severe electroendo-osmosis. These effects are minimized by prior purification of agar to remove SO_4 ions.

Partially hydrolyzed starch
Starch is partially hydrolyzed under carefully controlled conditions. The degree of hydrolysis determines the pore size of starch gel. Therefore, by controlling the degree of hydrolysis, the starch may be obtained which when gelified gives desired pore size.

Partially hydrolyzed starch when boiled gives a solution which upon cooling forms a gel. The porosity of the gel can be varied by changing the concentration of starch to prepare the gel from 5% for very porous gels to 20% for very small pore gels. However, for a given concentration of starch, the pore size of the gel is neither uniform nor can be accurately determined. However, pores of the gel facilitate separation, besides electric charge, by molecular sieving. As a result, separation on these gels is far more superior than on paper or agar gels. Both two dimensional and preparative electrophoresis can be done on starch gels.

Agarose
Purified agarose is now used extensively for the separation of nucleic acid and DNA restriction fragments because agarose gels are without charge and thus lack endo-osmosis. Since the pore size of agarose gels is very large there is negligible molecular sieving.

Polyacrylamide gels
The gels are strongly hydrophilic, do not have any charge thus there is no adsorption of solutes and there is absolutely no electroendo-osmosis. Gels have uniform pore size which can be varied at will during preparation of gels. Separation of solutes in these gels is both due to charge differences and molecular dimensions. Due to these properties separation on polyacrylamide gels is very rapid with high degree of resolution and very sharp separated zones are obtained. Also, by modifying electrophoretic conditions, it is possible to separate solutes entirely on the basis of molecular size and weight only by molecular sieving action.

Buffers suitable for starch and PAG Electrophoresis

For successful conduct of electrophoretic separation of the zone type in which the individual solutes move further and further apart from one another, presence throughout the system of charged molecules other than those undergoing separation is essential. Because of uniform pH needed throughout the migration channel, a buffer is needed. Various buffer systems have been employed for improving the resolution.

Same buffer in gel and electrode compartment
1. pH in the range of gel where separation occurs should be such that there is maximum difference in mobility of the solutes to be separated. Thus a buffer of proper pH is selected by trial and error.
2. Ionic strength and the conductivity of the buffer must be as low as possible, because at low conductivity higher mobilities can be achieved with minimum production of heat. Although buffers of low conductivity have the advantage that higher potential gradients can be applied for rapid separation, the degree to which a buffer can be diluted is strictly limited, because on dilution of the buffer the probability of molecular association increases. Moreover, upon dilution probability of adsorption of solutes, though minimal in these gels, increases.

3. The buffer should be such that the possibility of interaction between solutes (especially proteins) is minimized. Such interactions are less likely in buffers of higher ionic concentrations.
4. The buffer should be such that interaction between it and solutes undergoing separation does not lead to formation of more than one band for a single protein.

Different buffers in the gel and electrode chamber

We will be considering systems in which there are changes in buffer composition and concentration along the length of the gel and electrode compartments.

As employed by Ornstein and Davis, contents of each gel consisted of three layers of acrylamide with different porosity and buffers composition. The systems constituted, was named by originators as disc electrophoresis, because of very narrow bands or discs of separated zones and also use of discontinuous buffer system employed. The purpose of upper or sample gel is prevention of loss of sample by convection. The middle or spacer gel permits concentration of the sample prior to separation. Only in the lower gel does the separation actually occur. Because of difference in buffer composition in large and small pores gel, the sample is caused to concentrate sharply at the gel interface. Only after this the solutes separate in the third gel. The great advantage of the technique is that it permits large volume of the sample to be applied.

Buffers composition of Ornstein and Davis system

Gel layer	**Buffer solution**					
	In Gel			**In Electrode vessel**		
	Buffer ion	**M**	**pH**	**Buffer ion**	**M**	**pH**
Sample (large pore)	Tris Cl	0.062 0.06	6.7	Tris-Glycine	0.005 0.038	8.3
Spacer (large pore)	Tris Cl	0.062 0.06	6.7			
Small pore gel	Tris Cl	0.37 0.06	8.9			

The behaviour of slower and faster ions are determined, in separate zones, by so-called Kohlrauch regulating function. If such a system is present in the gel and current is passed downwards, a sharp boundary will be maintained between the so-called “leading” and “trailing” ions. In addition, an increased potential gradient will come in to existence where the faster ion has been replaced by the slower. Under these circumstances a protein with the intermediate mobility, if present in the upper part of the system will migrate rapidly down and form a concentrated disc at the top surface of the small pore gel. Subsequently proteins enter and separate in small pore gel maintained at somewhat higher pH.

Narrowing of the starting protein zone can also be obtained by simpler methods than described above e.g. (a) applying the mixture to be separated, in dilute buffer and/or following the sample with water or diluted buffer results in band sharpening because of higher potential gradients produced in the region of the column occupied by the sample. The above principle has been used to obtain comparable degree of resolution and band sharpening wherein the gel was prepared in 0.37 *M* Tris at pH 9.5. The same buffer was used in electrode compartment, but the sample was diluted with 2.5 vol. with water before use. Under these conditions proteins move into gel from the region of lower conductivity with the result that the starting zone becomes narrower. Although a considerable degree of band sharpening of the sample can be achieved in this simple manner the band sharpening can also be attained by buffer front consisting of ions of a different mobility. In one of such systems borate (slower ion) follows citrate (faster ion) leading to considerable increase in potential gradient that provides considerable degree of band sharpening. Here band sharpening occurs only after all the solutes have left the origin i.e. some degree of separation has already taken place (Gel buffer: Tris 0.068 *M*, citrate 0.025 *M*, pH 7.2; Electrode buffer Borate 0.05 *M* pH 8.6).

Starch gel electrophoresis

Depending upon the pore size required starch gels are prepared by preparing 5-20% suspension of partially hydrolyzed starch in suitable buffer. The suspension is heated at about 90^{o}C and suction is applied to cause boiling of the suspension till all the air is removed and a clear solution is obtained. While still hot, the solution is poured in rectangular plastic trays with thin bottom to permit ready dissipation of heat. When cooled, the solution is transformed into white gel. Generally, sample is applied as a narrow zone in a slit cut out in the gel. After applying the sample the slit is sealed. Electrophoretic run can be carried out by connecting the ends of the gel to electrode compartments containing suitable buffer by filter paper wicks. Electrophoretic migration can be carried out both horizontally or vertically. Horizontal electrophoresis has the disadvantage of electro-decantation where in the shape of separated bands may be irregular. After electrophoresis is over, the gel is sliced in two halves and freshly exposed surface is stained by suitable dye to visualize the separated solute zones.

Polyacrylamide gel electrophoresis

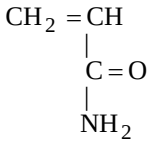
Two solutes of different size but same charge density may not separate by paper electrophoresis, whereas provided that the size difference is large enough, they could be separated by starch and polyacrylamide gel electrophoresis because of the sieving action of pores in these gels.

The extent of molecular sieving depends upon how close the gel pore size approximates the size of the migrating particles. The pore size of the agarose gels is sufficiently large so the molecular sieving of most proteins is minimal and separation is based mainly on charge density. In contrast, starch and polyacrylamide gels have the pores of same order of size as protein molecules and these contribute to molecular sieving effect.

Pore size of starch gels, though dependent upon concentration of starch used to prepare the gel, is neither uniform nor well defined. On the other hand, polyacrylamide gel can be prepared from highly purified reagents in a reproducible manner. These gels are chemically inert, highly

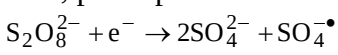
hygroscopic, devoid of any charge and stable over a wide range of pH and temperature and are transparent. Pore size of the gels is uniform and can be varied by varying the composition of the gel.

Acrylamide has the following structure :

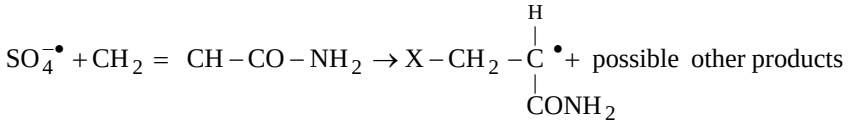


when a free radical forming agent such as ammonium persulphate or riboflavin and light is added in its aqueous solution in absence of oxygen a free radical end to end polymerization occurs.

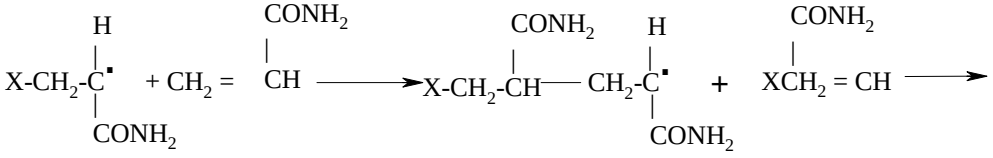
First, persulphate ions form sulphate ion free radicals :

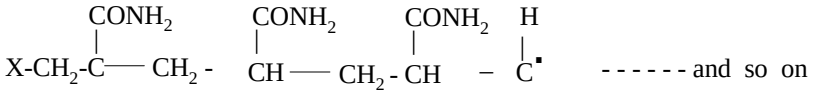


Latter form acrylamide free radicals :

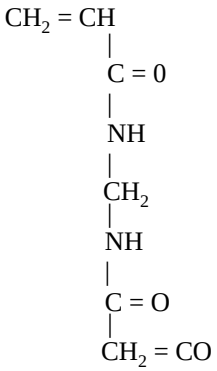


Where X represents original sulfate free radicals. Acrylamide free radicals then react with acrylamide molecules in a series of reactions to form long chain acrylamide free radical:

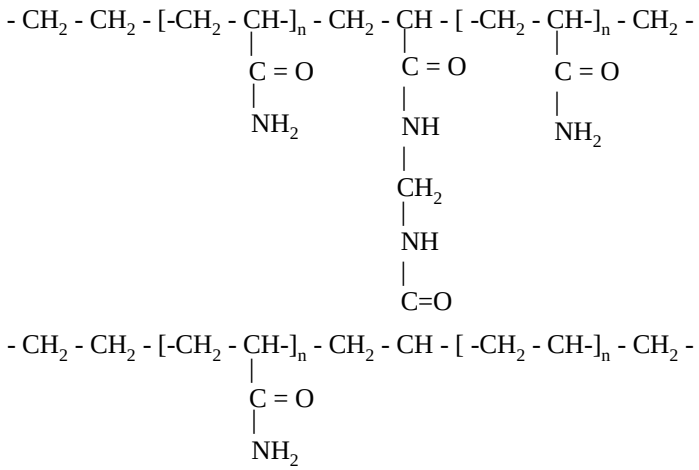




Although gelation occurs in the presence of ammonium persulfate or riboflavin, addition of tertiary amines such as N,N,N′,N′-tetramethyl ethylene diamine (TEMED) or diethyl amino propionitrile acts as catalyst to polymerization. The resulting solution contains long unbranched polyacrylamide chains which is viscous but not a gel. Addition of methylene-bis acrylamide causes cross linking in otherwise independent chains to form a cross linked porous gel.



N,N'-methylene bisacrylamide



Cross linked polyacrylamide

The carboxyamide residues of polyacrylamide gel are chemically stable, strongly hydrophilic but devoid of charge.

Gelation is slow at low pH values and can be delayed by holding the solution at $0^{o}C$. After gelation is complete, excess persulfate or riboflavin and unpolymerized acrylamide can be removed by soaking the gel in excess buffer or passing electric current.

The pore size of a gel is dependent upon total acrylamide concentration (acrylamide plus bis acrylamide). With increase in acrylamide concentration the pore size decreases.

For most purposes 5% bis acrylamide to total acrylamide concentration is very suitable. However, Davis recommends 2.6% bis acrylamide for small pore and 5% for large pore gels.

Gels can be made from 2.5-40% acrylamide. With increase in concentration, pore size decreases. If one desires to separate all components of a protein mixture, the best way is by using gradient gel electrophoresis. In this technique concentration of acrylamide gel increases (thus pore size decreases) along the direction of protein migration. Gradient gels have two advantages over uniform concentration gel: (i) they are able to fractionate protein over a wider range of molecular weight, and (ii) the gradient in pore size causes significant sharpening of protein bands during migration. The result is that the gradient gels are unsurpassed in the resolution of

mixture covering a wide range of molecular weight. A useful gradient gel for initial SDS-PAGE analysis is 5-20% linear gradient slab gel.

Both step and continuous gradients are used. The disadvantage with step gradients being that they can give multi-component bands at the interface between two layers.

Gradient gel can be formed both as a slab or in tube. Two containers of identical dimensions are placed at leveled surface and solution filled to the same height. The container connected to tube or gel cassette has concentrated solution and the other diluted solution respectively and connected through two way stopper. The concentrated acrylamide solution runs down first and settles at the bottom of the tube. As the concentrated solution from container enters the tube, where gel is to be formed, equal volume of dilute solution enters into this chamber. So the concentration of acrylamide in the tube decreases and a gradient is formed from bottom to top of the tube. Ammonium persulfate or the catalyst are added in lower than needed quantity to delay gelation.

Electrophoresis an example

Separation of serum proteins

A stock solution of acrylamide and bis acrylamide in suitable proportion may be stored in dark at 2^{o}C. For preparation of gels containing 12.5% acrylamide, the following solution may be added: 14.3% acrylamide (within addition desired concentration of bis acrylamide, usually 2.5% of the total) in 0.37 *M* Tris buffer, pH 8.8. Immediately before use, dissolved air must be removed from the solution *in vacuo*. To each 10 ml of this solution is next added 0.72 ml of 0.5% TEMED and 0.72 ml of 0.06% solution of ammonium persulfate. Without delay this solution is run in glass plate sandwitch with spacers (Fig. 2). Pipetting by mouth is dangerous as the acrylamide solution is neurotoxic; after gelification, it is relatively harmless. The spacer comb is inserted into the solution to desired depth to form sample wells. As the gel is formed, comb is removed and the wells are filled with electrode buffer.

Electrode buffer

0.05 *M* Na borate pH 9.2.

Preparation of sample

Sample should contain 7.5 mg/ml sucrose or a drop of glycerol to increase density and serum containing protein concentration of 1-2 mg/ml. A volume of 20-50 μl gives satisfactory result. A marker dye such as bromophenol blue is also added to track electrophoretic mobility.

Application of sample

The sandwitch is placed in the electrophoresis apparatus. This apparatus has the upper cathode compartment and lower anode compartment. Electrode buffer in the lower compartment should be to such a height that the gel is below the level of buffer in the chamber to ensure uniform cooling of the gels. The desired volume of sample is very carefully layered over the gel in the form of a narrow band.

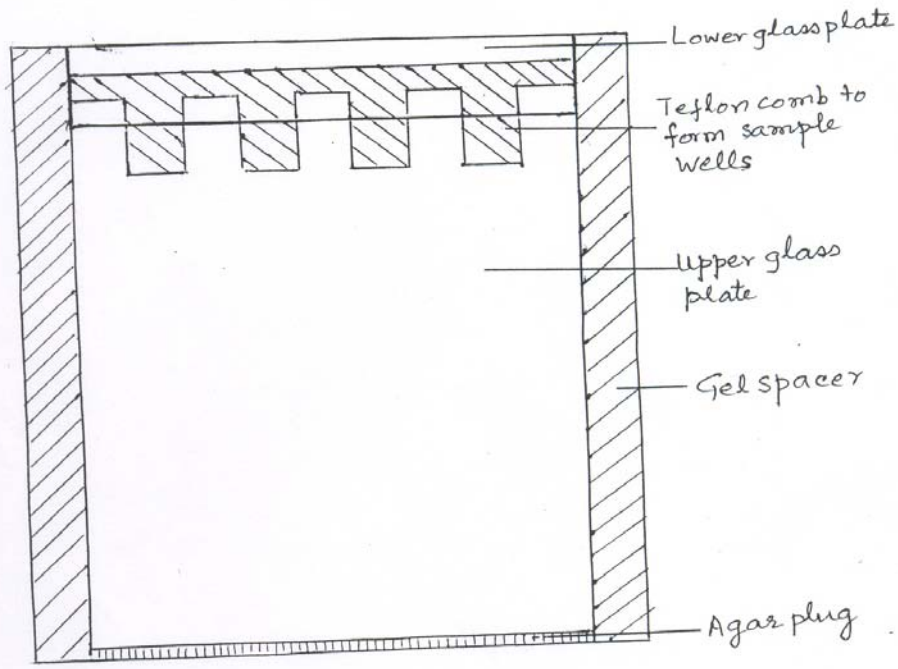


Fig. 2. Arrangement to cast acrylamide gel slab

Electrophoresis

Constant current of 2 mA/sample well at 150-200 V is applied till the proteins enter into the gel as noted by movement of marker dye. Then the current may be increased up to 20 mA/sample well, if there is provision for efficient cooling. Electric current is passed till the marker dye travels down the bottom of the gel.

Staining and destaining

The gels are taken out of and stained with suitable dye to detect proteins. Number of dyes are used to detect proteins e.g.

Coomassie brilliant blue

Dissolve Coomassie blue in water : methanol : glacial acetic acid (5:5:2 v/v) to prepare 0.1 % solution. Filter off un-dissolved material. Stain the gels for 24 hr at room temperature and remove the back ground color by repeated washing with 10% CH_3COOH. This stain is far more

sensitive than amidoblack and up to 0.5 μg protein in a sharp band can be detected. Staining is quantitative up 15 μg protein.

Silver stain

This stain is up to 100 times more sensitive than Coomassie blue. After electrophoresis place the gel in sufficient amount of fixative solution (50% (v/v) methanol, 10% (v/v) acetic acid) for 1 hr. Gels may be left in this solution overnight. The gel is then transferred to rehydration solution (10% (v/v) methanol, 5% (v/v) acetic acid) and left for 10 min. Rehydration solution is discarded and replaced by glutaraldehyde solution (1% (w/v) glutaraldehyde). Soak the gel in this solution for 30 min. Discard glutarladehyde solution and wash the gel with deionized water for 15 min. Discard the wash and add enough dichromate solution (34 m*M* potassium dichromate, 32 m*M* nitric acid) and leave for 5 min. Discard dichromate solution and add enough staining solution (0.118 *M* silver nitrate), leave for 25 min. Now discard the staining solution and wash the gel briefly (approximately 15 sec.) with 50 ml reducing solution. Followed by washing with 500 ml reducing solution (0.283 *M* sodium carbonate, 7 m*M* formaldehyde). Continue development till proteins are sufficiently stained. Stop staining by placing the gel in 3% (v/v) acetic acid. Finally wash the gel with 10% methanol.

Preparative electrophoresis

Besides analytical uses, electrophoresis can be used for purification of macromolecules by upgrading and increasing the size of the gel.

Enzyme electrophoresis

For localization of enzymes after electrophoresis, on the gel, it may be incubated with the substrate of the enzyme and then treated with some color forming agent with the product after washing off the substrate (e.g. by incubating the gel with starch and treatment with I_2-KI reagent locates the position of amylase).

Isoelectric focusing

The method requires a stable pH gradient between anode and cathode in an electrophoresis cell. This is obtained by electrolysis of solution of mixture of suitable low molecular weight ampholyte called carrier ampholyte. When proteins are allowed to move by passing electric current, each protein will migrate to, and focus at its isoelectric pH. This makes the following possible:

1. Separation of protein with high degree of resolution for analytical as well as preparative purposes. Proteins differing in isoelectric pH with as little as 0.01 of pH unit may be separated.
2. Characterization of each protein by its isoelectric pH. pI values are obtained simply by measuring the pH of gel where concentration of protein in question is maximum.

This technique requires simple apparatus and is easy to handle. The volume of sample to be applied is not critical and dilute solution in large volumes can be loaded as a given protein would focus at a suitable place in the gel. This is a clear advantage over other conventional electrophoretic and chromatographic techniques. Molecular size is not important for separation.

The present technique, like other electrophoretic methods, requires means to counteract convective mixing of separating components. Three stabilizing techniques are used (a) density

gradient, (b) use of polyacrylamide, agarose or dextran gel and (c) zone convection electrofocusing. Technique (b) is very simple and most common.

pH gradients are produced by incorporating synthetic carrier ampholyte into the gel. The natural pH gradients can also be generated with ordinary buffers with different pKs. Care should be taken to ensure that gradient remains stable for at least a period till electrophoresis is complete.

Initially strongly acidic anolyte and strongly basic catholyte solutions were used as electrode solutions. The rationale for this was carrier constituents would protonate or deprotonate when they migrated to the ends of the gel and made contact with extremes of pH, and thus reverse the direction of their migration and remain within the gel. However, pH gradients are generated equally well if one uses terminal carrier ampholytes of the pH gradient as anolyte and catholyte in which case there is additional advantage that the stability of pH gradients with time is enhanced.

Two dimensional gel electrophoresis

Two dimensional gel electrophoresis is a powerful approach to resolve proteins and polypeptides in complex mixtures. Initially the technique was used to double the length of migration channel. To afford this the sample to be separated, was loaded on one corner of the rectangular gel slab and electrophoresis was carried. After this first electrophoresis, electrophoresis was carried out by passing electric current through the gel placed at 90^{o} clockwise to the first run.

Now a days separation in the two directions is affected by employing two different separation techniques. Most common technique is to carry out separation by native or SDS-PAGE in one and isoelectric focussing in the other dimension.

Usually isoelectric focusing is carried out on the strips of gel to resolve the proteins on the basis of their differing isoelectric pH. Strips are then loaded on the gel slabs, equal to the thickness of the strips and electrophoresis carried out. These gel slabs may be meant for native, SDS or gradient gel as the need be.

Isotachophoresis

Like electrofocusing, this technique is a moving boundary electrophoresis. The principle underlying isotachophoresis is used in the stacking gel system in SDS PAG electrophoresis. The name of the technique, derived from Greek, refers to the fact that ions being separated while travel (phoresis) at the same (iso) speed (tacho). Separation of ionic components of the sample is achieved by stacking them in discrete zones in order of their mobilities, producing very high resolution.

For separation of a mixture of anions, a leading ion (e.g. Cl^{-}) is chosen which has higher mobility than the sample ions and a tailing or terminating ion (e.g. glutamate) which has lower mobility than sample ions. All the anions must have a common cation usually tris. When current is switched on, the leading ions would move to appropriate electrode, sample ions would follow in order of their mobility and tailing ions would follow behind the sample ions. Once equilibrium is reached all the ions would move at the same speed in discrete bands in order of their mobilities.

Determination of molecular weight of macromolecules

Principle

When proteins are treated with anionic detergents such as sodium dodecyl sulphate (SDS), it binds to proteins in proportion to their size. When treated with SDS and a reducing agent, polypeptides become rods of negative charge, but with equal charge densities. Intrinsic charge of polypeptides is effectively overwhelmed by SDS binding thus allowing their migration and separation solely according to their size.

This method is rapid, versatile and requires very little sample and the sample need not be pure.

Procedure

Marker proteins with known molecular weights and unknown are heated in 1% SDS and 2% 2-mercaptoethanol at 100^{o} C for 10 min. They are then electrophoresed in 7-10% acrylamide gel containing 0.1% SDS. Electrode compartments have suitable buffer containing SDS. Electrophoresis staining and destaining is carried out in usual fashion. Using the distances traveled by marker proteins, a graph is plotted between molecular weight and electrophoretic mobility. By interpolating the distance traveled by the protein of unknown molecular weight, its molecular weight is determined with accuracy of $\pm$ 5%.

Suggested Readings

1. Separation Methods in Biochemistry *by* C.J.O.R. Morris and P. Morris
2. Practical Biochemistry and Techniques *by* K. Wilson and J. Walker
3. Biochemical Methods-A concise guide for students and researchers *by* A. Pingoud, C. Urbanke, J. Hoggett and A. Jeltsch
4. Laboratory techniques in biochemistry and molecular biology *by* T.S. Work and E. Work
5. Methods in enzymology Vol. 182 Guide to protein purification M.P. Deutscher, ed.