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# HISTORY OF ELECTROPHORETIC METHODS

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## SUMMARY

Electrophoresis is the migration of electrically charged particles or ions in solutions due to an applied electric field. The ability to separate very similar substances including different proteins for analytical and preparative purposes has increased, especially since 1950, owing to the introduction of zone electrophoresis in paper and later in gels of polyacrylamide or agarose. After 1960, disc and displacement electrophoresis (isotachophoresis) and isoelectric focusing offered much increased resolution. Electrophoretic methods nowadays promote advances in biochemistry and molecular biology and will continue to be very important in science and for numerous applications in genetics, gene technology, sequencing of nucleic acids and proteins, studies of diseases and malfunctions including cancer, and in the identification of species and individuals, *e.g.*, in forensic medicine.

## INTRODUCTION

At least one separation process is almost mandatory in analytical or preparative studies of molecules of chemical or biological interest. Techniques based on the use of migration of electrically charged particles or ions in solutions due to an applied electric field between an anode and a cathode are collectively called electrophoretic methods. Within the last 30 years these have evolved rapidly to offer very high resolution separations useful for numerous purposes in chemistry, analytical chemistry and especially biochemistry and the biological sciences for research and numerous applied purposes. One could describe this as a virtual explosion in their use. The methods are now used by several thousands of researchers and laboratory workers. More than half of the scientific papers currently published in biochemistry depend on some use of electrophoretic methods. However, for many years the progress was very slow. The purpose of this paper is to give an overview of the development of electrophoretic methods from very primitive separations of ions by electrolysis to the refinement of various principles.

In moving-boundary electrophoresis, the ionized components of a sample migrate with sharp boundary fronts in a buffer solution. Usually on the basis of this principle only the fastest migrating component could be isolated. This method was replaced after 1950 by zone electrophoresis, where the moving components migrate as well defined delimited zones. During electrophoresis in free buffer solution there is

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a tendency for mixing of components to occur during separation because they often have a density higher than that of the buffer and because the applied electric current causes heating of the solution, thus making it less dense, which may cause thermal convection disturbances. Therefore, many principles have been tried and found useful to counteract this.

Zone electrophoresis can be performed in the solution space of various supporting media such as paper or cellulose powder. A few different types of gels can also often be used with advantage for zone electrophoresis and for most electrophoretic methods. In contrast to the use of these supporting media are principles that utilize separations in free solution in, *e.g.*, density gradients of solutes such as sucrose, capillaries of, *e.g.*, glass or plastic tubing, a revolving horizontal tube or narrow cells with a vertical free flow of solution.

In zone electrophoresis, a single buffer connecting the anode and the cathode is used. In displacement electrophoresis, a buffer with several ions, which in the electric field first orientate in a series according to their transport numbers, is used. Thereafter all these ions migrate towards one of the electrodes with the same speed.

The name isotachophoresis is derived from Greek: iso = equal; tacho = velocity. After 1960 this principle was developed into isotachophoresis, disc electrophoresis and related methods. In the former method the compound to be studied corresponds to one of the ions in the series. In disc electrophoresis, by special arrangements of discontinuous buffers of different composition, the displacement principle is used to create a narrow starting zone, which is then subjected to zone electrophoresis. The separations are performed in gels of mainly polyacrylamide. This method has been found very useful for the separation of complex mixtures of ionized molecules such as proteins and oligonucleotides. Electrophoresis with sodium dodecyl sulphate (SDS) in gels is very useful for the determination of the molecular weights of proteins.

In isoelectric focusing, a pH gradient is created so that the pH increases continuously from the anode to the cathode by use of special buffers, called carrier ampholytes. Each protein is transported to reach a pH value equal to its isoelectric point. The invention in 1964 of suitable carrier ampholytes paved the way for the widespread use of isoelectric focusing, allowing the convenient determination of the isoelectric points of proteins and very high-resolution separations. A good example is two-dimensional gel electrophoresis. Here proteins are first separated according to charge by isoelectric focusing in one gel and then separated by electrophoresis in a gel slab according to size.

In this paper, not only principles for the separation of components but also methods useful for their detection will be stressed. Developments of different principles are described under separate headings, which in some instances do not always present the developments in chronological order. Another consequence is that some of the methods used today could have been described under more than one heading, because they depend on the combined use of more than one principle.

## EARLY HISTORY AND MOVING-BOUNDARY ELECTROPHORESIS

Let us start with the early history. In 1791, Faraday presented his laws of electrolysis. In the nineteenth century, important experiments were made and some

theories formulated that promoted electrophoresis[1]. Interesting observations were made in applying an electric field at the ends of a horizontal glass capillary filled with an aqueous solution of a salt also containing charged particles. The inside surface of the tube wall acquired a negative charge. The layer of solvent close to the wall and the surface of a charged particle acquired an opposite charge, as pointed out by Helmholtz[2]. The resulting movement of liquid close to the tube wall towards one of the electrodes was called electroosmosis and could be described by an equation.

By carefully conducted experiments, the conductance and the radii of several small ions could be measured and calculated. Hittorf[3,4], Nernst[5] and Kohlrausch[6] registered for small ions transport numbers, *i.e.*, the fraction of the total current carried by the ion.

Very essential, especially for the much later development of displacement electrophoresis (later called isotachophoresis), was the formulation of the Kohlrausch regulating function describing the order of electrophoretic migration of ions and their relative concentrations[6]. He also formulated equations governing the behaviour in an electric field at a boundary, *i.e.*, a sharp front of moving colloidal particles. This was later of importance for large ions and proteins. The understanding of how to obtain moving boundaries with sharp fronts was important for achieving separations of components in a sample and also to be able to observe them. At the turn of the century, it was found that sharp electrophoretic moving boundaries of ionized species could be obtained in U-shaped glass tubes and that there was somtimes an electric potential drop over the boundary. Arrhenius[7] increased the understanding of ions in water solutions by his dissociation theory; in 1903 he was awarded a Nobel Prize.

In 1926, Svedberg obtained the Nobel Prize for his work on proteins and especially the ultracentrifuge[8]. He encouraged Tiselius to work on electrophoresis. In moving boundary electrophoresis and ultracentrifugation, the migration of protein boundaries are observed and recorded, but neither method gives a complete separation of different protein molecules into zones. In 1930, Tiselius published his thesis, "The Moving Boundary Method of Studying the Electrophoresis of Proteins"[9], which was important as it represented a new technique for studies of the physico-chemical properties of proteins. The experiments were performed in a quartz U-tube and the protein boundaries were detected by photography using ultraviolet light. The apparatus was improved by Theorell[10], which allowed him and others to separate serum into albumin and globulin. One problem was that the boundaries were often blurred by thermal convection caused by electrical heating in the solution. In 1937 Tiselius[11] reduced this effect by introducing an electrophoresis cell with a rectangular cross-section and efficient cooling at 4°C, where water has its maximum density. The improvements made moving-boundary electrophoresis an accurate analytical method. Thus for serum Tiselius described four moving boundaries corresponding to albumin and $\alpha$-, $\beta$- and $\gamma$-globulin (*cf.*, Fig. 1). The $\gamma$-globulin was found to increase after infections and immunization. In 1948 Tiselius won the Nobel Prize for the development of the moving boundary method and chromatographic adsorption analysis.

It is easy to understand why methods for the observation and measurement of the mobilities of ions and other substances during electrophoretic experiments have been very important. As early as 1866 Toepler published his schlieren (or shadow) method. Tiselius adopted this method for detecting moving boundaries[11]. Based on

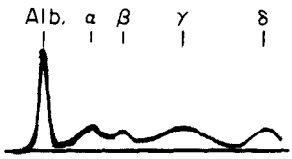


Fig. 1. Concentration distribution of blood plasma proteins following electrophoresis in a modified Tiselius moving-boundary apparatus as revealed by direct photography of the refractive index gradient by the Philpot–Svensson method. Alb. = Albumin; $\alpha$, $\beta$ and $\gamma$ = $\alpha$-, $\beta$- and $\gamma$-globulin; $\delta$ is a stationary boundary.

this, in 1938 Philpot[12] published the astigmatic schlieren camera method. Shortly afterwards improved procedures were presented by Longsworth[13] and Svensson[14]. The method permitted direct recording on a photographic film of refractive index gradients, which are proportional to the protein concentration gradient against the height in the cell, as illustrated in Fig. 1. This moving-boundary electrophoresis apparatus, developed by Tiselius, was later marketed worldwide by LKB and other companies.

Svensson, a pupil of Tiselius, also soon made very important contributions to the theory of electrophoresis[15]. The works of Svensson[14], Longsworth[16] and Dole[17] were of fundamental importance for the development also of zone and displacement electrophoresis. Longsworth[18] made careful measurements of transport numbers of ions and introduced them as variables. The theory showed that all ion constituents change their concentrations at all boundaries.

## DISPLACEMENT ELECTROPHORESIS: ISOTACHOPHORESIS, ZONE AND HIGH-PERFORMANCE ELECTROPHORESIS IN FREE SOLUTION

Most electrophoretic principles can be described on the basis of Kohlrausch's[6] equation for ionic migration, including zone electrophoresis, moving-boundary electrophoresis and isotachophoresis.

Hardy[19] discovered that the mobilities of proteins depend largely on the pH of the electrolyte solution in which they are present. The characterization of substances on the basis of their electrophoretic properties, *e.g.*, isoelectric points (p*I*), increased interest in electrophoretic separation techniques. Here it could also be mentioned that Michaelis[20] found that enzymes could be characterized by their isoelectric points, measured in electrophoretic migration experiments performed at various pH values.

It was not until about 1923 that a principle of electrophoresis other than boundary electrophoresis was described. Kendall and Crittenden[21] succeeded in separating rare earth metals and some simple acids by, as they called it, the "ion migration method", which was, in fact, isotachophoresis. It was stated that the ions not only separate, but also adapt their concentrations to the concentration of the first zone according to the Kohlrausch's regulating function[6]. Kendall[22] stressed the desirability of observing the separation in a convenient way. Thus he suggested the use of a coloured ion, with a mobility intermediate between those of the ions of interest. Later such intermediate ions, which could improve separations, were called spacers. Kendall proposed some detection methods, *e.g.*, utilizing thermometry, conductivity and spectrometry, especially for analysing metals[22]. In 1942, Martin separated

chloride, acetate, aspartate and glutamate by "displacement electrophoresis", named in analogy with displacement chromatography. Although he did not publish until he worked together with Everaerts, he told researchers about the experiments[23]. The "moving-boundary method" of MacInnes and Longsworth[24], which was used for the determination of transport numbers of ions, was also based on Kohlrausch's[6] theory.

In a Tiselius moving-boundary apparatus, Longsworth[18] introduced a mixture of cations between two other zones, called the leading and the trailing ions. On electrophoresis the intermediate ions were arranged in decreasing mobility from the leading towards the trailing ion. He also found that a steady state was obtainable. The importance of the pH of the trailing solution was also recognized.

From 1960, displacement electrophoresis was often performed in tubes with inner diameters (I.D.) of 0.2–0.5 cm. Reduction of the tube diameter improved the resolution, provided electroosmosis could be eliminated, *e.g.*, by coating as described by Hjertén[25]. He devised a micro U-tube moving-boundary electrophoresis cell made of quartz with an I.D. of 0.3 cm. A device recorded the derivative of the light absorbance at 280 nm. Electroosmosis was eliminated by coating the inner wall of the U-tube with methylcellulose and thermal convection was suppressed by efficient cooling. Protein concentrations as low as 0.1% could be determined. This apparatus reduced the amount of protein required and increased the accuracy considerably.

In 1963, Everaerts started work together with Martin on displacement electrophoresis in a glass tube of I.D. 0.5 mm filled with an electrolyte containing a linear polymer, *e.g.*, hydroxyethylcellulose. A thermocouple was used as a detector[26,27].

In 1964, Ornstein and Davis[28] introduced "disc electrophoresis", named after the use of discontinuous buffers. They placed a protein mixture between one electrolyte with an anion of high mobility and another electrolyte with an anion of low mobility (the trailing ion; see above). Owing to the concentration phenomenon of displacement electrophoresis, the proteins were stacked closely spaced as very narrow bands (thin as "razor blades"), between the two electrolytes ("steady-state stacking"). To separate the bands the principle of zone electrophoresis was subsequently utilized. Gels of cross-linked polyacrylamide was used as a stabilizing medium and for molecular sieving. The mobilities of the proteins could be much influenced by varying the pore size in the gel. It was very important to be able to see the protein bands in the gel after electrophoresis. Amido Black, first used, was soon replaced by Coomassie Brilliant Blue, which offered much lower detection limits for proteins[29].

In 1967, Vestermark published results on "cons electrophoresis" which was displacement electrophoresis with spacers[30]. This later proved important for increased resolution, *e.g.*, by use of carrier ampholytes to separate proteins. Preetz and Pfeifer[31,32] described an instrument for the measurement of potential gradients and also continuous counter flow equipment. Everaerts and Verheggen built an instrument for displacement electrophoresis and introduced it at the Karolinska Institute, Stockholm, in 1968. This was the basis of the LKB isotachophoretic equipment. LKB propagated the principle of displacement electrophoresis under the name of isotachophoresis. In the steady state, isotachophoresis is based on the important phenomenon of identical velocities of the zones of the separated ions. The name has been adopted by most researchers in the field and is derived from Greek: iso = equal; tacho = velocity; phoresis = to be dragged.

In 1967, Hjertén presented a thesis describing an apparatus for free zone electrophoresis in a revolving tube[25]. This permitted studies of, for instance, inorganic and organic ions, peptides, proteins, nucleic acids, viruses and bacteria. Mobility determinations could be made with high accuracy. The equipment can also be used for isoelectric focusing and isotachophoresis.

Isotachophoresis has been much promoted by the development of suitable detectors. In 1970, Arlinger and Routs[33] introduced a UV absorption detector, and in 1972, Verheggen *et al.*[34] a conductivity detector, which were built into commercial instruments. The imprecision may nowadays be as low as about 1% even at nanogram analyte levels. Some reviews on isotachophoresis have been published[35,36]. In isotachophoresis on-tube detection of the separated components and very high voltages are often used (up to 400 V/cm). By using a high voltage it is also possible to obtain an improved resolution in zone electrophoresis in narrow bore capillaries (I.D. often $<0.2$ mm), a technique called high-performance capillary electrophoresis (HPCE)[37–39]. Isotachophoresis and HPCE are useful for, *e.g.*, determinations of the concentrations of various ionic substances for, *e.g.*, water quality assays, and monitoring drugs and metabolites, partly because ionized substances can be analysed without derivatization, which is often necessary in gas–liquid chromatography.

Off-tube detection systems have been applied for analytical and micropreparative purposes. The compounds migrate out of the tube by electrophoresis or are transported by pumping liquid through an high-performance liquid chromatographic UV monitor and then to a fraction collector. By use of light absorption at about 185 nm, very low concentrations of proteins and several other substances can be monitored[40].

HPCE apparatuses have similar broad application areas as the revolving tube zone electrophoresis apparatus, but lack a scanning system. In a scanning free zone electrophoresis apparatus, but not in HPCE, the light absorption of the substances under study can be monitored during the course of separation. The scanning system also permits accurate mobility determinations, and every run can give many mobility values. Interactions between two solutes of different mobilities can also be studied.

## ANALYTICAL AND PREPARATIVE ZONE ELECTROPHORESIS USING SUPPORTING MEDIA AND FREE FLOW ELECTROPHORESIS

As mentioned in the Introduction, electrophoresis in free solution is very prone to disturbances by thermal convection. Therefore, some different principles have been tried in order to counteract this. Electrophoresis in aqueous solutions in the spaces of filter-paper as a supporting medium, paper electrophoresis, became a success from about 1950, especially in the routine biomedical analysis of serum, thanks to contributions by Wieland and Fischer[41] and others[42–44]. There are some special explanations for this. First, up to this time the boundary electrophoresis according to Tiselius was almost the only commercially available type of electrophoresis apparatus. It was expensive and required many square metres of floor, whereas the equipment needed for paper electrophoresis was simple to construct, relatively cheap and required less than 1 $m^2$ of bench space. Second, with earlier apparatus, complete separation of proteins was difficult to achieve and many milligrams were required, whereas with electrophoresis in paper, components of mixtures could be separated into zones that

were easy to isolate or reveal by staining for proteins, lipids or carbohydrate, etc. For analytical or diagnostic purposes this required much less than 1 mg of sample and the principle was therefore much used.

Svensson was responsible for LKB's development of a paper electrophoresis apparatus, which became very popular especially in routine biomedical analyses of serum, owing largely to his and Valmet's invention of the "fakir mattress" as a support for the paper strips[45]. Later, Hjertén developed a zone-sharpening method, which allowed the application of dilute protein solutions[46]. Amino acids, peptides and nucleotides were later often separated first in paper by chromatography followed by electrophoresis.

In 1950 Haglund and Tiselius[47] described a preparative apparatus in which the vertical electrophoresis tube was filled with glass beads or powder material to counteract convection. This method was improved by Flodin and Kupke[48], who replaced the glass beads with ethanol–HCl-treated cellulose. Stabilization against convection was improved and electroosmosis and the adsorption of protein was strongly suppressed. Important methodological studies of zone electrophoresis in vertical, cylindrical columns packed with cellulose were made by Porath[49]. He also designed a large cooled column, which could effectively fractionate more than 100 ml of serum[50].

Svensson and Valmet[51] developed a method for electrophoresis in sucrose density gradients. The use of a density gradient instead of a powder or gel to stabilize against thermal convection and to carry protein zones has the advantage of eliminating disturbing adsorption phenomena. Later, Svensson and subsequently many others used sucrose density gradients for isoelectric focusing.

Early arrangements for continuous-flow electrophoresis for preparative purposes were presented in 1949[52,53]. Thus, in a vertically elongated cell filled with glass powder, a vertical flow of buffer and sample solutions was applied. An electric field was applied perpendicularly and horizontally, leading to fractionation of the sample constituents towards the bottom of the cell. Equipment for free-flow electrophoresis using flat vertical cells with the electric field perpendicular to the buffer flow but without glass powder were described many years later[54]. This has been improved and used for preparative (allowing processing of many litres per day) and analytical purposes for proteins and cells, *e.g.*, to study cancer[55]. Similar apparatus is nowadays commercially available and used for, *e.g.*, free-flow isoelectric focusing[56] and even in many manned space shuttle flights[57].

## ELECTROPHORESIS IN GELS

Gels of gelatin and agar have been used as supports for electrophoresis for about 100 years, but progress was very limited and up to 1955 only a few papers had been published (*e.g.*, ref. 21).

Smithies[58] pioneered the electrophoresis of proteins in starch gels, which produced much improved resolutions of, *e.g.*, proteins in serum. Later he also showed that on electrophoresis, molecular sieving in a gel could provide considerably higher resolution than was attainable in free solution[59]. Flodin prepared granulated dextran gels as the bed material in zone electrophoresis[60]. Coloured proteins and low-molecular-weight compounds were electrophoresed as model substances. Hjertén[60] dis-

covered that on elution after electrophoresis migrated down the columns faster than the low-molecular-weight compounds. Hjertén thus made the primary discovery of the molecular-sieving properties of dextran gels (later named Sephadex). The granulated dextran gels were not suitable as supporting media in electrophoresis because the separation obtained by electrophoresis was superimposed on gel filtration during elution. As it was difficult to obtain reproducible results with starch gels, Hjertén searched for well defined gels, the pore size of which could be chosen for particular separation purposes. He found that polyacrylamide gels fulfilled these requirements[60].

While these experiments were in progress, Raymond and Weintraub[61] and Davis and Ornstein[62] reported on the use of polyacrylamide gels for electrophoresis and therefore Hjertén did not publish his results. Hjertén[63] demonstrated the importance of testing different gel compositions. He also succeeded in adapting analytical gel electrophoresis to preparative purposes[64]. Substances migrating electrophoretically out of the gel column were transferred by a buffer flow to a fraction collector. This method often gave the same resolution as obtained with analytical gels. For detection of proteins, Hjertén also developed other methods based on, *e.g.*, localization of the proteins in polyacrylamide gels by UV scanning at two different wavelengths[65], or cutting out a gel segment containing the protein of interest and elution of the protein from gel pieces by electrophoresis into a sucrose–salt gradient[66]. The proteins were concentrated as a narrow band which was easy to isolate. A similar principle is widely used for recovery of DNA after gel electrophoresis.

Zone electrophoresis in agar gel was for many years a popular analytical method for serum proteins, especially when combined with immuno techniques[67]. The introduction of strips of cellulose acetate as a support was a definite improvement[68]. Unfortunately, agar contains many charged groups, which cause high electroosmosis. Therefore, Hjertén and co-workers introduced agarose, the neutral component of agar, for use in electrophoresis[69,70] and later also for chromatography. It is surprising that no-one had previously used agarose for such purposes. In 1937, Araki[71] reported that agar consists of two main components: agarose, with a low sulphur content, and the highly sulphated component agaropectin. In a review on agar his paper was barely mentioned, whereas many papers described agar as a polysaccharide with all chains containing sulphate groups[72,73]. This is not the first occasion in the history of science when a correct description of some fact has for many years been suppressed by misleading information. Much effort has been devoted to the development of methods for the preparation of good agaroses[72,73].

Hjertén[74] also developed an electrophoresis method using very low agarose gel concentrations ($<0.2\%$) for the fractionation of proteins and particles such as ribosomes and viruses. The agarose can be removed simply by centrifugation. Agarose has been widely used especially for routine biomedical separations of serum proteins for diagnostic purposes by Laurell and co-workers[75,76]. Very important also is the development of procedures using antibodies to obtain immuno precipitates in agarose gels by crossed immunoelectrophoresis, *e.g.*, for identification purposes[75] and "rocket" electrophoresis for the specific quantification of proteins[77]. An improved procedure for quantification has been described[78]. Axelsson[79] developed techniques for immunoelectrophoresis in agarose gel.

Adaptions of electrophoretic methods to the microscale have been presented[80–82].

In 1967, an important paper was published that described the relationship between the electrophoretic migration of proteins in homogeneous polyacrylamide gel (PAG) in the presence of SDS (sodium dodecyl sulphate) and the effective molecular radius or roughly the molecular weight[83]. SDS is generally useful because numerous such molecules are adsorbed on each protein molecule so as to give the SDS complex a pronounced negative net charge. In homogeneous gels and with excess of SDS, the limiting mobility of the complex has been found to be a linear, decreasing function of the logarithm of the molecular weight of proteins[84,85]. Electrophoresis in PAG with SDS is now widely used for, *e.g.*, the assessment of homogeneity and purity but notably for the determination of the molecular size and weight of proteins and polypeptides. For similar purposes gels made up of gradients of polyacrylamide[86], *i.e.*, to obtain a decreasing pore size towards the anode, are also used with or without SDS[84,86]. Here proteins migrate until they are trapped in the pores of the gel. In homogeneous PAG, if studies are made at different gel concentrations a relationship between the retardation coefficient of a protein and its molecular weight have been found, first for starch gels by Ferguson[87] and later for PAG[88].

For the solubilization of hydrophobic membrane proteins and in their purification, neutral detergents, *e.g.*, Triton X-100, have been used since many years. A drawback is that their solubilizing power is often insufficient to overcome the hydrophobic bonds between membrane proteins. However, SDS is much more effective than neutral detergents. Therefore, polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) commonly gives very good resolution of membrane proteins. The great disadvantage of SDS is that it has a strong denaturing effect. However, Hjertén and others have shown that hydrophobic membrane proteins are less susceptible to denaturation by SDS than water-soluble proteins, and they can often be reactivated after removal of SDS by electrophoresis[65] or by adding an excess of a neutral detergent[89]. High-resolution analytical SDS-PAGE is thus a powerful tool also for the analysis and isolation of membrane proteins. The use of different detergents has also been considered in detail[90].

Important electrophoretic separations of nucleic acids and their nucleotides (for a review, see ref. 91) have in recent years been extended to remarkable separations in agarose gels of DNA by pulsed field electrophoresis[92], and even separations of chromosomes have been achieved[93]. These methods are thus very important for studies of genes.

## ISOELECTRIC FOCUSING AND TWO-DIMENSIONAL GEL ELECTROPHORESIS

In 1941, Tiselius tried to separate serum albumin and haemoglobin by "isoelectric condensation" in a multi-compartment membrane apparatus with sodium sulphate as electrolyte. However, the centre of the apparatus became very hot, because as a result of electrolysis all salt ions were removed from regions around pH 7, resulting in a very low conductivity. Stationary electrolysis in similar types of apparatus had been used earlier by, *e.g.*, Williams and Waterman[94]. Amino acids and peptides could be separated into three groups, basic, neutral and acidic, but no separation within these groups was obtained. These shortcomings are nowadays well understood and will be described in the following text.

Kolin's first papers on isoelectric focusing appeared in 1954[95,96]. However,

Kolin's pH gradients were very short and unstable with time. This strengthened the desire of Svensson to develop a new isoelectric focusing method. In 1956 he wrote a paper on the concept of transport numbers of ampholytes[97]. Isoelectric ampholytes with zero net charge were found to have appreciable conductance if the p$K$ values of the dissociating groups were close. If many such ampholytes, isoelectric at various pH values, were available, then on electrolysis they would be able to dictate from the anode to the cathode a smooth pH course without any deep conductivity minima. This was the very basic concept of isoelectric focusing, and the desirable ampholytes were later called *carrier ampholytes*. A few years later, Svensson published the basic theory of isoelectric focusing[98] and in a later paper[99] experimentally verified that pure ampholytes had conductivities in agreement with theoretical predictions. The separation of haemoglobins was also described[100]. At the Karolinska Institute, Svensson formed a research group, which I had the opportunity to join. The search to find suitable carrier ampholytes had high priority. In spite of scrutinizing catalogues of commercially available chemicals, only a few suitable materials could be found. It was especially difficult to find carrier ampholytes with isoelectric points in the pH range 4–8. Nor did we receive any help from organic chemists; a typical reply was, "to get the many substances needed would constitute a huge task".

By partial hydrolysis of haemoglobin or whole blood we kept producing oligopeptides which were used as carrier ampholytes. This "bucket-scale" work, a laborious task, delayed us from extending isoelectric focusing to new fields, *e.g.*, focusing in gels. Despite treatment with carbon and other sorbents, we could not obtain colourless peptide preparations. However, isoelectric focusing showed that the colour could be focused in a few discrete zones. In 1963 Svensson left for a Professorship in Gothenburg and I obtained some laboratory space at the Karolinska Institute in Professor Theorell's laboratories at the Nobel Medical Institute. It was very annoying that the peptides that I used as carrier ampholytes gave zones of similar colour to myoglobins, which I studied. This promoted a search for uncoloured synthetic ampholytes with suitable buffer capacity and conductance. From my chemistry studies I had memorized the fact that owing to mutual influences within a molecule, identical protolytic groups in polyvalent acids and bases may have widely different p$K$ values. I made an extensive study of possible synthetic methods for amino acids. At first I was disappointed because the methods found were not suitable for one reason or another. Finally, in 1964, I tried to attach carboxylic acids to amines. By boiling under reflux a mixture of acrylic acid and polyvalent amines I obtained ampholytes with many protolytic groups having suitable p$K$ values and isoelectric points. The first syntheses were encouraging. I worked very hard and developed modified recipes giving improved properties of the carrier ampholytes, which finally fulfilled all the desired criteria. The advantages of these carrier ampholytes were soon obvious. With the aid of the new carrier ampholytes and focusing in density gradients of sucrose, two myoglobins could be separated although the difference in isoelectric point ($\Delta$p$I$) was only 0.05 pH unit. To resolve them, it was necessary to create a very shallow pH course. The resolving power, *i.e.*, the ability to separate proteins with close isoelectric points, was thus better than 0.05 pH unit. A theoretical resolving power of 0.02 was calculated[101]. We also designed and built apparatus for fractionation of the synthetic products and also columns for isoelectric focusing and separation of proteins in density gradients[102].

The resolving power for isoelectric focusing is very high and can be described by the equation

$$\Delta pI = 3\sqrt{\frac{D(\mathrm{dpH}/\mathrm{d}x)}{-E(\mathrm{d}u/\mathrm{dpH})}}$$

where $D$ is the diffusion coefficient, $E$ the electric field strength and $u$ the mobility of the protein[101].

Many proteins have subsequently been successfully studied by isoelectric focusing in density gradients and some of the results and procedures for making carrier ampholytes were presented in my thesis[102].

A few workers in LKB's research department became convinced of the outstanding capabilities of the new method and succeeded in the production and marketing of columns and carrier ampholytes, under the trade-name Ampholine. From that time, an increasing number of groups exhibited great enthusiasm for the use of the method.

After 1970, Svensson, who changed his name to Rilbe and his collaborators designed apparatus for rapid and convenient preparation and focusing in short density gradients[103]. Jonsson and Rilbe developed a method for isoelectric focusing, which permitted the convenient spectrophotometric evaluation of the separation[104]. This was continued by Fredriksson[105]. Procedures for the preparative purification of proteins have also been described[106]. Focusing in granulated gels such as Sephadex can also be mentioned[107]. A review of the carrier ampholytes has been published[108] and Rilbe wrote an interesting autobiography[109]. A description of some of the early developments of isoelectric focusing in polyacrylamide gels is presented below. Thousands of laboratories have benefited from the use of horizontally run flat-bed gels for various types of electrophoresis, and especially isoelectric focusing, using equipment and apparatus partially developed by myself[110] and others such as Multiphore (since 1973) and Ultrophore, which have been marketed by LKB and recently by Pharmacia-LKB (see Fig. 2).

Up to 1979, analytical isoelectric focusing in gels was usually performed in polyacrylamide gels. However, large proteins are difficult to study as these gels have molecular-sieving properties. Agarose, with large pores, has advantages but requires highly purified special agaroses with very low electroosmosis[111]. The development of equipment and methods for isoelectric focusing in polyacrylamide gels and agarose and sensitive staining of proteins are important[112,113]. Numerous reports have given the general impression that most researchers would obtain increased and valuable information by using not only wide pH ranges (>4 pH units) but also narrow ones (<2 pH units) or even ultra-narrow ones (<1 pH unit).

For the quantification of proteins, zone immunoelectrophoresis assay (ZIA) has been developed[79]. This can be used successfully not only for the quantification of proteins in solution but also after separation of proteins in gels by electrophoretic methods[114]. By use of special ZIA procedures, protein concentrations down to about 10 ng/ml can be measured[115].

A technique for isoelectric focusing in polyacrylamide gels with buffer substances fixed (immobilized) to the gel matrix has been developed by Bjellqvist *et al.*[116].

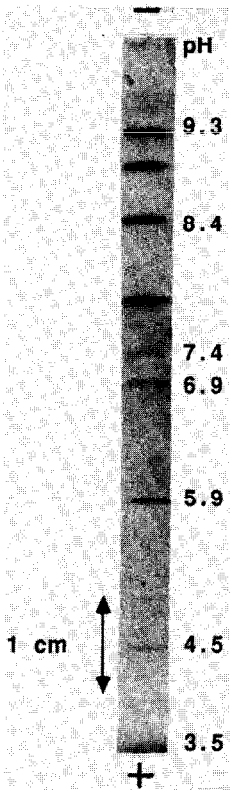


Fig. 2. Detection of proteins by Coomassie Brilliant Blue staining[110] after separation and isoelectric focusing in a flat-bed gel of polyacrylamide. A ready-made gel with Ampholine (PAG plate), isoelectric point (p*I*) marker proteins and chemicals were obtained from Pharmacia-LKB. Of the gel plate only one lane is shown, with p*I* values to the right.

Such pH gradients ensured stability of the pH course and allowed the focusing of proteins even in ultra-narrow pH ranges. A resolving power of 0.001 pH unit has been reported. Righetti and co-workers have invested much effort in developing procedures for isoelectric focusing in such gels (for a review, see ref. 117). During the early years there were many problems with the use of these gels. One remedy has been admixture of classical carrier ampholytes, a procedure called hybrid isoelectric focusing[117,118].

Microtechniques and sensitive staining protocols are important and widely used for, *e.g.*, studies of plant, microbial, animal and human proteins and in forensic medicine (*e.g.*, ref. 119).

*Two-dimensional gel electrophoresis and blotting of proteins*

The earliest application of electrophoresis in two dimensions in gels was published by Smithies and Poulik in 1956[120]. However, the starch gels were far from optimal. Margolis and Kendrick used polyacrylamide gel (PAG) preferably with a gradient[86]. Improvements were obtained by using isoelectric focusing of protein in

a gel rod of PAG for separation according to charge in the first dimension followed by electrophoresis perpendicularly in the second dimension in a PAG slab as described by Dale and Latner[121] and Macko and Stegemann[122]. In 1970, Stegemann[123] introduced IEF in PAG followed by electrophoresis of proteins in a gel slab containing SDS to increase the negative net charge of proteins and to utilize the relationship between their size and mobility.

High-resolution separation of proteins by two-dimensional gel electrophoresis was obtained after pretreatment of the samples in hot SDS–urea solutions and IEF followed by SDS-PAGE as described in 1975 by O'Farrell[124], Klose[125] and Scheele[126]. Proteins occurring also in very low concentrations could be made visible elegantly by autoradiography in the second-dimension gel[124,126]. A few years later, Anderson and Anderson[127] described the high-resolution separation of human serum proteins and the semi-automated ISO-DALT system allowing the parallel use of twenty two-dimensional electrophoresis gels[128]. Here again the introduction of sensitive procedures for revealing proteins in PAG have been very important for progress. Especially here could be mentioned silver staining, developed by Switzer *et al.*[129], Sammons *et al.*[130] and others[131]. It soon turned out that most protein samples of biological origin (plant, microbial, animal or man) were composed of large numbers, often over 1000, of proteins or polypeptides, which could be separated by two-dimensional gel electrophoresis and made visible as spots in each gel slab.

The transfer of proteins by electrophoresis from a gel slab on to a sheet of nitrocellulose or some other material is very helpful for the identification of protein spots or bands in gel slabs after one- and two-dimensional electrophoretic separations[132]. The procedure called blotting (for a review, see ref. 133) is very important. It also allows the transfer of proteins to membranes for subsequent automatic determination of their amino acid sequence[134,135]. Antibodies specific for the recognition of certain proteins are very useful for the identification of proteins on blotting membranes.

Trials to quantify the abundance of polypeptides in each of the numerous spots in gel slabs after two-dimensional gel electrophoresis required the development of special scanners. These are still under development. The required handling of all measured position and intensity data prompted the development of large computerized data-handling systems. Recently databases have appeared for the storage and retrieval of a large body of information on the studied proteins (for a review, see ref. 136).

Two-dimensional gel electrophoresis techniques are now of tremendous importance in various fields of the biological sciences, such as genetics, molecular biology, research on various diseases including AIDS (HIV) and gene technology. Without the power of these and allied electrophoretic techniques such as blotting, one of the largest projects ever in the biological sciences would not have been started, namely "mapping the human genome"[137,138]. Here can be mentioned again the importance of electrophoresis in gels of chromosomes, nucleic acids and their nucleotides, *e.g.*, for determination of their sequence, which ultimately determines the sequence of amino acids in each protein. This is the very basis for the great biological variability among all living forms, including microorganisms, plants, animals and humans. Two-dimensional electrophoresis and other electrophoretic techniques such as IEF are also important for studies in man and experimental animals of the effects of chemicals[139],

alcohol[140], radiation and mutations[139,141]. Further, mankind can acquire greater insight into details of cell proliferation, ageing and various diseases, including cancer (*e.g.*, refs. 137, 138 and 142).

From 1972, international symposia on isoelectric focusing and electrophoresis have been arranged annually or biennially by the International Electrophoresis Society. Transactions of the symposia and many monographs on isoelectric focusing have been published (*e.g.*, refs. 143–145). There are now many national societies for electrophoresis. It has given great satisfaction to witness the rapid growth in methodology and the great services to biochemistry and medicine that the isoelectric focusing method has offered.

## CONCLUSION

Electrophoretic methods have become some of the most widely used high-resolution techniques for analytical and preparative separations. Literature studies reveal that electrophoretic methods are used almost ubiquitously in research and applied biomedical studies. The methods have become increasingly diversified, and are now indispensable in many different areas of science and also for various even routine applications, especially for the separation of different proteins and nucleic acids. These two classes of substances represent a challenge not only because of their fundamental and important biological functions but also because of their large numbers and complexity. Separations of proteins in gels by electrophoresis and especially isoelectric focusing are performed in thousands of laboratories for, *e.g.*, identification of species (*e.g.*, ref. 146), genetic variants (*e.g.*, ref. 147) and individuals, *e.g.*, in forensic medicine (*e.g.*, ref. 148). Studies of differences in protein structure will become increasingly important[149].

A summary of some of the milestones in the development of electrophoretic methods have been reviewed here. The overall development and progress have been accomplished by numerous researchers and laboratory workers, many of whom remain unheralded. Owing to limitations of space it has been possible to mention only a fraction of the contributors. Those seeking more details are referred to special articles, *e.g.*, in the journal *Electrophoresis*, proceedings of international meetings (*e.g.*, refs. 143 and 144) and other reviews[60,109,145].

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