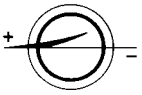
Electrophoresis '82
Advanced Methods
Biochemical and Clinical Applications



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Advanced Methods
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Proceedings of the International
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Athens, Greece, April 21–24, 1982

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PREFACE

The International Conference "Electrophoresis '82" was held at the School of Natural Sciences in Athens on April 21-24, 1982, under the auspices of the Ministry of Culture and Sciences and the Greek Atomic Energy Commission. It was the fourth of a projected series of international meetings organized with the objective of stimulating communication of ideas, information exchange and the advancement of knowledge in all areas of electrophoresis.

This Conference, held in conjunction with the Annual Meeting of the Electrophoresis Society, was attended by about 340 participants from 26 countries, representing a broad spectrum of scientific disciplines and research fields and included most of the recognized leaders in the field.

In an experimental approach, toward a present-day *agora*, we aimed at maximal level of communication in theory and ideas, laboratory expertise, new results, layman's applications, as well as speculation in future trends. Accordingly, leading companies in the field were asked to hold up-to-date "laboratory workshops" during the Conference. There, in the actual milieu of the laboratory, the participants could work *in situ* on the latest methodology. This integration of lectures, poster sessions, laboratories and round-tables within the same building on the slope of Mount Hymettus created a lively community and led to a very successful meeting.

This volume contains a large portion of the papers presented at "Electrophoresis '82". They are compiled in the following sections: I. Plenary Lecture. II. Theory and Methods. III. Two-Dimensional Separations. IV. Biological and Biomedical Applications. V. Round-Table Discussions. As it was already felt during the sessions and became fully evident now, very few manuscripts belong solely where they are listed; many ex-

pand thematically beyond one or even two sections. This high sophistication in a field of composite methodology, which characterized the whole meeting, was most vividly reflected in the final round-table discussions. These are also included here, thanks to the indefatigable efforts of the Chairmen who succeeded, each in his own manner, in conveying on paper the exciting uniqueness of each session. The editor contributed only his persistence.

This volume could not have been produced without the cooperation of all contributors in the preparation of their manuscripts and I would like to express my thanks to them. I also greatly appreciate the efforts of the staff of Walter de Gruyter towards the publication of this volume.

Athens Dimitri Stathakos

ACKNOWLEDGEMENTS

I am indebted to the members of the Organizing Committee of "Electrophoresis '82", Drs. C. Coutsogeorgopoulos, E. Frangoulis and C. Sekeris for their wholehearted and valuable collaboration. I would also like to thank the staff of the Biochemistry Department, School of Natural Sciences, and in particular Mr. L. Pastelakos of the Nuclear Research Center "Demokritos", for their splendid help in running the whole meeting. Furthermore, I wish to thank FMC-Marine Colloids, LKB Produkter, Pharmacia Fine Chemicals and Serva Feinbiochemica for their contribution through their excellent "laboratory workshops", and to assure them that, to my knowledge, all participants and especially the students share this statement.

Financial aid for the Conference was provided by the Greek Ministry of Culture and Sciences, the National Tourist Organization of Greece, Olympic Airways, AC&R Advertising and the Electrophoresis Society.

CONTENTS

SECTION V: ROUND-TABLE DISCUSSIONS

SECTION I PLENARY LECTURE

EVOLUTION OF IDEAS IN ELECTROPHORETIC DEVELOPMENTS - Selected Highlights

Alexander Kolin
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To begin with, I wish to thank Dr. Stathakos and the Organizing Committee of the International Electrophoresis Congress for their kind invitation to deliver this lecture in Athens, the cradle of western science and civilization. It is particularly gratifying to do so under the auspices of the research center that bears the name of the father of the atomic concept.

The content of this lecture is limited and determined to a great extent by shortage of time. It is impossible to do justice to developments of the past two centuries in about an hour. Consequently, instruments and techniques will not be described and we shall limit ourselves to methodological highlights. Their choice is obviously personal and to some extent arbitrary. Inclusion or exclusion of a method or an investigator's name is not a reflection on their deemed importance but, rather, on the limitation of time. Thus, for instance, continuous flow methods will not be discussed, although I have devoted much time and effort to methods of this type. Finally, the amount of time devoted to a subject will not be proportional to its importance. Significant and generally well known topics will get only brief treatment. Unfamiliar ones will be discussed more fully.

Antiquity

Appropriately for this location, we shall begin in ancient

Electrophoresis '82

Greece. Thales of Miletus (about 600 B.C.), who is generally considered to be the father of Greek science, described a curious transformation in amber ("electron" in Greek) when it was rubbed. It exhibited the ability to attract small light objects, like a piece of straw, and then to repel them after contact. Thales had the means at his disposal to discover electrophoresis in air. He could have observed dust particles in dark-field illumination by viewing sideways a beam of light penetrating into a dark temple through a crack in the wall. Approaching an electrified piece of amber to the light beam, he could have observed attraction of oppositely charged dust particles toward it and repulsion of particles of like charge from it. But there is no record of such an observation in antiquity.

The Discoveries of Reuss

Despite the potential for discovery of electrophoresis in air more than half a millennium B.C., it was not until 1809 that electrophoresis in liquids was discovered. Why did this discovery have to wait for two and a half thousand years? --The answer is very simple. There is essentially no energy required to maintain an electrical field in air by means of an object carrying an electrostatic charge. In a conductive liquid, however, an electrical current must flow in accordance with Ohm's law in a region where an electric field is maintained. A source of energy is needed to maintain such a current. Such energy sources did not exist prior to the discoveries of Galvani and Volta. The Voltaic battery (1800) provided the first electrochemical energy source for sustaining electrical currents in conductive solutions. It is therefore no mere coincidence that electrophoresis was discovered by Reuss in 1809, nine years after Volta's historic publication (1). To appreciate the state of knowledge at the time of

Reuss, one should keep in mind that the year 1800 was twenty-eight years ahead of the discovery of Ohm's law and thirty-two years before Faraday's discovery of his laws of electrolysis. Oersted's discovery of electromagnetism was nineteen years in the future so that electromagnetic current meters were not available. The intensity of electric currents was semi-quantitatively estimated by observing the rate of evolution of bubbles in an acid solution.

In 1800, the nature of the electric current was still mysterious. One spoke of an "electrical effluvium." As soon as voltaic batteries made maintenance of electric currents possible, they became a subject of intense investigation and electrical conduction through all kinds of materials was subjected to scrutiny. Professor F. F. Reuss of the University of Moscow was studying electrical conductivity of moist soil in a garden on the shores of the Moskva river. His electric battery was a voltaic pile. It was a multiple sandwich of ninety-two silver rubel coins separated from ninety-two Zink discs by layers of acid-soaked cloth. He estimated the current intensity by the rate of evolution of bubbles in an acid solution traversed by the current. He was surprised to note that the current was not greatly affected by the distance between his electrodes in the soil. He decided to repeat his experiments under better controlled conditions in the laboratory.

The simple apparatus of Reuss was a V-tube shown in Fig. 1. Instead of soil it contained quartz sand Q at the bottom. The effect of the electric current that he observed was a rise of liquid in the negative electrode leg and a drop in liquid level in the positive electrode leg. There was no effect in the absence of the sand. In this first experiment Reuss discovered electroosmosis and reported it on April 15, 1908 in a lecture entitled: "On a New Effect of Galvanic Electricity."

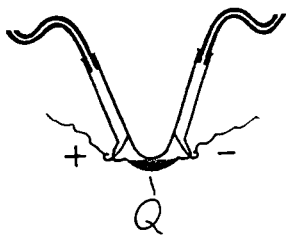
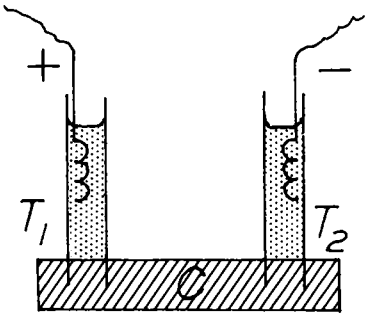


Fig. 1 *Reuss' apparatus in which he observed electroosmosis. Q: quartz sand. (From H. A. Abramson, Electrokinetic Phenomena. New York: Chemical Catalogue Company, 1934.)*

In a second experiment, he changed the texture of his soil. He replaced the coarse sand with very small clay particles. Fig. 2 illustrates the scheme of this experiment. He inserted two vertical glass tubes into a slab of wet clay and filled the tube with a conductive aqueous solution. As the current passed between the electrodes, clay particles migrated upward in the positive leg, but not in the negative leg. Reuss, thus discovered electrophoresis in his second experiment.

Fig. 2 *Set-up in which Reuss observed electrophoresis. C: moist clay slab. T_1, T_2 glass tubes.*



Prelude to Contemporary Developments

The early pioneers in the study of electrophoresis and electroosmosis were not analytical chemists but, rather, pure physicists. It is surprising that the unspectacular effects described by Reuss should have attracted the attention of some of the foremost physicists. Most notable among them was Helmholtz, the codiscoverer of the law of conservation of energy. He was followed by other first-rate physicists like Quincke, Smoluchowski, Lodge and later by Nobel Laureates like Stern, Perrin, Debye and Onsager. The early physicists may have been attracted by the insight that electrification of matter by contact is related to the structure of matter. The contact between a liquid and an immersed solid is much more reproducible and well defined than the contact between two solids used to separate electric charges by friction. Indeed, the work of Helmholtz and Smoluchowski led to a neat mathematical formulation of a common theory for electrophoresis and electroosmosis on the basis of the notion of an interfacial electric double layer (2, 3). This notion gradually evolved into a more sophisticated theory of the diffuse ionic atmosphere in the subsequent treatments of Guye, Chapman and Stern (4, 5, 6). And the Debye and Hückel theory of counter-ion atmospheres and ionic migration can be considered as an extension of the theories originally designed for plane surfaces to a spherical configuration (7).

The physicists of the past century were interested in studies of electrolytic conduction (let us call it "ionics") for similar reasons as contemporary solid state physicists study electronics. Such studies resulted in revolutionary changes in our notions. Thus, for instance, Helmholtz was able to infer the quantum nature of the electric charge from Faraday's laws of electrolysis (8). Arrhenius' theory of ionic dissociation was so controversial (revolutionary) that he barely

squeezed through with the lowest passing grade in his doctoral examination with a thesis for which he later received a Nobel Prize. His theory assumed that supposedly immutable atoms radically change their chemical properties by acquisition of an electric charge in becoming ions.

The development of theories went hand in hand with experimental studies, notably of the electrical conductivities of ionic solutions. Such measurements revealed lower conductivities for strong electrolytes than would be expected from complete dissociation. This discrepancy was resolved by Debye's and Hückel's theory on the basis of the interaction between a migrating ion and the cloud of counter-ions. Most notable among the pioneers in the study of electrolyte conductance was Friedrich Kohlrausch. The laws which he discovered for concentration shifts and boundary migration in electrolyte columns with concentration gradients and abrupt changes in electrolyte composition have provided a foundation for some of the most important recent developments in electrophoretic methodology (9).

Sharp boundary discontinuities in electrolyte columns were of importance and interest in connection with new methods for studies of ion mobilities which were being developed by Oliver Lodge (10). In 1886 Lodge was interested in direct observation and measurement of migration rates of ions in solution. He proposed the moving boundary method which was perfected for analytical use by Tiselius nearly half a century later. Lodge also suggested zone electrophoresis in gels. The ideas of Lodge present an interesting example of a phenomenon that we shall encounter on more than one occasion in the field of electrophoresis. We may call it "hybernation" of ideas which often lie dormant in the literature until they are retrieved or reinvented when a practical need for them arises. Another important instance of "hybernation" is the invention of paper chromatography by Tswett in 1903 (11) which was resurected by

Consden, Gordon and Martin in 1944. One cannot help wondering how many more important ideas may be lying buried in the literature.

Tiselius Initiates Analytical Use of Electrophoresis

Analytical chemists showed no interest in electrophoresis for 120 years. The ground for analytical uses was prepared by physicists (notably Lodge (10)) who showed how electrophoretic mobilities could be measured by the moving boundary method. In 1930 (12) this method was transformed into a powerful analytical tool by Tiselius. At that time fractionation of serum proteins by ultracentrifugation revealed only two components, the albumin and the globulin fractions. By means of electrophoresis, Tiselius was able to resolve the globulin fraction into several components (Fig. 3). We shall not discuss his

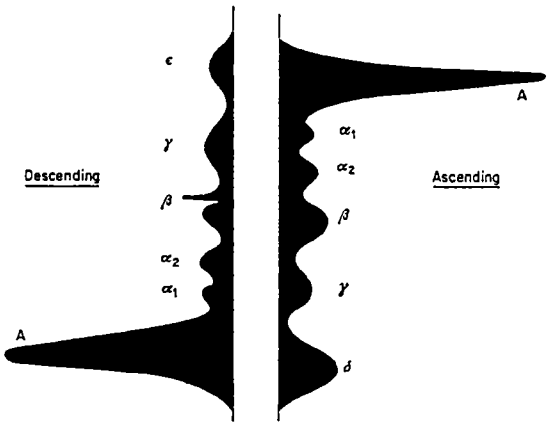


Fig. 3 *Descending and ascending electrophoretic separation patterns obtained for human serum proteins with a Tiselius apparatus. A: albumin; α_1, α_2, β and γ: globulins; δ and ϵ: salt boundaries. (From D. J. Shaw: Electrophoresis. New York: Academic Press, 1969.)*

well-known apparatus and will limit ourselves to the enumerations of the main innovations which Tiselius introduced in it: I) A method for generation of sharp boundaries. II) Suppression of thermal convection by carrying out electrophoresis at the density maximum of the solution near $4^{\circ}C$. III) An optical method for visualization of boundaries between colorless components.

Tiselius' apparatus was complex and costly. It represented a great leap forward in biochemical analysis. It had, however, serious limitations. Its usefulness was great in providing information about the number of fractions in protein mixtures and in characterizing the components by their electrophoretic mobilities. Its preparative capabilities were, however, very limited. Only a fraction of the fastest component and a portion of the slowest one could be isolated in pure form. The growing need for preparative isolation of all components of a mixture, as well as a desire for simplification of the apparatus, provided the stimulus for the development of "new" zonal methods. Actually these methods represent a revival of old ideas of Lodge (1886) (10) and of Tswett (1903) (11).

Evolution of Zonal Methods

The evolution of zonal methods from the Tiselius U-tube scheme is an almost self-evident logical step. If we imagine the U-tube straightened out, then the protein mixture in the lower portion of the U-tube will become a straight zone which we can make as narrow as we please. In the course of electrophoresis the protein components of this zone will get far enough apart to exhibit complete separation without overlap. The narrower the original zone, the less time will be required for a complete separation of the ingredients. We shall classify the zonal separation procedures in three types of methods. I) Non-

focusing methods, II) focusing methods and III) stacking methods. The following survey of zonal separation methods is intended to be illustrative rather than exhaustive.

Zonal Non-focusing Methods

DENSITY GRADIENT ELECTROPHORESIS

The classical moving boundary electrophoresis (12) was performed in liquid columns. Zone electrophoresis can also be performed in liquid columns stabilized by density gradients (13, 14, 15). A sucrose concentration gradient in a vertical liquid column will be accompanied by a concomitant density gradient (and viscosity gradient). A downward increase in density will stabilize the column against thermal convection and will permit to establish concomitant gradients of other parameters, such as pH and electrical conductivity (13, 14). Withdrawal of liquid from a level in the density gradient generates a density jump ("density shelf"). Reinjection of a sample whose density lies within the range of the density jump produces a stabilized narrow sample zone. The electrophoretic column becomes a racetrack and electrophoresis separates the original sample into migrating zones of different electrophoretic mobilities.

HJERTÉN'S ROTATING TUBE METHOD

Density gradient electrophoresis has certain drawbacks. Because of the electrophoretic mobility-and electrical conductivity gradients that are concomitant with the density gradient, it is not suitable for analytical characterization of the electrophoretic components by measurement of absolute values of their electrophoretic mobilities. Such measurements would require an homogeneous electrophoretic column. An ingenious solution to this problem was provided by Hjertén in which thermal stability

is obtained without recourse to a density gradient (16). Gravitationally induced movements, like sedimentation of denser particles or thermal convection, are inhibited by rotation of a fluid about a horizontal axis (17). In Hjertén's "free zone electrophoresis," a horizontal buffer-filled tube rotates slowly (about one revolution per second) about a horizontal axis. The solution in the tube is homogeneous (i.e., devoid of concentration gradients). Fig. 4 shows Hjérten's apparatus in which four dye stuffs have been separated into four distinct zones.

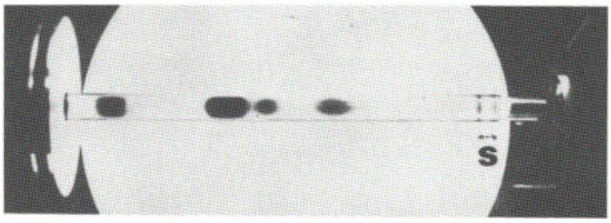


Fig. 4 *Hjertén's rotating tube "free zone electrophoresis" apparatus separation pattern obtained in 2 hours from a sample introduced at S containing: naphtholgreen, phenol red, bromo thymol blue and methyl orange. (From: S. Hjertén in Protids of the Biological Fluids. 7, 28, 1959.)*

The mixture is introduced at the marked location (S) by means of a fine plastic tube and the separated fractions can be withdrawn in similar fashion. This method is capable of separating molecules as well as cells and subcellular particles and it provides an elegant means for absolute determination of electrophoretic mobilities.

STABILIZATION BY ELECTROMAGNETIC ROTATION

Hjerten's rotating tube apparatus involves exacting mechanical precision work. This factor, as well as its effectiveness, stimulated attempts to achieve fluid rotation by non-mechanical means. These attempts failed in a horizontal tube but succeeded in the annular space (about 1.5 mm in width) between two horizontal plastic cylinders of different diameters (18). The buffer solution in the annulus is traversed by the axial electrophoretic current. By penetration of the annulus with a radial magnetic field, electromagnetic forces are engendered at right angles to both the electric and the magnetic field; i.e., these forces are tangential and the fluid is set into a circulating (rotational) motion about the horizontal cylinder axis. When a liquid sample containing electrically neutral particles is injected as a pulse centrally between the plastic cylinders into the annular interspace, the particles are aligned in a thin short streak and go into a circular orbit. If the particles are electrically charged, they exhibit simultaneously an axial electrophoretic migration, so that their actual path becomes helical. With each revolution two particles of different electrophoretic mobilities will gain an increment S in separation, so that their separation after n revolutions will be n·s. Clearly visible separations can be achieved in less than five seconds. The separated zones can be intercepted and retrieved by a collector at the end of the migration path. Fig. 5 illustrates the scheme of the electromagnetic endless belt electrophoresis apparatus as applied to zonal separation. (The main purpose of this principle is continuous-flow separation which will not be discussed (19)). It should be mentioned in this context that zonal separations can also be achieved by pulsed injection into a fluid curtain electrophoresis apparatus (20).

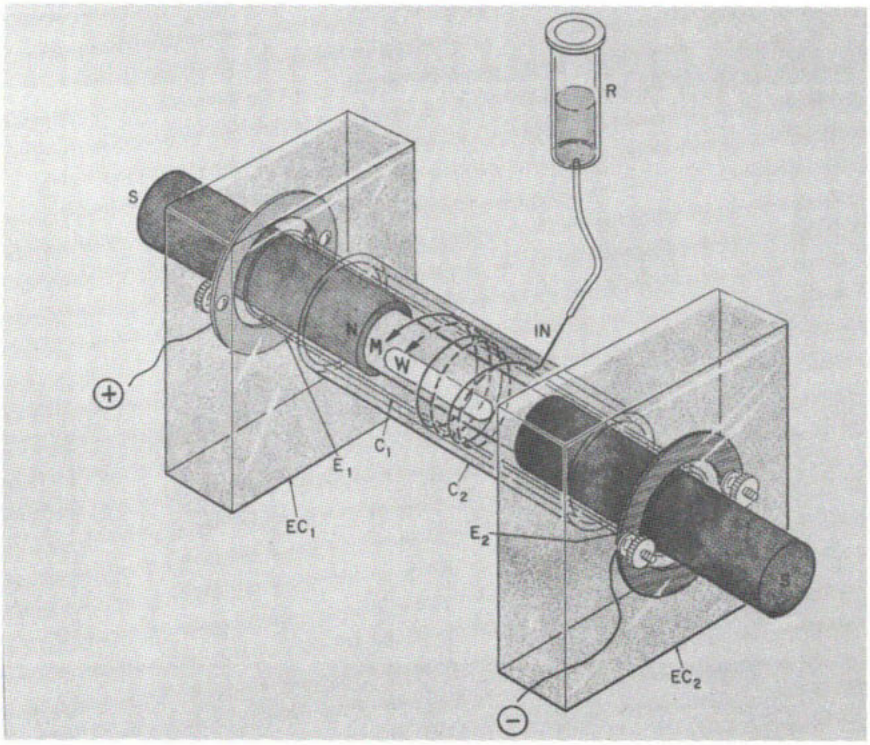


Fig. 5 *Scheme of rotational stabilization in a fluid endless belt. N-S : Bar magnets; m : soft-iron cylinder; E_1, E_2 : electrodes; EC_1, EC_2 : electrode compartments; C_1 : inner lucite cylinder; C_2 : outer lucite cylinder; IN : injector; R : reservoir for mixture to be analyzed.*

ANTICONVECTION STABILIZATION BY FLUID ENTRAPMENT IN THE MESHWORK OF A MATRIX - Filter Paper and Gel Electrophoresis

These methods, which came into general use only relatively recently, have their roots in the distant past. Deposition of mixtures of pigments to be separated in form of a narrow zone

on filter paper was initiated by Tswett in 1903 in connection with the method of chromatography which he invented (11). This idea was transferred to electrophoresis in 1937 by König in Brazil who used it for fractionation of snake venoms (21). König's use of filter paper electrophoresis actually preceded by several years the revival of filter paper chromatography by Consden, Gordon and Martin (22). The successes obtained in these uses of sample zones on filter paper stimulated an avalanche of applications of filter paper zone electrophoresis beginning about 1950.

The advantages of simplicity of technique and completeness of zonal separations on filter paper were gained at the expense of emergence of new artifacts. Pronounced electroosmosis and streaming caused by evaporation of water, as well as non-uniformity of buffer concentration and temperature along the paper strip made filter paper electrophoresis unsuitable for measurement of electrophoretic mobilities for characterization of components. Adsorptive interaction between filter paper and some of the components caused not only retardation and possibly immobilization of some of the zones but also "tailing" which broadens the zones and degrades the separation pattern. This led to replacement of paper by other materials, such as cellulose acetate in which some of these problems are less severe. The use of a thin supporting matrix introduced, however, one advantage: The possibility of cooling the paper strip. This permitted the use of high potential gradients with resulting gain in speed of the separation process ("high voltage electrophoresis") (23).

The search for materials in which the above-mentioned artifacts were less pronounced than in paper and in thin plastic films led to the discovery of many different types of gel with suitable properties, most notably polyacrylamide gel. But this again was more a revival than an innovation. Oliver Lodge

(10) originated this approach to electrophoresis in 1886 but analytical chemistry did not adopt it until recent times. The transition from a paper- or thin-film matrix to a gel had the important advantage of adding a significant third dimension which permitted an increase in the sample quantity for preparative separations. But the gel layer can also be made very thin so as to permit cooling and application of high potential gradients to achieve rapid separations. The filter paper- and gel methods have one factor in common. Both inhibit thermal convection in the liquid buffer by viscous shearing stress in the interstices of the fiber-or molecular network of the matrix in which the liquid is entrapped. There is, however, one property of gels which can be utilized concurrently with electrophoresis which makes them superior to other matrices; it is the possibility of superimposing a molecular sieving effect upon electrophoresis, thus adding the capability of resolution on the basis of differences in molecular dimensions. This type of interaction will be considered later in connection with other methodological aspects.

Zonal Focusing Methods

ISOELECTRIC FOCUSING

It would be logical to surmise that electrophoretic focusing methods resulted from the quest of analytical biochemists for an increase in resolving power of zone electrophoresis. But this is not the case. The concept was originated by a physicist and was described in 1954 as a physical effect in the Journal of Chemical Physics (13) with indications of the analytical potentialities of an electrophoretic method based on this phenomenon. In fact, analytical biochemists came very close to this concept in designing "electrodialysis apparatus" with compartments separated by membranes and containing buffers of different pH (24, 25). But, in spite of the time-

lapse between 1929 (resp. 1941) and 1954 they did not make the essential transition from a pH step-function to a continuous pH gradient. This conceptual transition was apparently not self-evident since even the prolific innovator Tiselius did not make it. The conceptual step required was analogous to the transition from algebra to infinitesimal calculus.

The consideration that led to the conception of isoelectric focusing was an imaginary experiment ("Gedanken experiment") in which the titration of a solution containing one single protein molecule was visualized. A positive protein ion in an originally acidic solution was seen to acquire a negative charge after passing through the neutral state at the isoelectric point as the pH was gradually changing from acidic to basic. In a "thought-experiment" it was possible to accomplish this titration (i.e., change in the pH of the environment of the molecule) without modifying the buffer solution. The buffer column was imagined to have a continuously monotonically changing pH. The pH of the surroundings of the protein molecule could be changed by mechanically pulling it through the pH gradient in the buffer column (13).

Two additional steps in this train of thought led to the concept of "isoelectric condensation" or "isoelectric focusing." First, the entire column was populated with protein molecules of the same species. It was at once clear that there was variation in the charge of the protein ions in the pH gradient. The ampholyte had to have opposite charges on either side of the isoelectric zone. In the second step the mechanical propulsion of the ions was replaced by electrical transport, i.e., by electrophoresis. Here, an unusual situation presented itself. Ions of the same substance were moving in opposite directions in the same electric field, depending on their location in the electrophoretic column.

There had to be three related effects: I) "Isoelectric condensation" in a current flowing in the direction from low pH to high pH. In this case, the ampholyte ions had to be swept toward the isoelectric zone where they would loose their charge and accumulate. II) "Isoelectric evacuation" in an oppositely directed current. Under these conditions, the oppositely charged ions located on either side of the isoelectric zone would move away from this zone, creating a "protein ion vacuum" on each side of it. Protein molecules removed from the isoelectric zone by diffusion or stirring would acquire an electric charge and would be swept away by the electric field. III) The isoelectric condensation zones had to exhibit electrochemical stability. Molecules removed mechanically or thermally from such a zone in a current directed toward increasing pH values would acquire a charge in the pH gradient of such a sign as to be returned to the isoelectric zone.

The isoelectric focusing effect was presented as a basis for a new analytical approach in which the parameter of electrophoretic mobility would be replaced by the isoelectric point as a separation and characterization parameter. The predicted effects were demonstrated in a simple U-tube cell shown in Fig. 6 (13). In order to produce the anticipated effects, some additional new ideas had to be introduced: 1) stabilization of the electrophoretic column against hydrostatic imbalance and thermal convection by density gradients, 2) coupling pH and/or other physical parameters with a concomitant density gradient, and 3) making the electrical conductivity in the sample volume much lower than in the adjacent buffer solution so that most of the voltage applied to the cell electrodes appeared as a voltage drop across the sample zone producing a high electric field intensity and rapid electrophoretic migration.

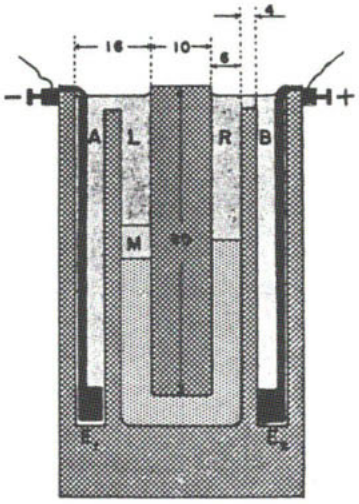


Fig. 6 *Cell which was used to demonstrate isoelectric focusing and velocity gradient focusing. E_1, E_2: electrodes; A, B: electrode compartments; L, R: left and right legs of U-tube; M: middle layer containing sample. (From Ref. 13.)*

M is the sample volume ("M-layer") in Fig. 6. Its density is intermediate between the densities of the acidic buffer below and the basic buffer above it. Channels A and B harbor the electrodes E_1, E_2. A pH gradient is established in the sample volume M by diffusion and/or by stirring its boundaries. Fig. 7 shows simultaneous demonstration of isoelectric "focusing" and "evacuation" effects in the cell of Fig. 6. In this case (Fig. A) two M layers containing a hemoglobin solution are symmetrically placed in the left and right legs L, R of the U-tube section. The current is flowing, as indicated, by the polarity of the electrodes in Fig. 6, from high to low pH in the R leg and in the direction of increasing pH in the L leg. As seen in Fig. 7B, there is a clear-cut condensation ("focusing") of hemoglobin in the left leg and an evacuation zone in the right leg after passage of a current of 9 mA for 40 seconds. Fig. 8 (26) shows an "isoelectric spectrum" (i.e., sorting pattern) produced within four minutes in the same apparatus in an M-layer containing four proteins. The three lower zones have been obtained by isoelectric focusing,

the top zone by non-isoelectric focusing to be discussed below.

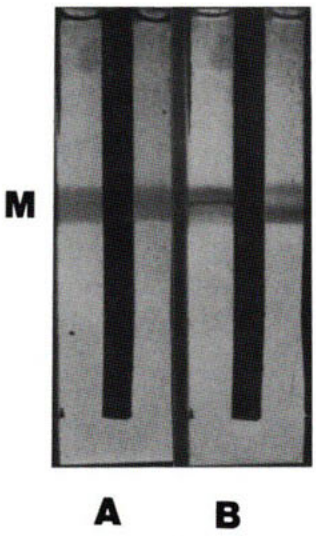


Fig. 7 *Demonstration of isoelectric focusing and evacuation of hemoglobin in the cell shown in Fig. 6 in a pH gradient (pH 2.6-9.6) within 40 sec. A: U-tube with hemoglobin belts M in both legs before passage of current. (The polarity is indicated in Fig. 6.) B: Focusing of hemoglobin by a current flowing in the direction of increasing pH in left leg of U-tube and evacuation of hemoglobin in right leg by a current flowing in the direction of decreasing pH. (From Ref. 13.)*

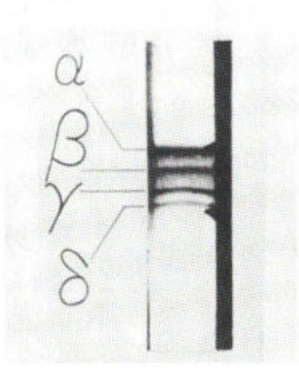


Fig. 8 *Isoelectric focusing of a mixture of proteins obtained in 4 min. The top componenet (α), cytochrome c, is focused non-isoelectrically in a conductivity gradient. The lower ones, hemoglobin (β), catalase (γ), and Azocoll (δ), are focused isoelectrically in pH gradients ranging from pH 4.8 to 7.7. (From Ref. 26.)*

This method gave impetus to several developments in electrophoresis: 1) density gradient electrophoresis, 2) electrophoresis in discontinuous multiphasic buffer systems (e.g., disc electrophoresis), 3) non-isolectric focusing, and 4) iso-

electric focusing. As an approach to isoelectric focusing (27), it represented just a first step. It was an ephemeral link in an evolutionary chain. The prepared pH gradients on which it relied proved short-lived under passage of current. The survival of the method as a practical analytical tool required stable pH gradients that would show no objectionable drift in analytical runs extending over many hours. The achievement of pH gradients of adequate stability and durability was a decisive contribution of Svensson (Rilbe) and Vesterberg (28, 29, 30, 31).

In 1961 Svensson (28) introduced his so-called "natural pH gradients." He extended Tiselius' analysis of steady-state electrolysis (25) and showed, at first theoretically, that a stable stationary pH gradient could be established and maintained by prolonged (e.g., a day or longer) passage of a current through an electrolyte column containing a mixture of low molecular weight ampholytes ("carrier ampholytes") of closely spaced isoelectric points. Each carrier ampholyte migrates until it reaches a pH corresponding to its pI and becomes stationary. Eventually the zones of isoelectric carrier ampholytes form a continuous chain as the electric field ties them to their isoelectric zones and diffusion causes them to broaden and penetrate the neighboring ampholyte zones. Proteins to be subjected to isoelectric analysis present in small concentration ("trace ampholytes") will migrate in this stable pH gradient and condense or "focus" in very sharp thin zones at locations where the pH equals their isoelectric pH. The synthesis of appropriate carrier ampholytes was crucial to the success of this technique. This was accomplished by Vesterberg (31) who synthesized aminocarboxylic acids of a wide and quasi-continuous spectrum of pI values which have been made commercially available by LKB under the trade name of "ampholines." Fig. 9 shows Svensson's "natural pH gradient" column apparatus displaying an isoelectric separation pattern. Resolution of

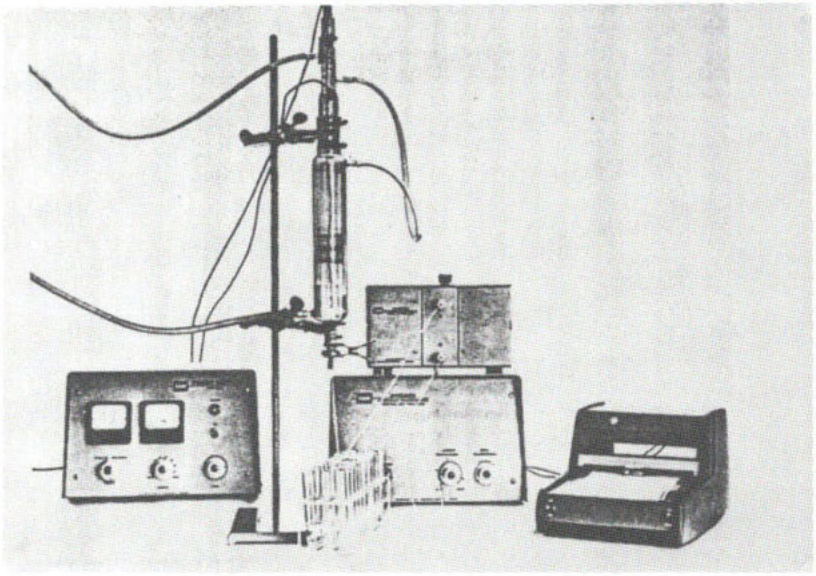


Fig. 9 Svensson's isoelectric focusing equipment consisting of electrofocusing column (LKB 8100-10) (110 ml), power supply (LKB 3371), UV monitor (LKB 8300), recorder. (From H. Haglund, Meth. Biochem. Analysis. 19, 1-104, 1970.)

componenets differing as little as 0.01 pH unit in their pI values is currently possible. (A more extensive review of the genesis and early evolution of isoelectric focusing can be found elsewhere (32, 33)).

A drawback of this powerful technique is the contamination of the separated fractions by the "ampholines" and by sucrose which have to be removed. Ideas to avoid "ampholines" by generating pH gradients by means of temperature gradients have been suggested but not worked out (34, 35).

NON-ISOELECTRIC FOCUSING: VELOCITY GRADIENT FOCUSING

Isoelectric focusing is a special case of a general principle which we shall call "velocity gradient focusing." Let us

consider a zone of width w in an electrophretic column. This zone width will be preserved if the rear of the zone migrates with the same velocity as the front of the zone. Let us assume now that the electrical conductivity σ of the buffer in the column increases in the direction of migration. Since by Ohm's law the electric feild intensity is $E = J/\sigma$ (where J is the current density), the electric field intensity will have to decrease in the direction of the zone migration. As a result, the ions forming the front of the zone will move more slowly than the rearguard ions and the zone will become progressively narrower i.e., we will observe non-isoelectric focusing (14). The final width of the zone will be determined by a balance between the tendency of the zone to diminish in width in the electrophoretic velocity gradient and the tendency of diffusion to broaden the zone. The gradient that determines the ion velocity need not be shallow. Zone narrowing will occur equally well when the zone migrates across a boundary at which the ion velocity changes abruptly. Such non-isoelectric focusing was first demonstrated in the same cell in which the first isoelectric focusing experiments were performed (13, 14) (Fig. 6). In Fig. 8 the top component has been focused non-isoelectrically because of the sharp increase in buffer conductivity across the upper boundary of the M layer. Fig. 10 shows non-

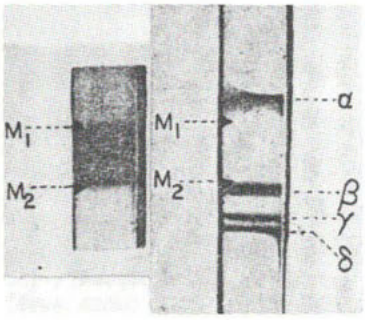


Fig. 10 *Velocity gradient focusing in the cell shown in Fig. 6. Separation of a mixture of dyes (time, 10 minutes). α: basic fuchsin, β: chrome hematoxylin, γ: brom thymol blue, δ: brom phenol blue. The original M layer is shown on the left of the separation pattern.*

isoelectric focusing of dyes which migrate upward (α) and down-

ward (β, γ, δ) from a sample zone of low electrical conductivity (between the marks M_1, M_2) into buffer solutions above M_1 and below M_2 of higher electrical conductivity. (Such zone narrowing has later been employed in other methods, such as "disc electrophoresis" (29).

Another means of achieving velocity gradient focusing is to use a molecular sieving effect. If the zone of ions must traverse a boundary between two gels of different porosity which are arranged so that the front of the zone migrates from a wide-pore gel into a small-pore gel, the front of the zone will be retarded by molecular sieving while the rear boundary is still migrating almost unimpeded with greater speed causing a narrowing of the zone. This effect is used along with other focusing means in "disc electrophoresis" (29) where zones can be compressed to the thickness of a few micra. Electrophoresis of ion zones in gentle gel-pore gradients also results in narrowing of migrating zones (37).

Finally, the ion velocity gradient can be created by means of a pH gradient in the electrophoretic column. Due to the pH-dependence of the ion mobility, the ions would move with different velocities at different points in the column even if the electric field is uniform throughout the column (13). Thus, if the pH gradient diminishes the ion mobility in the direction of migration, the rear of the zone will travel faster than the front and the zone will be narrowed (i.e., "focused") progressively. This method of modification of ion velocities has also been used in "disc electrophoresis" (see below). The converse will, of course, happen if the zone migrates in the direction of increasing ion mobility in the pH gradient. It will become progressively wider.

We can now easily see that isoelectric focusing is a special case of zonal velocity-gradient focusing where the ion mobility is controlled by a varying pH in the migration column.

We obtain isoelectric focusing if the pH gradient encompasses the isoelectric pH of the migrating protein and if the direction of migration is toward the isoelectric point. In this case the ion velocity relative to the pH distribution function is decelerated, reaching zero at the isoelectric point and the front of the protein zone moves more slowly than its rear boundary so that maximal condensation (or "focusing") is achieved when all protein ions have lost their charge in the isoelectric layer. In the special case where the isoelectric point lies inside the protein zone, its boundaries migrate toward each other if the current flows in the direction of increasing pH. We obtain again a focusing effect. If, on the other hand, the current flows in the direction of decreasing pH values, the velocities of the two zone boundaries are still opposite to each other, but they are directed away from the zone center and we obtain zone broadening ("defocusing"). The special features of isoelectric focusing, when viewed as a velocity-gradient focusing effect, are thus the existence of a plane of zero ampholyte velocity relative to the buffer and the condition that the zero mobility in this plane is brought about by the local pH value.

Stacking Methods

ISOTACHOPHORESIS

Ordinary zone electrophoresis does not have a clear-cut completion point beyond the stage where the original mixed-sample zone has been split into several perceptibly separated zones of different electrophoretic mobilities. The separation between the various components can, in principle, be increased indefinitely by continued electromigration which proceeds with different velocities for the individual components. Isoelectric focusing analysis, on the other hand, can be considered completed when the components of different pI values have

become stationary at their isoelectric points in the pH gradient. The separation method to be considered now sorts the components of a mixture according to differences in electrophoretic mobilities and arranges them as contiguous zones in the sequence of their mobilities. Unlike isoelectric focusing, however, these zones do not become stationary and, in contrast to zone electrophoresis, these zones do not separate from each other in the course of further electromigration because they continue to move indefinitely with the same speed. (Hence the name "isotachophoresis," derived from the Greek words "isos" for equal and "tachos" for speed.) Because of the resemblance of the contiguous separated zones to a stack of coins, we have classified isotachophoresis as a "stacking method."

The recent emergence and cultivation of isotachophoresis (ITP) is another example of "hybernation" of a scientific idea. It was first proposed by Kendall in 1923 (32) for electrochemical separation of isotopes. Its theoretical foundation is based on Kohlrausch's publication of 1897 (9). Kendall did not succeed in separating isotopes, but later others did so on the basis of his idea (39). (Kendall's principle is encountered in the literature under a variety of names, such as isotachophoresis, displacement electrophoresis, cons electrophoresis, transphoresis, etc.) The key to ITP is the following insight which was pointed out by Kohlrausch. Let us assume that we have an electrolyte column in which a sharp boundary exists between an electrolyte P^+L^- and P^+T^-. (We assume, for simplicity, all ions to be monovalent.) The positive ion species is continuous and the boundary is a discontinuity in the negative ion composition. Let us further assume the mobility of the L^- ion (leading ion) to be greater than that of the T^- ion (terminating ion) ($\mu_L > \mu_T$). When a current flows through this column, the boundary between the L^- and T^- ions migrates with the speed of the leading ions. This implies that the terminating ions migrate at the boundary with the same speed in

spite of their lower mobility. This can happen only if the electric field intensity on the T-side of the boundary is higher than on the L-side. Kohlrausch showed that this inequality in field intensity is caused by a readjustment of the ion concentration which alters the electrical conductivity in the column in such a way that a certain function of the ion mobilities and concentrations (the Kohlrausch "regulating function") has the same value on both sides of the boundary. This statement is valid not only for the case of one single boundary but is applicable to a column containing an arbitrary number of boundaries between ions of equal sign having a common counter-ion.

Fig. 11 illustrates the ion configuration in isotachophoresis. The long electrolyte column terminates in large reservoirs with electrodes at both ends which are not shown. The common positive ions P^+ are not indicated in the figure which shows only the negative ion species that migrate to the left. The L^- ions have a higher mobility than the T^- ions. Let us assume at first that $(ABC)^-$ is a species of monovalent ions of an intermediate mobility ($\mu_L > \mu_{(ABC)} > \mu_T$) (Fig. 11a). Then, changes in concentration and in potential gradients will occur at each boundary of the zone, as mentioned above, to insure equality of the Kohlrausch regulating function across the boundaries. The concentration changes will lead to a change in the width of the zone. After establishment of a steady state, both boundaries will migrate with equal speed to the left and the electric field intensities in the column will change abruptly across the boundaries which are demarcations of discontinuous changes in ion composition.

Let us now assume, that the ABC zone actually contains three different ion species A^-, B^-, C^- whose mobilities lie between those of the L^- and the T^- ions. Sharp boundaries will then develop between the individual ion species which will migrate with equal speed in the final steady state. The distances between the boundaries (i.e., the widths of the zones A, B, C)

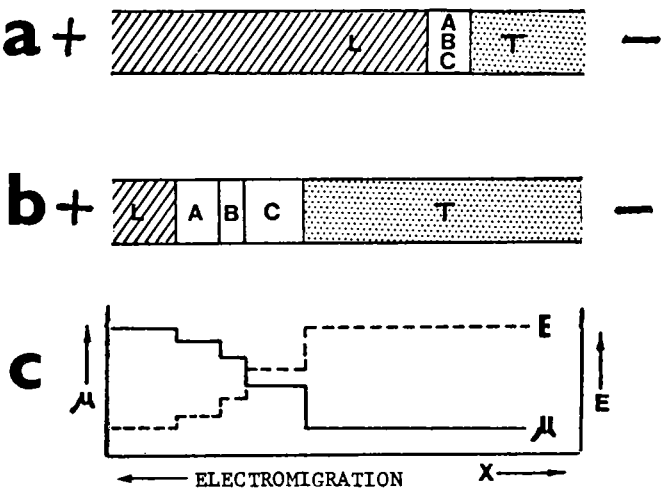


Fig. 11 *Illustration of isotachophoretic stacking. The current polarity is indicated in a and b. (a) The zone containing the mixture of anions to be separated is sandwiched between the leading anions L and terminating anions T. (b) Stack of contiguous zones of ionic species A, B, C transported to the left by isotachophoresis. (c) Correlation between the electric field intensity E and electophoretic mobility μ in the migration column. (Redrawn from M. Bier and T. Algyer in Electrokinetic Separation Methods, P. G. Righetti et al, Eds. (New York: Elsevier, 1979.)*

will change until the concentrations satisfying the Kohlrausch condition have been established (Fig. 11b). The sequence of the ions in these zones is such that their mobilities increase from T to L. Once the species A, B, C have been separated, their boundaries migrate with equal speed without further separation. Fig. 11c shows how the variations in the electric field intensity E within the various zones are coordinated with variations in the ion mobilities μ.

Isotachophoresis has been ingeniously and very effectively utilized, along with other principles, in the method of "disc electrophoresis" (36). Ornstein presents a calculation based

on a modified Kohlrausch equation to show how a 1cm wide protein zone sandwiched between ions of glycinate and chlorine can be reduced to a thickness of 25 micra and concentrated by a factor of 380. In addition to isotachophoresis, disc electrophoresis utilizes principles of velocity gradient focusing (14, 26) and discontinuous buffer systems (13, 14, 26). The scheme of disc electrophoresis shown in Fig. 12 has some common

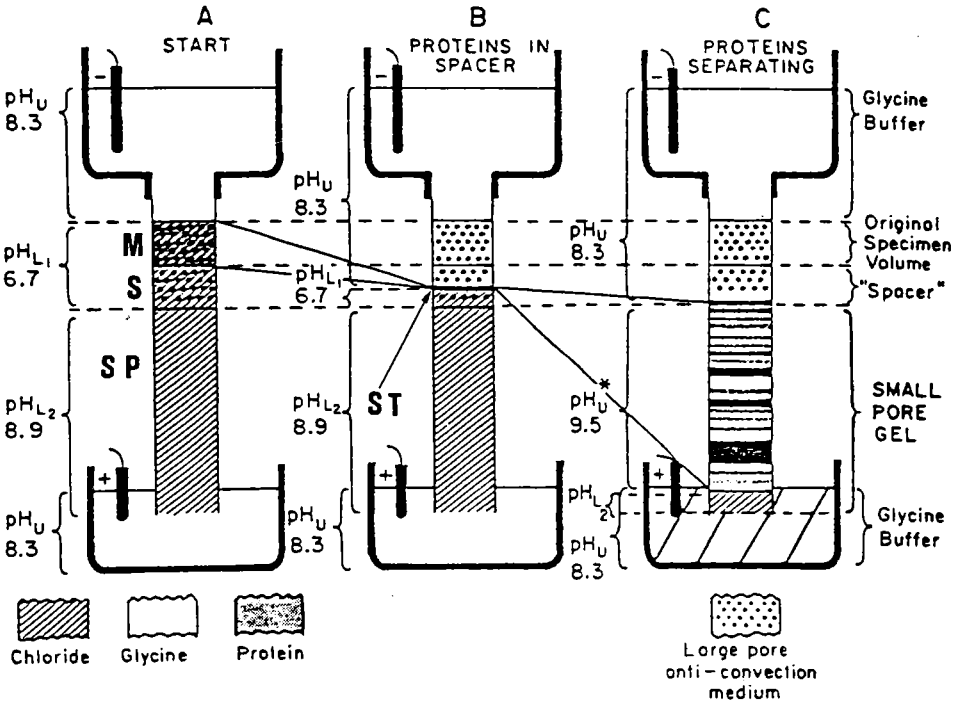


Fig. 12 *Scheme of Disc Electrophoresis showing 3 stages of the process.* **A:** *Initial stage. The sample is in zone M.* **B:** *The components of the sample have migrated into the "spacer gel" and are condensed by isotachophoresis into a narrow stack ST.* **C:** *The chloride ions have left the small-pore gel and separation proceeds in it by normal zone electrophoresis combined with molecular sieving. (Slightly modified from Ref. 36.)*

features with the scheme of velocity gradient electrophoresis shown in Fig. 6 (14, 16). An important difference is the use of a polyacrylamide gel for inhibition of thermal convection

in disc electrophoresis instead of density gradients used in the scheme of Fig. 6. The second important difference is the use of molecular sieving in a small-pore gel in disc electrophoresis to achieve velocity gradient focusing. The process of disc electrophoresis is illustrated in Fig. 12. The sample is contained in the large-pore gel section M. The sample ions are sandwiched between the low-mobility "terminating" glycine ions and the high-mobility "leading"chlorine ions so that the migration of the sample ions results in an isotachophoretic sorting or stacking process. The original sample zone M is thus compressed into a thin stack ST of contiguous separated zones by isotachophoresis. In Fig. 12c the chlorine ions are shown to have left the small-pore gel where the pH has risen from 8.9 to 9.5. Transition of the zones from the large-pore gel into the small-pore gel leads to further zone narrowing due to velocity gradient focusing and from then on, the electromigration through the small-pore gel is governed, in addition, by molecular sieving. Thus, the contiguous zones are separated from each other in accordance with the differences in molecular shape and dimensions of the ions in the zones.

Quest for Higher Resolution

TWO-DIMENSIONAL METHODS

Even with modern high-resolution separation methods, one comes across closely-spaced components in electropherograms that are difficult to separate. To get such narrow multipletts further apart, a practice that had been used in chromatography since 1948 may have provided the stimulus for introduction of two-dimensional techniques into electrophoresis (40). Durrum (41) appears to have been the first to suggest a two-step process in which zonal filter paper electrophoresis is carried out first in one direction and then in the transverse direction after drying the square sheet and replacing the previous buffer

by a new solution of different composition and, if useful, of different pH. The advent of new materials (such as various gels) and new electrophoretic techniques opened up new, almost unlimited possibilities of two-dimensional combinations of consecutive transverse separation processes with the consequence of greatly enhanced resolution. The separation criteria of the two transverse procedures must be different.

In view of the large and growing number of possible method combinations, which is augmented by the desirability to specify which method was used as the first step, it will be very helpful to represent two-dimensional methods by a diagram (Fig. 13). This diagram may also have a heuristic value by helping to conceive new combination methods. In analogy to the complex-number plane in mathematics, we define a method-plane in which the primed numbers along the vertical axis, as well as the unprimed ones along the horizontal axis are each correlated with a method. The unprimed numbers (horizontal axis) designate the first-step method and the primed ones the second step method. (In some instances, like electromagnetophoresis combined with sedimentation, the two processes can occur simultaneously.)

Thus, a point with integral coordinates in the method plane represents a combination method. Points along the 45^{o} line represent cases where the same method is used in both transverse directions (but with different parameters). For instance, the point (1, 1') represents two-step chromatography where two-different elution fluids are used in two mutually perpendicular directions. If a combination method AB can be carried out with either the method A or the method B forming the first step, we will obtain two points symmetrical with respect to the 45^{o} axis representing the combination method. In view of the large number of possible combination methods, which is practically limitless considering the rapid evolution of new techniques, we cannot review them all in the limited scope of this lecture.

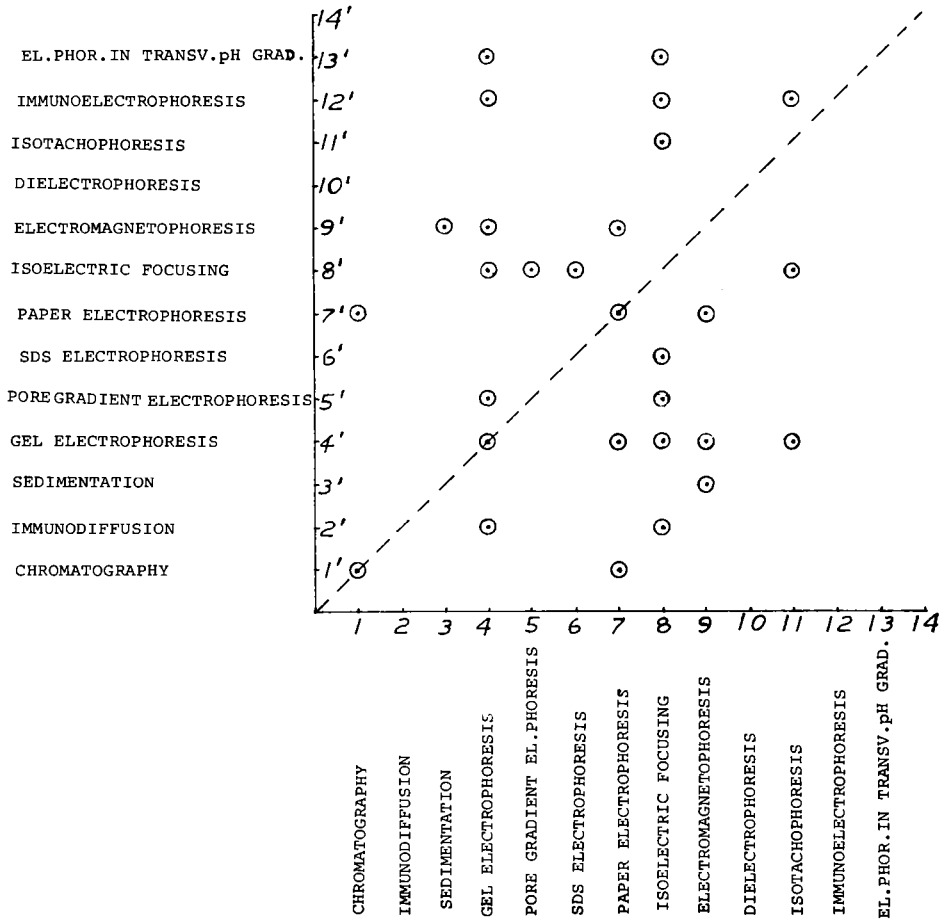


Fig. 13 *Coordinate representation of two-dimensional methods. The methods listed along the abscissa represent the first step of a two-dimensional procedure, the methods listed along the ordinate represent the second step.*

It will suffice to make a few comments on less well-known hybrid methods represented in Fig. 13. This figure is not intended to be comprehensive and the arrangement of methods along the coordinate axis is rather arbitrary. The methods will be designated below by the coordinates of the points representing them in Fig. 13.

Examples

(1, 7') - (1', 7). This is the well-known technique of "fingerprinting" (42). It proved particularly effective in resolving mixtures of many amino acid and polypeptide components, that could not be adequately separated by a one-dimensional process, into a two-dimensional map of clearly separated fractions. It exemplifies the basic advantage of two-dimensional methods.

(4-2'). Classical immuno-electrophoresis after Grabar and Williams (43).

(4-12') (44). The first step consists of isoelectric focusing of the antigen mixture in a polyacrylamide gel. The second step subjects the separation pattern to transverse electrophoresis into an antiserum-containing agarose gel where precipitation zones of antigen-antibody complexes are formed according to the diffusion-free immunoelectrophoresis method of Laurel (45).

(9-7') - (7-9'). Simultaneous application of transverse electric and magnetic fields imposes simultaneous electrophoresis and electromagnetophoresis upon changed particles in a quasi-two-dimensional matrix of filter paper or thin gel layer (46, 47).

(8-13'). Titration curves. This is a particularly interesting and beautiful procedure in which the amphoteric ions delineate their own pH-mobility curve (37, 48). Fig. 14 shows two examples of this process in A and B. To begin with, a stable

pH gradient is generated in the ampholine-containing gel by prolonged passage of current in the horizontal direction, as indicated by the double arrow (IEF) in Fig. 14B. Then, the

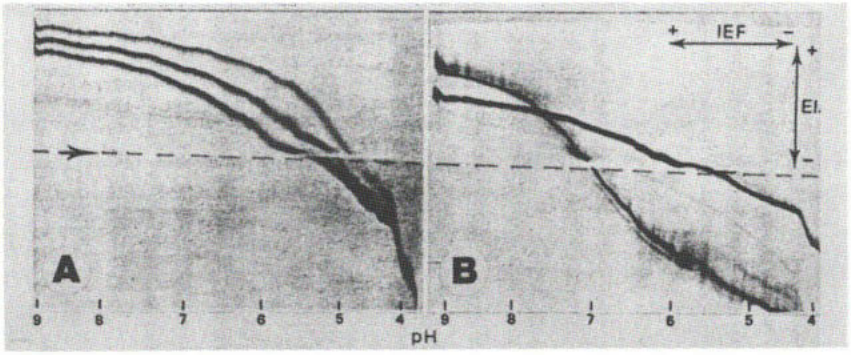


Fig. 14 *Vertical electrophoresis across a horizontal pH gradient. The sample is placed after the first step (establishment of the horizontal pH gradient) into a linear horizontal trough in the gel (dashed line). Electromigration along the vertical axis in the second step distorts the zone as shown, delineating the pH-mobility curve (titration curve). The intersection of this curve with the dashed line marks the isoelectric point of the component. The two electropherograms represent titration curves of Hp-Hb complexes in a 3:1 molar excess of Hp (A) and in a 3:1 molar excess of Hb (B). The three curves in A are: free, excess Hp (pI 4.5), Hp-Hb, 1:0.5 molar, complex (pI 5.0) and Hp-Hb, 1:1 molar, complex (pI 5.5). The two curves in B are: Hp-Hb, 1:1 molar, complex (pI 5.5) and excess free Hb (pI 7.0). The two arrows and positive and negative symbols represent the direction and polarity of isoelectric focusing (IEF) and electrophoresis (El.) The arrowhead indicates the sample application zone (i.e, the zero-mobility or isoelectric plane) (slightly modified from Ref. 37).*

electrode axis is turned 90° (as indicated by the double arrow (EL) in Fig. 14B) so that a current can be passed in the vertical direction at right angles to the established pH gradient. (The electrofocused ampholines are, of course, not affected by this current since they are located at their isolation points in the pH gradient). A long trough is now cut into the gel about centrally in the direction of the pH gradient as indicated by the arrow and dashed line in Figs. 14A and B. The sample is placed into this trough and the gel is subjected

to a vertical electric field. If we consider one particular protein in the sample, it will not move at the intersection of the dashed line with the vertical line which passes through the pI value of the protein on the horizontal pH scale. On both sides of this intersection point, the protein ions will carry opposite charges and, hence, move in opposite directions (upward on the left and downward on the right side of this point in Fig. 14). Since the rate of migration depends on pH, the vertical distance traversed by the ions will vary from point to point of the original horizontal zone which is thus deformed from a straight line into a curve depicting the pH-mobility function of the given ion species. This method provides a characterization which is superior to the mere specification of the isoelectric pH or of the ion mobility at a given pH value.

The basic principle of this method was conceived by Michl in 1952 (49) before the advent of isoelectric focusing (13) in 1954! But, using ordinary buffers, he was unable to obtain good results. The successful revival of this idea (48) became possible after invention of the ampholines (31). This method was further developed by Righetti and Gianazza (37).

It is safe to conclude that two-dimensional techniques will enjoy increasing use and development because of the obvious advantage of wider distribution of separated fractions in two dimensions than can be accomplished in a one-dimensional process. It is not unlikely that further improvements will be achieved in the future by development of three-dimensional methods.

Quest for New Separation and Characterization Parameters

MOLECULAR DIMENSIONS (DETERMINATION BY SDS-PAGE)

In the methods that we surveyed, separation and characterization of the molecular species were based on ion mobilities and isoelectric points. Is electrophoretic methodology necessarily limited to these parameters? --Developments have shown that there is no such restriction. One of the main parameters of chemistry, the molecular weight, can be linked with the electrophoretic process. If we had a means of imparting the same electric charge density to a variety of molecules (nucleic acids, proteins) of similar conformation (e.g., random coil) but different in size, their migration through a gel (e.g., polyacrylamide gel) of suitable porosity could provide information about their molecular dimensions. In this case, the electrophoretic mobility of the molecular species under study in the gel can be compared to mobilities of standard molecules (ions) of known molecular weight (MW). This would lead to a determination of relative molecular size from which the (MW) could be inferred. To achieve uniform electrical charge densities, the unknown as well as the known standard molecules are treated (by "cooking") with sodium dodecyl sulfate (SDS) (50). Electrophoresis is performed in a crosslinked polyacrylamide gel of suitable concentration.

MOLECULAR DIMENSIONS (DETERMINATION BY ELECTROPHORESIS IN GEL-PORE-GRADIENT)

Another approach to (MW) determination is based on electrophoresis in a polyacrylamide gel of graded porosity. If a zone of a molecular species migrates in such a gel parallel to the pore-size gradient in the direction of diminishing pore-size, it will become progressively narrower (due to greater retardation of the front of the zone in the more concentrated gel matrix as compared to the rear boundary (velocity gradient focusing (14)). If the original zone contains a mixture of proteins, they split into progressively narrowing zones as they travel. Studies on mixtures of known molecular

weight have established (51) that the relative migration speeds of the proteins (based on the slowest component serving as a standard) become constant after all components have passed beyond a certain gel concentration ("pore limit"). The plot of the logarithm of the molecular weight versus the logarithm of the relative migration speed becomes linear. Thus, if a mixture of proteins of unknown molecular weights with two components of known (MW) is examined, the line fixed in the log log plot by the two known components permits the determination of the (MW) of the unknown fractions (51).

IMMUNO-ELECTROPHORESIS (Immunochemical Specificity as Analytical Parameter)

Many proteins are hard to differentiate because of closeness of their electrophoretic mobilities, isoelectric points and/or enzymatic or staining reactions. This is especially true of proteins derived from genetically related plants and animals. The idea of Grabar and Williams (43) made such a differentiation possible with immunochemical specificity. Their method is a two-step process where electrophoresis is followed by transverse immunodiffusion. The end result is a two-dimensional pattern which enables one to identify antigens and/or antibodies.

It goes beyond the scope of this lecture to review all the variants of this method. Fig. 15 illustrates one typical use.

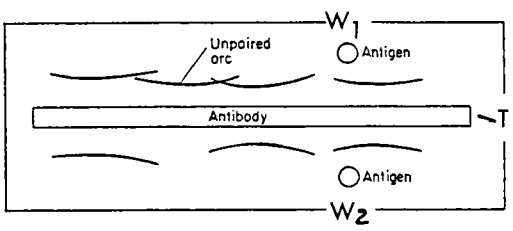


Fig. 15 Schematic representation of immunoelectrophoretic comparison of antigen mixtures. (Slightly modified from D. J. Shaw, Electrophoresis. New York: Academic Press, 1969.)

Let us assume that a mixture of unknown antigens is placed in the circular well W_1 of the gel. Electrophoresis of the antigen ions toward the left has resolved this sample into four invisible circular protein zones. The problem of visualizing them is analogous to the development of a latent photographic image. The idea of Grabar and Williams was to accomplish it by "development" with a mixture of antibodies so that an immunoprecipitate would reveal and identify an antigen present in the mixture. But this is not a trivial experimental problem since the formation of the precipitate requires a certain antigen/antibody concentration ratio. This difficulty was overcome by the ingenious idea of creating concentration gradients of the reactants by diffusion. Thus, there will be arc-shaped loci within the diffusion field where the concentration ratio will be optimal for the precipitation reaction.

After termination of the first step of electrophoresis in Fig. 15, a mixture of antibodies is placed in a long trough T in the gel. The macromolecules of the antigens and antibodies are now allowed to diffuse at the low rates determined by their diffusion coefficients for a long period of time (e.g., 2 days). The radial diffusion patterns of the circular antigen zones and the linear diffusion front which proceeds from the antibody trough penetrate each other and characteristic precipitation arcs are formed at proper locations within the concentration gradients of the diffusion fields for those antigens whose antibodies are contained in the trough. By concurrent electrophoresis of a mixture of known antigens placed in the circular well W_2, one obtains a second pattern of arcs below the trough T. Thus, the paired ones in the two patterns permit identification of antigens present in the unknown mixture and the presence of an unpaired arc in the unknown sample reveals an antigen without a counterpart in the known antigen mixture. This is a highly simplified description of a powerful method that is much more complex in actual practice. The most time-consuming part of the method is the preparation of the anti-

serum which may require many weeks.

The loss of time due to the slow diffusion process has been eliminated by development of new immunoelectrophoretic methods in which the diffusion step is eliminated. This is achieved by incorporating the antibodies in the gel under electrochemical conditions which immobilize them in an electric field while the antigens migrate and form precipitate arcs with the antibodies. Such methods permit a quantitative estimation of the amounts of antigens in the sample (e.g., Laurel's method (45)).

DIELECTROPHORESIS (Charge-Independent Migration)

Dielectrophoresis is the oldest known electrical phenomenon (except for lightning). It is the effect through which electrical attraction has been discovered in ancient times. A small electrified object produces a diverging electric field at a distance. A distant object subjected to this field is polarized, i.e., becomes an electric dipole with zero net charge. As early as in 1600 Gilbert observed the deformation of a hanging drop of water exposed to an electric field. A, say, positively charged object induces a negative charge on the proximal side of the sphere facing it and a positive charge on the distal side. The attraction of the proximal opposite charge on the sphere and the simultaneous repulsion of the like distal charge distort the hanging drop. Suppose now that the drop is detached. The greater force of attraction on the proximal charge will surpass the repulsion force exerted on the distal charge and the drop will move toward the electrified object under the predominant action of the attractive force. The direction of this force depends, however, on the relative values of the dielectric constants of the drop (or other polarizable object) and the surrounding medium. The force exerted by the source of a radial electric field upon a dielectric particle will be attractive if it is embedded in a medium of lower dielectric constant.

It will be repulsive if the dielectric constant of the medium (ε') surpasses that of the particle (ε'') and will be zero if the two dielectric constants are equal. This interaction is described by the equation (52):

$$\vec{F} = 2\pi a^3 \varepsilon' \left[\frac{\varepsilon'' - \varepsilon'}{\varepsilon'' + 2\varepsilon'}\right] \vec{\nabla} |E|^2. \qquad (1)$$

($\vec{F}$ is the force, a the radius of the spherical particle, and E the magnitude of the electrical field intensity. The force upon a conductive sphere in a conductive medium is given by a more complex equation and will not be considered here (53)).

Thus, attractive or repulsive forces can be exerted upon suspended particles in a non-uniform electric field depending on the relative magnitudes of the dielectric constants of the particle and the medium. The nature of the force (attraction or repulsion) is not affected by the direction of the electric field. Thus, alternating fields can be used.

Practical analytical use of this effect in cell biology is made difficult by the required non-uniformity of the electric field. Particles located in different portions of the non-uniform field experience forces of different magnitude. This creates a problem in design of a separation method of good resolving power and throughput. In addition, non-uniform heating of the fluid in the non-uniform electric field engenders thermal convection.

ELECTROMAGNETOPHORESIS (Charge-Independent Migration)

There is a growing interest in separation of cells, cell organelles and viruses in connection with research in cell biology and molecular genetics. Electrophoresis is capable of accomplishing such separations not only zonally but on a continuous-flow basis as well (18, 19, 20). However, electrophoresis is

not an ideal method for cell separations. Good resolution and fast separations require a high electric field intensity. Avoidance of overheating the buffer and the cells requires a low current density. This leads to the use of low ionic strength buffers in cell electrophoresis which subjects the cells to the hazards of clumping and deterioration during the separation process. Ideally, one would wish to maintain the cells in their native body fluid that would have a much higher ionic strength than the commonly used buffers. The solution of this problem may be offered by a relatively recently discovered electrokinetic phenomenon, electromagnetophoresis (EMP) (54, 55, 56), which requires for optimum performance media of high ionic strength and would operate well in an electrolyte such as blood serum. The reason for the preference of a high ionic strength medium in EMP is that the particle velocity in EMP is proportional to the current density whereas in electrophoresis it is proportional to the voltage gradient.

Electromagnetophoresis does not require the particles to be electrically charged and their migration direction does not coincide with the electric field. The effect requires, in addition to an electric current, the presence of a magnetic field. Only the magnetic field component perpendicular to the current contributes to the force exerted upon the particle and the migration is perpendicular to both the electric and the magnetic field. Reversal of either field reverses the direction of the particle migration. Simultaneous reversal of both fields does not reverse the direction of particle migation. Thus, unidirectional EMP can be obtained with an alternating electric current, provided it is accompanied by a perpendicular alternating magnetic field of equal frequency and phase.

The force $\vec{F}$ and the migration velocity $\vec{v}$ for a spherical particle of radius a and electrical conductivity σ' suspended in a fluid of electrical conductivity σ'' which is traversed by an electric current of current density $\vec{J}$ and a magnetic field of

intensity $\vec{B}$ is given by equations (2) and (3):

$$\vec{F} = 2\,\pi\,a^3\,\left(\frac{\sigma'-\sigma''}{\sigma'+2\,\sigma''}\right)\,[\vec{B}\times\vec{J}]\,. \qquad (2)$$

$$\vec{v} = \frac{a^2}{3\eta}\,\left(\frac{\sigma'-\sigma''}{\sigma'+2\,\sigma''}\right)\,[\vec{B}\times\vec{J}]\,. \qquad (3)$$

As we see, the particle velocity does not depend on the particle charge, but rather on the electrical conductivities σ' and σ'' of the particle and the suspension medium. If the particle moves to the right for $\sigma' > \sigma''$, it will move to the left for $\sigma' < \sigma''$ and will remain at rest for $\sigma' = \sigma''$. The particle speed increases with the square of its radius. The force upon a non-spherical particle depends on its shape and orientation relative to the electric and magnetic fields (54). In addition, to the electrical conductivity of a particle, its dielectric constant and/or its magnetic susceptibility can be used as a separation parameter in addition to particle size and shape (55, 57).

Fig. 16 shows an arrangment in which the EMP effect can be demonstrated. The Helmholtz coils C_1, C_2 generate a uniform magnetic field $\vec{B}$ in the central region of their interspace where a plastic cell with electrodes E_1, E_2 is placed. They form the top and bottom surfaces of the cell which contains an electrolyte, such as physiological saline or blood serum, with suspended particles. $\vec{J}$ is the current density vector and $\vec{F}$ indicates the direction of the force exerted upon the current-carrying electrolyte as well as on suspended particles whose conductivity exceeds that of the electrolyte. Particles of lesser conductivity than the electrolyte's experience a force in the opposite direction.

The force experienced by a conductive particle consists of two components. The first one is an electromagnetic force exerted by the perpendicular magnetic field upon the moving ions of the current passing through the particle. Its nature is the same

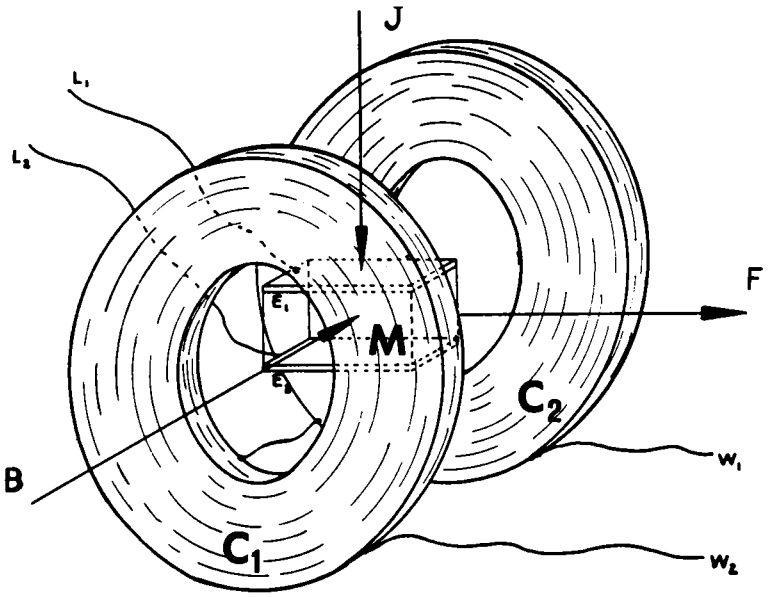


Fig. 16 *Scheme of electromagnetophoresis apparatus. M is the migration cell with electrodes E_1, E_2. C_1, C_2 is a pair of Helmholtz coils generating a uniform magnetic field within the cell M. $\vec{B}$: magnetic field vector. $\vec{J}$: electric current density vector. $\vec{F}$: electromagnetic force vector. W_1, W_2: magnet coil lead wires. L_1, L_2: electrode lead wires. (From Ref. 19.)*

as that of the force exerted upon a wire in the rotor of an electric motor in its magnetic field. The second component is a hydrostatic force exerted upon the particle surface by the electrolyte surrounding the particle. It is due to a hydrostatic pressure gradient which is set up in the electrolyte by the interaction of the ion current traversing it with the transverse magnetic field. This latter force component is very similar to the force of buoyancy exerted upon a floating object, whereas the first force component is roughly analogous to the gravitational force. The resultant force on the particle is the sum of these two opposing force components. A perfectly non-conducting particle experiences only the force of "electro-

magnetic buoyancy." The principle of Archimedes which is valid in the gravitational field can be qualitatively extended to electromagnetophoresis by assigning to the electrical conductivity in the case of EMP the same role as is played by the specific gravity in the gravitational case.

Having seen the lapse of time between the discoveries of chromatography (1903) and electrophoresis (1809) and the advent of their practical analytical uses, we should not be surprised that electromagnetophoresis has not yet reached this stage. The growing interest in electrokinetic separation of biological particles and the availability of superconducting magnets of field intensisites two orders of magnitude above those used in the initial demonstration of electromagnetophoresis will undoubtedly have a catalytic effect upon further developments in this field.

It is reasonable to expect that being a physical effect like electrophoresis, EMP will give rise to a comparably large variety of methods and techniques as we now see in the numerous implementations of electrophoresis. It has been shown, for instance, theoretically (55, 57) that the electrical conductivity is not the only parameter according to which particles can be sorted by EMP. The dielectric constant and the magnetic susceptibility can also be used as separation parameters. A further example will show the variety of ways in which electromagnetophoresis can be implemented. It can, for instance be used to achieve electrokinetic centrifugation. Fig. 17 shows an arrangement using the simplest conceivable electromagnet, namely, a wire w carring an electric current I. The magnetic field it generates is depicted by circular field lines H. The electrolyte-filled cell C containing suspended particles surrounds this wire and carries a vertical current of current density J between its electrodes E_1, E_2. This field configuration of circular magnetic field lines with an axial electric cell-current parallel to the wire generates a force

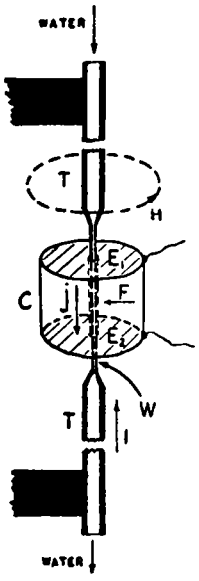


Fig. 17 *Scheme of non-rotational electrokinetic centrifuge based on electromagnetophoresis. T_1: watercooled copper tube carrying the current I which generates the magnetic field $\vec{H}$ within the cylindrical migration cell C with electrodes E_1, E_2. W is the narrowed portion of the copper tube that runs along the central axis of the cell C. $\vec{F}$ electromagnetic force (centripetal or centrifugal) exerted upon particles suspended in the cell C. (From Ref. 55.)*

upon suspended particles that is perpendicular to the electric and magnetic field lines. This force is thus radial and is directed toward the wire (centripetal force) or away from it (centrifugal force) depending on the phase relationship between the alternating current and alternating magnetic field, which optimally, should be either in phase or phase opposition. We have thus in this EMP variant an electrokinetic centrifuge without rotational motion.

We can now remove needless restraints from our imagination and remember that a plane electromagnetic wave (such as a radiowave) has an electric field vector and a magnetic one perpendicular to the electric field and in phase with it. This is the configuration for generation of the EMP effect and the force should be perpendicular to these two vectors, i.e., it should point toward the source of the wave or away from it. Going one step further, we allow the frequency of the electromagnetic wave to reach the domain of visible light. A particle

in the path of such a wave should exhibit "photophoresis" in the direction of the light ray. This prediction of electromagnetophoresis at optical frequencies reminds us, of course, of the well-known effect of "light pressure." In fact, it was this mode of propulsion by light pressure of microscopic organisms through interstellar space that Arrhenius assumed in his hypothesis of panspermia to explain the origine of life on earth. Thus, if Arrhenius' intuition is correct, electromagnetophoresis may play a role in disseminating life in the universe by intergalactic photophoretic transport between distant inhabited and inhabitable worlds.

EPILOGUE

We can confidently close this lecture with the conclusion that discoveries of new effects and inventions of new instruments will continue to provide new building blocks to the ingenious analytical chemist to create new electrokinetic separation methods. But what we have shown in this lecture also suggests that potentially fruitful ideas, discoveries and inventions may be lying buried in the massive literature until some perceptive scientist will disturb their winter sleep or until they are resurrected through an independent act of creative imagination.

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SECTION II THEORY AND METHODS

A UNIFIED MATHEMATICAL THEORY OF ELECTROPHORETIC PROCESSES

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Introduction

The last thirty years have seen the proliferation of a rather bewildering number of electrophoretic instruments and methods. Most of these remained confined to the laboratory of the developer, but some gained rapid and widespread acceptance. As a result of the diversity of existing methods, their rational classification is rather difficult and somewhat subject to the personal bias or interest of the individual reviewer. Nevertheless, most authors have emphasized four classical modes of electrophoresis: zone electrophoresis (ZE), moving boundary electrophoresis (MBE), isotachophoresis (ITP) and isoelectric focusing (IEF). The reason for this emphasis is that elements of one or another of these four modes are usually recognizable in most electrophoretic techniques.

Past theoretical treatments of electrophoretic processes have been usually confined to a single mode, such as the reviews of Longsworth on MBE (1), the work of Martin, Everaerts, and others (2-4) on ITP, and the papers of Svensson, Almgren, Cann and others on the theory of IEF (5-7). This compartmentalization of the theory of electrophoresis is not only unneces-

Electrophoresis '82

sary but counterproductive, as it sets artificial barriers to the understanding of the underlying processes. The object of the present paper is to show that each of these electrophoretic modes is based on the same general principles and can collectively be described in terms of a single set of equations. The mathematical model so derived is an amplification of concepts first utilized in our model of the steady state in IEF (8) and has been previously described in general terms (9).

For a qualitative understanding of the characteristic features of the four modes the pioneering work of Kohlrausch in 1897 (10) and the still earlier work of Hittorf (11) is essential and is here briefly summarized. Kohlrausch was the first to demonstrate that the passage of an electric current induces no concentration changes in a system with uniform electrolyte distribution. He has further shown that any concentration boundary, i.e. boundary resulting from dilution of the electrolytes will not migrate under the influence of the electric field but will gradually broaden due to diffusion. Such 'stationary' boundaries are commonly observed in MBE there being a slight change in electrolyte concentration at the protein-buffer interface. It is also observed in ITP as a result of the concentration readjustment of samples and terminators to conform to the Kohlrausch regulatory function.

Kohlrausch has also considered species discontinuities, where one of the ionic constituents is replaced by another species across a boundary. This boundary may migrate in two different modes: if the leading ion has a net mobility higher than that of the trailing ion, a steady state, governed by the well-known Kohlrausch regulatory function, will be reached (3). If there are several such boundaries in the system, they will all migrate at the same velocity. Moreover, the structure of the boundaries will remain constant, once steady state is reached, and they will not broaden as a result of diffusion. This is the essential feature of ITP. Alternatively, if the trailing ions have the higher net mobility, no steady state

will be reached, each boundary migrating at its own characteristic velocity and broadening as a result of diffusion.

Finally, electrodes are present in all forms of electrophoresis. Assuming that the solutes undergo no electrochemical reactions at the electrodes, cations will accumulate at the cathode and anions at the anode, as first studied by Hittorf (11). This is one of the methods whereby a natural pH gradient can be established in IEF, if no, or minimal electrode buffer volumes are utilized. Most other electrophoretic systems comprise large electrode buffer reservoirs, thereby avoiding the necessity of considering these electrolytic changes.

The above considerations encompass the essential features of the various modes in electrophoresis, which are seen to differ only in terms of initial conditions (i.e., presence of concentration gradients and/or species discontinuities) and boundary conditions (i.e., the presence or absence of large electrode buffer volumes). The mathematical model presented in the following pages is applicable irrespective of the imposed initial or boundary conditions.

Model construction

The model was designed to predict the evolution of the four classical electrophoretic modes as a function of time. The modeled system is one-dimensional, and the effects of electroosmosis, temperature gradients, or any bulk flows of liquid are neglected. It was further stipulated that the only electrolytes in the system are monovalent buffers or biprotic ampholytes, such as simple amino acids. The model is based on equations describing the components' dissociation equilibria, the mass transport due to electromigration and diffusion, electroneutrality, and the conservation of mass and charge. The resulting model consists of a system of coupled partial differential and non-linear algebraic equations.

The model is far too complex for an analytical solution and a program was developed for its numerical solution. The equations were put in dimensionless form to provide proper physical scaling (9). Discretization of the spatial derivatives at a set of grid points generated a set of ordinary differential equations with time as the independent variable. The resulting equations were integrated numerically using DARE-P software (12) and the calculations were carried out with a CDC-Cyber-175 processor.

Simulation studies

Our first objective was to verify the versatility of our model to simulate the typical features of ZE, MBE and ITP. A three component system, comprising cacodylate, tris hydroxymethylaminomethane (TRIS), and histidine, i.e., a weak acid, a weak base and an ampholyte, was sufficient for this purpose. The initial conditions for ZE and MBE were similar: histidine was assumed to be the sample, migrating in a buffer of TRIS-cacodylate. For the simulation of ITP, cacodylate was assumed to be the leading ion, histidine the terminator, and TRIS the common counterion. In all three cases, unrestricted permeability of column ends to all ions was specified as the boundary condition. This is the situation prevailing in experimental runs, where large electrode buffer volumes are generally utilized. All simulations were run at constant current, and the model was able to predict the characteristic behavior of each mode, such as Kohlrausch adjustment of terminator concentration, the diffusional spreading of stationary boundaries, etc. Unfortunately, space does not permit us to describe in detail all the simulations performed.

Because of our prior studies with the simulation of the steady state in IEF (8), we were more interested in the simulation of transient states in focusing. Uniform distribution of the same three compounds was stipulated as the initial

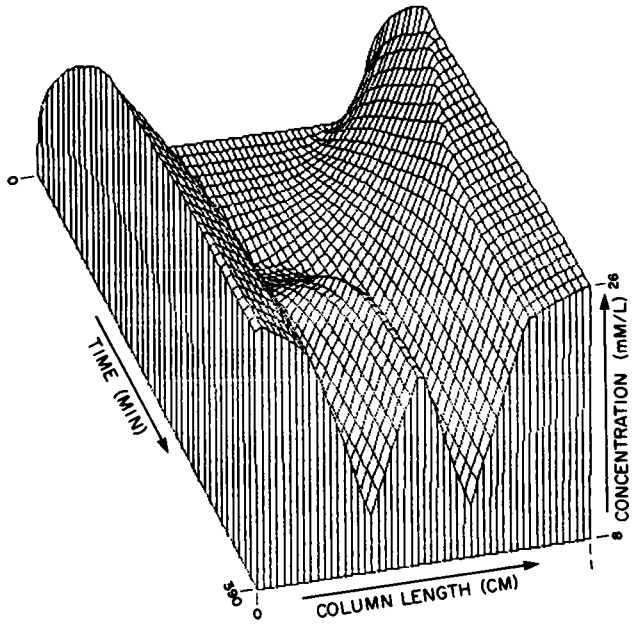

Fig. 1. Time dependent evolution of concentration profiles resulting from isoelectric focusing at constant current. Only the predominant component along the column axis is shown, cacodylate focusing at the left, histidine in the middle and TRIS at the right end of the simulated column.

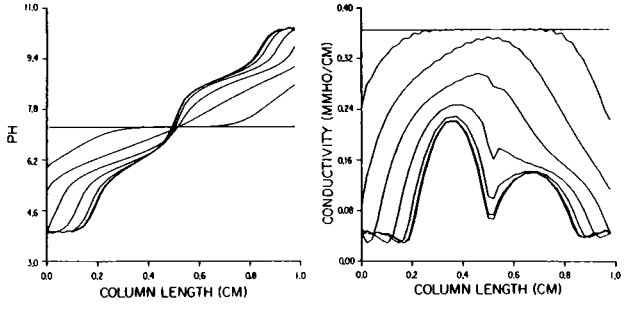

Fig. 2. Plots of pH and conductivity corresponding to the simulation presented in Fig. 1. The horizontal lines in both plots represent the initial condition in the column, as a uniform distribution of all components was specified. The other lines represent the plots at 30, 90, 150, 210, 270, 330 and 390 minutes of focusing.

condition. As the isoelectric point of histidine (pI = 7.60) is intermediate to the pK values of cacodylate (pK = 6.21) and TRIS (pK = 8.30), histidine was expected to focus towards the center of the column. IEF, however, requires a different set of boundary conditions than the other three modes: the ends of the column were assumed to be permeable to hydrogen and hydroxyl ions only. These conditions prevail in experimental flat bed IEF with electrodes in close contact with the gel surface.

Again, a constant current was assumed. The time-dependent progress of focusing is reproduced in the three-dimensional plot in Fig. 1. It can be seen that the focusing starts with the migration of cacodylate and TRIS to the anode and the cathode, resp., followed by the later focusing of histidine in the center of the column. The concomitant changes in pH and conductivity are shown in Fig. 2. The horizontal lines in both plots represent the initial conditions, as uniform initial concentration of all components was assumed. The remaining lines represent the simulation at 30, 90, 150, 210, 270, 330 and 390 minutes of constant current. The pH plots show that the pH gradient is generated at the electrodes and slowly propagate towards the center. The conductivity plots show the development of three 'conductivity gaps' corresponding to the accumulation of relatively undissociated acid and base at the electrodes, and of isoelectric histidine in the center of the column. Most gratifying, however, was to find that the predicted profiles of concentration, pH and conductivity, derived from the transient model, matched those previously derived from our older steady state model. This verifies the numerical integrity of the two programs.

Discussion

Our mathematical model was found useful for computer simulation of the four classical modes of electrophoresis, i.e., ZE, MBE, ITP and IEF. It not only correctly predicts the characteristic features of each mode, but gives details of concentration, pH and conductivity profiles not easily amenable to direct experimental measurement. While the present program is limited to simple chemical systems, ongoing work is directed to its expansion to more complex mixtures. The practical applications of such a model are somewhat limited by the paucity of reliable data on components dissociation constants and mobilities. It is well known, in particular, that anionic and cationic ampholyte subspecies differ in their mobilities, but exact data are virtually unavailable.

We wish to propose that our model provides the basis for a rational classification of all electrophoretic techniques, and is not limited to ZE, MBE, ITP and IEF. These four classical modes differ only in terms of the initial distribution of components along the column axis and ionic permeabilities of column ends, i.e. the previously described Kohlrausch-Hittorf constraints. These two conditions define an 'electrophoretic plane', in which the classical modes are but idealized points within a virtual infinity of possible conditions. To model the manifold electrophoretic techniques in actual usage, such as immunoelectrophoresis or density gradient gel electrophoresis, other constraints have to be incorporated. Some of these constraints are presented schematically in Fig. 3. Accordingly, a technique is characterized by its location within the illustrated multidimensional 'electrophoretic space'. Our model can easily be adapted to encompass those additional non Kohlrausch-Hittorf constraints which involve only changes of transport characteristics of the molecules. For instance, to model molecular sieving in porosity gradients, the mobility of the components would have

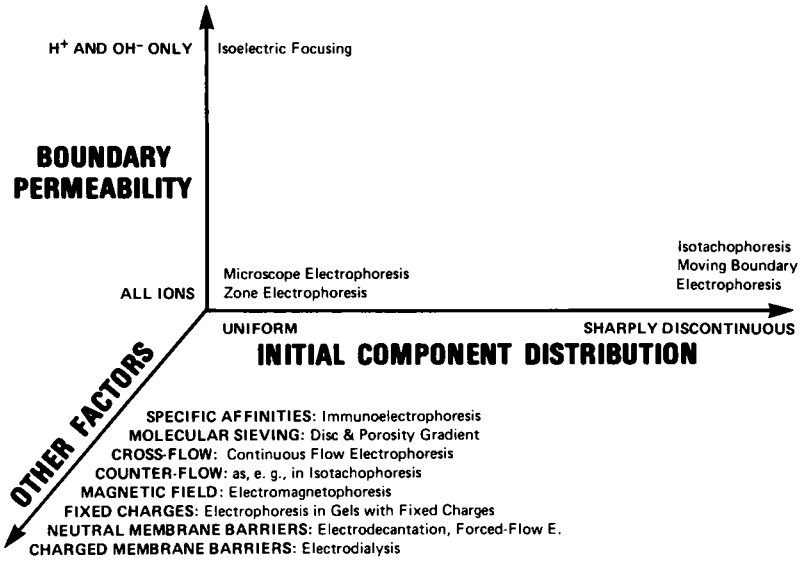

Fig. 3. A graphic representation of the suggested classification of electrophoretic processes. For an explanation see text.

to be made a function of their location along the column axis. We have already modeled ITP in gels with immobilized counterions, as well as electrodialysis, i.e. the transport of ions across a membrane incorporating fixed charges. In both instances, it was sufficient to assign a zero mobility to the stationary species. On the other hand, some of the other constraints, such as incorporation of cross-flow fluid motion (13), as in continuous flow electrophoresis, would be far more difficult. Thus, we foresee that our model may be most useful in evaluating the possibility of developing new separation techniques involving conditions not yet tried in the laboratory, or even of conditions which may not be easily obtainable in practice. An example of such conditions would be the

modification of boundary conditions, rendering the ends of the column permeable to selected ions only.

Acknowledgements

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ISOELECTRIC FOCUSING IN IMMOBILIZED pH GRADIENTS. I: PRINCIPLE AND METHODOLOGY

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Introduction

In 1966 LKB introduced the first commercial ampholyte mixture, based on Rilbe's isoelectric focusing (IEF) theory (1). Since then the technique of IEF in carrier ampholyte generated pH gradients has developed to one of the most commonly used analytical and preparative methods in work with amphoteric molecules such as proteins and peptides. The resolution given by analytical IEF is amongst the highest available from present biochemical separation techniques.

Sixteen years of experience with IEF show that, notwithstanding the extreme success of the method, the carrier ampholyte based technique has certain inherent limitations and problems. The problem most frequently discussed in the literature is the so called "cathodic drift" (2) or "plateau phenomenon" (3-5), that is to say the continuous slow change of what was expected to be a stable pH gradient with time. This causes problems mainly when narrow pH gradients are used, especially at high pH values, where the drift is most pronounced. In experiments using a matrix for convectional stabilization, a large part of these phenomena could be ascribed to electroendosmosis caused by minor amounts of charges bound to the matrix (6), but there

Electrophoresis '82

also exist good reasons to believe that it is partly connected with more basic features of the carrier ampholyte pH gradient. Therefore IEF, as it is commonly used today, is a non-equilibrium method. One of the consequences of this is that the pH gradient obtained in an experiment depends on the salt content of the sample. To avoid displacements of the pH gradient in the anodic or cathodic directions, either the salt content or the sample volume must be kept small. In flat bed IEF runs, with a number of parallel sample tracks separated by sample-free zones, the effect of high salt concentrations, as a consequence of diffusional mass transport perpendicular to the focusing direction, can be seen as distorted zones (7). Conventional IEF can give curved zones also for a number of other reasons, such as skewed end-electrolyte distribution, uneven cooling and bad contact between the platinum wires and the electrode strips in flat-bed techniques. In no case introduced disturbances are eliminated as a result of focusing and, if large amounts of persulphate have been used for polymerization, disturbances even seem to be amplified (6).

Another inherent weakness of the carrier ampholyte concept is that the pH gradient is generated with the aid of a large number of amphoteric compounds. With techniques available today, it could neither be guaranteed that these compounds are present in equal amounts nor that essential properties, such as buffering capacities, are identical. There exists overwhelming evidence for the fact that a large number of the individual species in commercial carrier ampholytes are focused as sharp zones (8). As a consequence of this, not only the buffering capacity but also the ionic strength, which directly influences pK- and pI-values, might vary in a dramatic manner along the pH gradient. The schlieren pattern (9) resulting in polyacrylamide gels containing focused carrier ampholytes is a clear indication of the fact that the conditions in the pH gradient vary.

From what has been described above, present day IEF techniques show certain limitations and problems; therefore, there is a need for a complementary technique solving some of the above problems. Different possibilities to create stable pH gradients without carrier ampholytes have also been tried (10-12) but no work, except the LKB patent by Gasparic *et al.* (13), has until now been published on what might seem as the most obvious possibility, namely to bind the buffering groups generating the pH gradient to the ma-

trix used for conventional stabilization. There is probably a number of reasons for this. Thus, it might seem unrealistic that sufficient conductivity would result in a gradient of this type; moreover, bound charges could result in electroendo-osmosis and the ion-exchange character of the matrix might lead to binding of proteins. On the contrary, we have found that the demand on electric conductivity is relativey small and that neither electroendo-osmosis nor binding creates any problem, and thus any matrix suitable for generating the necessary conventional stabilization in IEF could be modified to generate an immobilized pH gradient suitable for IEF.

The present paper will describe the production and use of immobilized pH gradients made with the aid of Immobilines™. These are a number of buffering acryloil monomers developed by LKB Produkter AB for the purpose of immobilizing pH gradients in polyacrylamide gels. This work as well as the following ones (14, 15) will show that the use of Immobiline™ based pH gradients not only solves the problems associated with pH drift and distorted protein bands, but also allows isoelectric focusing under controlled conditions with increased resolution and loading capacity compared to what can be achieved with carrier ampholyte generated pH gradients.

Materials and Methods

Apparatus. The IEF experiments were carrier out using the LKB Ultrophor apparatus together with the LKB 2197 constant power supply and for cooling the LKB 2209 Multitemp. For pH measurements, a LKB 2117-111 surface pH electrode was used, together with a Beckmann model 3500 digital pH meter.

Chemicals. Immobilines™, acrylamide, N,N'-methylene bisacrylamide, ammonium persulphate, TEMED, Repelsilane and Coomassie Brilliant Blue R-250 were all from LKB Produkter AB. Sulfosalicylic acid and trichloroacetic acid were from Merck AG. All the standard proteins used were purchased from Sigma. All other chemicals were of analytical grade.

Gel casting. The gel casting has been made according to Görg *et al.* (16) using the LKB 2117-901 Gradient Gel kit. The gels were 250x110x0.5 mm and, for sample application, they contained slots of the size 5x2x0.2 mm. Before use, the U-frame with the slots was coated with Repelsilane to avoid that

the gel could stick to the glass surface when opening the cassette. Acrylamide and N,N'-methylene bisacrylamide have been used as separate stock solutions with the concentrations 29.1% (w/v) and 0.9% (w/v), respectively. The chambers of the gradient mixer were filled with 7 ml of each solution containing acrylamide and Bis corresponding to a 5%T, 3%C gel and Immobilines^{Tm} in concentrations adjusted to give the desired pH gradient. The acidic solution was always chosen as heavy solution and contained 20% (v/v) glycerol. The gel solutions were not degassed as the gradient mixer was used with both chambers open. The catalysts (5 µl TEMED and 5 µl of 40% persulphate per chamber) were added directly to the gradient mixer immediately before filling the gel in the cassette. The gels were polymerized for one hour at 50°C (17). After removing the gels from the cassette they were placed in one liter distilled water for half an hour whereafter excess water was allowed to drain from the vertically standing gels for another half hour at room temperature. The drying step is essential as a gel containing too large amounts of water will sweat in the focusing step giving water droplets on its surface. These droplets result in blue spots when the gel is stained as described below.

Running conditions. In most experiments the electrodes have been applied directly on the gel surface, alternatively 10^{-2}M H_3PO_4 and NaOH could be used as electrode solutions.The samples were applied without pre-running, with the power set at 10 W, the voltage at 2500 V and the current at 25 mA. Focusing was continued overnight at 10°C for any given pH range selected. With pH values higher than 7, two strips of absorbent cloth 200x20x5 mm were soaked in 1M NaOH and placed inside the safety lid of the 2217 Ultrophor chamber in order to minimize the influence of CO_2. The gels were fixed, stained and destained as with conventional carrier ampholytes PAG-plates using Coomassie Brilliant Blue R-250 (18).

Results

1) General physico chemical properties of Immobiline^{Tm}

The Immobilines^{Tm} are acrylamide derivatives with the general structure (where R contains either a carboxyl or a tertiary amino group):

TABLE I

PROPERTIES OF IMMOBILINES™

Apparent pK-values[1] $I = 10^{-2}$

	H_2O		Polyacrylamide gel[2] T=5% C=3%		Polyacrylamide gel[2] T=5% C=3% Glycerol 25%-w/v		Physical state at room temperature
	10°C	25°C	10°C	25°C	10°C	25°C	
Acids with carboxyl as buffering group							
Immobiline pK 3,6	3,57	3,58	-	-	3,68±0,02	3,75±0,02	solid
Immobiline pK 4,4	4,39	4,39	4,30±0,02	4,36±0,02	4,40±0,03	4,47±0,03	solid
Immobiline pK 4,6	4,60	4,61	4,51±0,02	4,61±0,02	4,61±0,02	4,71±0,03	solid
Bases with tertiary amine as buffering group							
Immobiline pK 6,2	6,41	6,23	6,21±0,05	6,15±0,03	6,32±0,08	6,24±0,07	solid
Immobiline pK 7,0	7,12	6,97	7,06±0,07	6,96±0,05	7,08±0,07	6,95±0,06	solid
Immobiline pK 8,5	8,96	8,53	8,50±0,06	8,38±0,06	8,66±0,09	8,45±0,07	liquid
Immobiline pK 9,3	9,64	9,28	9,59±0,08	9,31±0,07	9,57±0,06	9,30±0,05	liquid

[1] pK-values measured with glass surface electrode given without any corrections.

[2] Mean-values of 10 determinations. Due to the slow response of the electrode the pK-values for the amines are uncertain.

$$CH_2 = CH - \underset{\underset{O}{\|}}{C} - \underset{\underset{H}{|}}{N} - R$$

Provided that the correct polymerization conditions are chosen, these monomers are efficiently incorporated in the gel. As a result of their presence, the gel (in the absence of other compounds taking part in protolytic equilibria) will have a pH defined by the concentrations and dissociations constants on the Immobilines^{Tm}. The gel will also have a conductivity not only due to H^+ and OH^- ions, but also connected to the amount of incorporated Immobilines^{Tm} and their buffering capacity.

Table I lists the relevant physico chemical data of Immobiline^{Tm}: at present three acids and four bases are available, with pK's spanning the pH range 3.6 to 9.3. Immobiline^{Tm} pK 9.3 has been used as counter ion for determination of the pK values of the acids; conversely, Immobiline^{Tm} pK 3.6 has been used for the pK determination of the bases. pK measurements in gels have been performed by including 10^{-2}M buffering Immobiline^{Tm} and $5x10^{-3}$M Immobiline counter ion in the solution used to generate a gel of the size 240x110x1 mm. For catalyst removal, the gel was electrolyzed on the Ultrophor overnight and then the pH was measured. All pH measurements were made with a glass surface electrode calibrated with NBS standard buffers. The distance between the double bond and the group taking part in the protolytic equilibrium has in all cases been chosen long enough so that the influence of the double bond on the dissociation constant can be neglected. As a result, the pK difference between the Immobiline^{Tm} monomer and the buffering group fixed in the immobilized pH gradient when used for IEF is mainly due to media effects and temperature variations.

2) Band eveness

As can be seen from Fig. 1, the present technique gives straight iso-pH lines covering the whole width of the gel. In order to obtain this type of result it is essential that the pH gradient is approximately linear and the buffering power even. The result shown in Fig. 1 is also typical in that no distorted protein bands result when using Immobiline^{Tm} gels. This will be true also when the sample contains large amounts of salt. If the salt content in the samples is high and variable, protein bands from samples with high conductivity will spread out over an appreciably wider part of an Im-

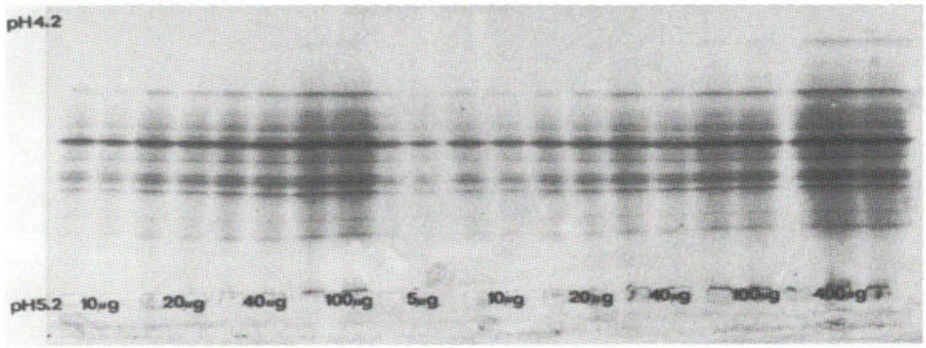

Fig. 1. Varying amounts of ovalbumin focused in an Immobiline^{Tm} gradient pH 4.2-5.2. Immobiline^{Tm} concentrations used for gradient mixing: acidic solution: $5x10^{-3}$M Immobiline^{Tm} pK 4.6 and $1.4x10^{-3}$M Immobiline ^{Tm} pK 9.3; basic solution: $5x10^{-3}$M Immobiline^{Tm} pK 4.6 and $4x10^{-3}$M Immobiline^{Tm} pK 9.3. Running conditions: overnight at 250 V/cm and 10°C.

mobiline^{Tm} gel than what corresponds to the width of the application zone. Thus, when running this type of samples it is important to use individual gel strips for each sample track.

3) Resolution

The band width and resolution obtainable in Immobiline^{Tm} pH gradients are comparable to conventional carrier ampholyte pH gradients. Thus narrower pH gradients decrease the band sharpness, but the resolution is increased. Fig. 2 shows the results of electrofocusing ovalbumin in a carrier ampholyte pH gradient with an approximate slope of 0.2 pH units/cm and in Immobiline pH gradients with the following slopes: 0.1, 0.02 and 0.01 pH units/cm, respectively. In the two last experiments, part of the gels were cut off and stained after 16 hours in order to localise the position of the main band. After 18 hours the distance between the electrodes was shortened to 5 and 2.5 cm, respectively, to allow delivering 500 V/cm to one gel and 1000 V/cm to the other. The experiment was then continued for 3 more hours. In going from 0.1 pH units/cm to 0.02 pH units/cm, the ovalbumin bands are resolved into doublets. With the slope 0.01 pH units/cm, the distance between the bands in the doublets is approximately 2 mm, which should correspond to a pI difference of 0.002 pH units. From this we estimate that it should be

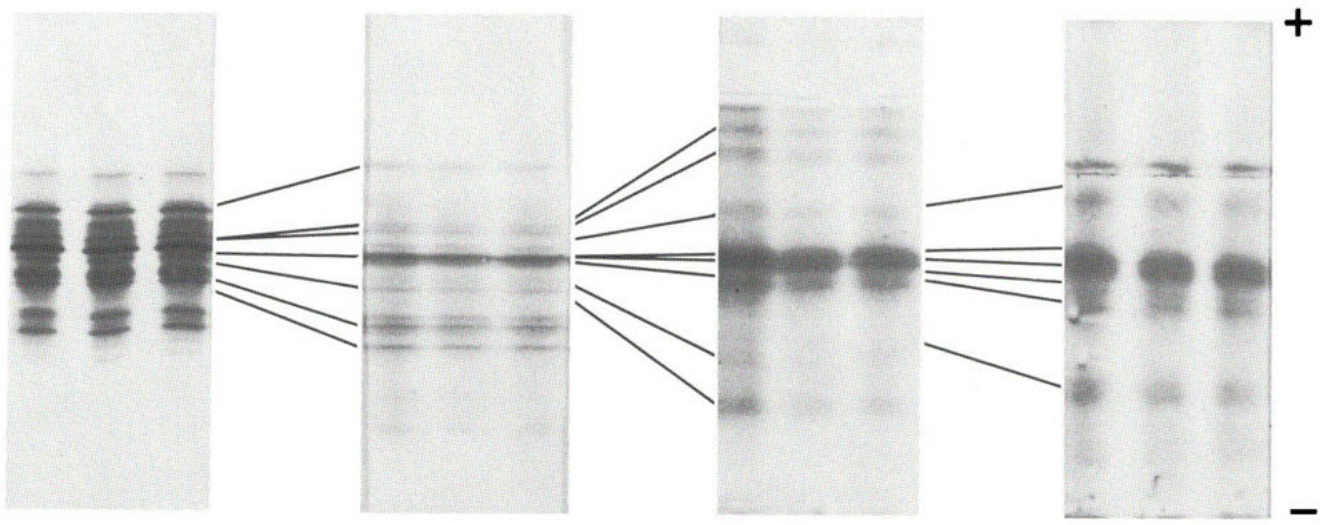

Fig. 2. Ovalbumin focused on a narrow Ampholine pH gradient (A) and on Immobiline^{Tm} gradients with varying pH slopes (B-D). Strips B-D contain $5x10^{-3}$M Immobiline^{Tm} pK 4.6 titrated with Immobiline^{Tm} pK 9.3 to the respective pH slopes. Ovalbumin load in the sample tracks (from left to right): 40, 20 and 20 µg.

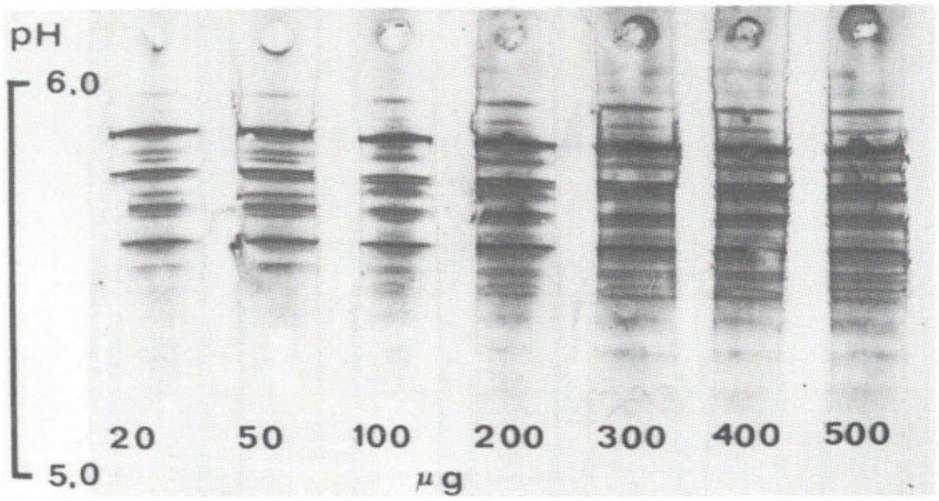

Fig. 3. Varying amounts of carbonic anhydrase focused on Immobiline^{Tm} gels in the pH range 4.3-6.8. Acidic end concentrations: $10x10^{-3}$M Immobiline pK 4.3 and $5x10^{-3}$M Immobiline^{Tm} pK 6.2; basic end concentrations: $3x10^{-3}$M Immobiline^{Tm} pK 4.3 and $10x10^{-3}$M Immobiline pK 6.8.

possible to resolve bands differing by as little as 0.001 pH units. From the early work of Vesterberg and Rilbe (19) the resolution limit for carrier ampholyte gradients has often been given as 0.02 pH units. In reality even slightly higher resolution is possible as long as the pH drift and conductivity gaps do not interfere if narrow pH ranges and high field strengths are used. Allen *et al.* (20) have as an example claimed the resolution of α_1-antitrypsin bands to be of the order of only 0.005 pH units in a narrow Ampholine pH gradient.

4) Loading capacity

Fig. 3 shows the effect of loading increasing amounts of carbonic anhydrase on 110 mm long, 10 mm wide and 0.5 mm thick Immobiline^{Tm} pH gradient strips. Up to 500 µg can be loaded on a gel strip of this size, which is appreciably more than what can be loaded on a carrier ampholyte gel. The low current in Immobiline^{Tm} pH gradients makes it possible to increase the gel thickness 5-10 times without problems with the cooling capacity. As also narrower pH ranges increase the loading capacity, it should be possible to handle more than 100 mg in one run in a gel with an area corresponding to

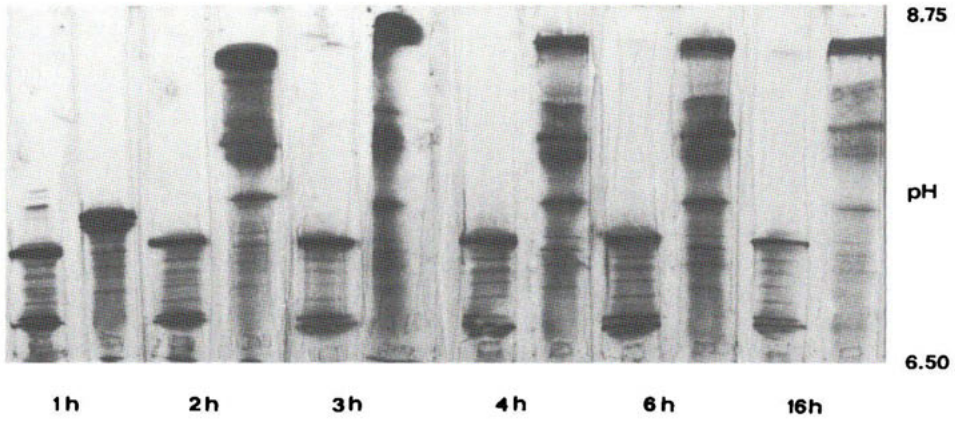

Fig. 4. Horse heart and sperm whale myoglobins, in alternate tracks, focused for varying times on an Immobiline^{Tm} gel, pH 6.5-8.75. The gradient was created as follows: a mixture of 2.5×10^{-3}M Immobiline^{Tm} pK 6.2, 5×10^{-3} M Immobiline^{Tm} pK 7.0 and 5×10^{-3}M Immobiline^{Tm} pK 8.5 was titrated with Immobiline pK 3.6 to the stated pH interval (pH 6.5 in the dense and pH 8.75 in the light gradient solutions).

an Ampholine PAG plate (110 x 265 mm): thus Immobiline^{Tm} pH gradients are clearly of interest in preparative runs. In this connection Immobiline^{Tm} gels are also advantageous because they do not contain any other extractable components than the focused proteins. Moreover the band position can be easily localized after focusing without any staining as ridges appear in the gel at the positions of focused proteins when large protein loads are used. It is thus very easy to cut out the parts of the gel containing the protein components of interest.

5) Behavior of proteins. pH gradient stability

Due to the low conductivity of Immobiline^{Tm} pH gradients, the sample components will show a different behavior than on a carrier ampholyte pH gradient. If the sample is introduced in the pH gradient after the unbound ions have been electrolysed away, the conductivity of the sample will be high compared with the background conductivity and the proteins will stay at the application area for a long period of time, with an increased risk of precipitates. It is thus normally preferable to apply the sample without pre-running the gel, so that it can move together with the salt background present in the gel plate. When coloured proteins are used, they can be seen

as sharp bands moving with the refractile line marking the rear border of an ionic compound moving towards an electrode. The protein band stops as it reaches the pH zone in the gel where it is isoelectric, becoming rather diffuse and then sharpening again as the focusing is continued.

The time needed by a protein to reach its pI position depends on a number of factors: slope of its titration curve around its pI, slope of the immobilized pH gradient, voltage gradient applied, medium viscosity, etc. Once a protein has reached its equilibrium position in an Immobiline^{Tm} gradient, however, it will stay in this position indefinitely. Fig. 4 shows the behavior of sperm whale and horse heart myoglobins in a pH 6.5 to 8.75 Immobiline^{Tm} gradient. The proteins are focused in four hours and after 16 hours at 250 V/cm they are still in the same position. Under these conditions most proteins,in conventional carrier ampholyte systems,would have drifted out in the cathodic compartment (21).

Discussion

A comparison of IEF in Immobiline^{Tm} pH gradients and in carrier ampholyte generated pH gradients shows a number of advantages for the Immobiline^{Tm} gel:

a) true equilibrium method - no drift;
b) higher resolution;
c) higher loading capacity;
d) better control of form, width, ionic strength and buffering capacity;
e) possibility of generating extremely shallow pH gradients;
f) easier separation of buffering species from proteins in preparative runs;
g) insensitivity to salts and buffers in the sample.

IEF with good quality carrier ampholytes is still an excellent analytical method which in most cases gives results which correspond to the demands of the user. Immobiline^{Tm} pH gradients require longer focusing time and, although it is easy to cast a gradient gel, it is still easier to cast a carrier ampholyte containing gel or use a ready-made PAG plate. Thus, for most routine uses, a carrier ampholyte based pH gradient is still the natural choice and Immobiline^{Tm} should be used mainly when its advantages are

needed. IEF in ultra-narrow pH gradients when very high resolution is required is an obvious case when Immobiline™ pH gradients ought to be used. This type of application is further exemplified and discussed in the second and third articles of this series (14, 15).

In order to detect the presence of minor components in a sample, it is often necessary to heavily overload major components, which disturb the IEF process in carrier ampholyte pH gradients. The high loading capacity of Immobiline™ pH gradients makes them the natural choice also for this type of application. The situation described frequently appears in two-dimensional applications using isoelectric focusing as first dimension. The other advantages with Immobiline™ pH gradients, such as insensitivity to disturbances and ease of control of form and width of pH gradients, are also of major importance to 2-D techniques and thus Immobiline pH gradients should be preferred as first dimension in 2-D runs. This important application is exemplified and described in detail in the third article of this series (15).

In the not too uncommon case when the samples contain very large amounts of salt, it should be more convenient to use Immobiline™ pH gradient gels than to first remove the salt before applying the samples to a conventional isoelectric focusing gel. Due to the fact that none of the naturally occurring amino acids have side chains with groups buffering in the pH range 7.5-9.5, many proteins isoelectric in this range will approach their pI relatively slowly. This phenomenon can be directly visualized with the aid of electrophoretically run titration curves on prefocused Ampholine pH gradients (22). Carrier ampholyte pH gradients in the pH range 7.5-9.5 are characterized by a fairly large pH drift, especially in the absence of urea (23). The combination of the slower focusing and the pH drift can result in less sharp bands and bad focusing also in pH gradients as wide as two pH units. In this case Immobiline™ gradients represent a valid alternative.

Another field in which the use of Immobiline™ should be of great interest is in preparative isoelectric focusing. The high load capacity, the ease with which a narrow, specially designed pH gradient can be generated and the fact that the problem of separating the focused proteins from the carrier ampholytes is avoided, clearly ought to make the Immobiline™ pH gradient attractive for preparative work.

To state that Immobiline™ pH gradients revolution the art of isoelectric

focusing is perhaps too strong, but quite clearly it is a technique with large future possibilities. Immobiline™ pH gradients are complementary to carrier ampholyte gradients and will certainly increase the usefulness of IEF as an analytical and preparative tool in biochemistry.

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