ELECTROPHORESIS

THEORY, METHODS, AND APPLICATIONS

Edited by

MILAN BIER

Electrophoresis

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PREFACE

The contribution of electrophoresis to our knowledge of proteins is second to no other method. Its impact is felt in biochemistry, physiology, and medicine.

Recent years have witnessed a broad development of new experimental techniques and their application to a variety of other substances. It appeared therefore, that an authoritative presentation, dealing with the theory, methods, and applications in their proper perspective would fill a gap in the existing literature. It is to be hoped that this has been accomplished in the present collaborative endeavor, and that the collected essays will be of value not only to experimental and theoretical workers in the field but also to all researchers concerned with broader aspects of proteins, polyelectrolytes, and colloids in general.

The emphasis in the exposition is on the fundamental principles involved in electrophoresis, the problems encountered therein, and the means of solving them. Detailed laboratory procedures are included inasmuch as they serve to illustrate these problems. All major areas of application were covered, encompassing clinical medicine, studies of proteins, viruses, bacteria, and other biological materials, analytical use in organic and inorganic chemistry, and preparative and industrial uses.

The editor takes pleasure in thanking all contributors for their arduous collaboration, and in acknowledging the advice especially of Dr. L. G. Longsworth in the preparation of the book.

M. Bier

New York City
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Introduction

ARNE TISELIUS

The term "electrophoresis" or "cataphoresis" was originally introduced to mean the migration of charged colloidal particles in an electric field. Just as the definition of a colloid, and particularly the distinction between colloidal and molecular systems, necessarily is rather vague, there has been some diversity of opinion about how wide use the term "electrophoresis" should have. This has become particularly evident in the many applications in chemistry (particularly biochemistry) of the migration of substances in an electric field. Some authors apparently prefer the term "ionophoresis" for substances of lower molecular weight, particularly when the experiments are made in stabilized media such as gels and powders. This term has even sometimes been used to distinguish such stabilized migration from migration experiments in free solutions, which does not appear logical.

During the last decade there has been a particularly rapid development in the application of methods of this type, covering the whole field from the largest protein molecules down to, for example, amino acids, sugars, purines, pyrimidines, and even simple inorganic ions, using essentially the same types of apparatus and procedures, irrespective of the molecular size of the substances studied. For this reason—and possibly also for historical reasons—most research workers in the field seem to prefer the well-established term "electrophoresis" as a common name for all these procedures, especially if separation problems are the chief purpose of the investigation. The situation is somewhat analogous to the use of chromatography, which nowadays is certainly not limited to colored substances.

In the physicochemical theory of the migration of small ions there is, according to Debye-Hückel, an "electrophoretic" factor which depends upon the diffuse double layer in the ionic atmosphere surrounding each ion. The role of this factor becomes the more pronounced, the larger the size of the ion and the higher the ionic strength of the solution. There is thus a continuous transition from free ionic migration to electrophoretic phenomena, where surface potentials and double layer dimensions are decisive.

From the very beginning of electrophoretic studies on colloids, the relationship between the mobility and the surface charge or surface potential

came into the foreground. The electrophoretic migration could be studied with relatively simple means and gave a convenient way of determining these properties, which are of fundamental importance for the stability of many colloidal systems, especially those of a hydrophobic type. The early investigations by Picton and Linder (1, 2), Hardy (3, 4, 5), Ellis (6), and Powis (7) give examples of this type of work. The discovery by Hardy that many biocolloids, like proteins, enzymes, etc., show characteristic electrophoretic mobilities, which largely depend upon the pH of the solution, greatly stimulated the interest in electrophoretic work (3, 4, 5). Now the *characterization* of such substances by means of their electrophoretic properties, and particularly of their isoelectric points, began to attract the interest.

An early example is the classical work of Michaelis (8), who determined the isoelectric points of enzymes by migration experiments at different pH's. It is interesting that this characterization could be done before pure enzymes were available: Michaelis determined the direction of the migration in a U-tube by analyzing the two limbs of the tube for enzymatic activity after current had passed through.

At that time very little was known about the chemical nature and physical properties of proteins and enzymes. It is only natural that their electrophoretic properties aroused interest, since these properties appeared to be to a certain extent characteristic and lent themselves to quantitative studies, even though accurate measurements proved to be difficult to make.

Since then electrophoretic methods have been successively refined. The conditions for disturbance-free migration have been established, and accurate quantitative measurements of migration velocities in different media have been made for a large number of biologically important substances, particularly for proteins. One important method depends upon the study of the migration of the boundary of a protein dissolved in a buffer solution of accurately defined pH and layered below a buffer solution of the same composition (the "moving boundary method"). The introduction of highly sensitive optical methods for observing the migration of the boundaries and of their resolution into several components in complex systems not only facilitated accurate mobility measurements but also opened up possibilities for an "electrophoretic analysis" of mixtures. Such systems are very frequently encountered in biochemical work, where the substances are obtained from extracts of biological material. The improved methods gradually led to results which demonstrated the high degree of specificity of the electrophoretic properties. Substances which had been believed to be reasonably well defined by common criteria, e.g. crystallization, sometimes were found to yield two or more components in electrophoretic

analysis, and the method has now found wide use as one important criterium of purity for substances which are otherwise difficult to define.

The information derived from electrophoretic investigations has been considered particularly reliable in such cases on account of the gentleness of the method. The application of other separation procedures, involving precipitation or the use of nonaqueous solvents or other aggressive chemicals, may easily cause damage to substances as unstable as are many biocolloids. Besides this, coprecipitation and adsorption phenomena are apt to interfere in fractionation work by such methods when one deals with crystals or precipitates of substances of large molecular weight. In electrophoretic separation (as in ultracentrifugation and similar "transport methods") the migrating substances remain in the same medium during the entire process and the observation method itself is apt to give warning of eventual irreversible changes accompanying the separation.

The diversity of electrophoretic behavior among biochemically important substances is very gratifying in relation to separation. It is evidently unusual for two different proteins to show the same mobility over the entire pH range accessible for investigation, and even small differences in migration may be utilized with the modern methods of investigation.

Thus the *separation* aspect has come into the foreground to an increasing extent. Electrophoretic analysis by the moving boundary methods, using refractive index observation, gives a high resolution and is suitable for characterization of the observed components by their mobilities, which can be measured under well-defined conditions. The method gives, however, only a boundary separation, and at the end of an experiment the components are only partially separated. Only some of the fastest and slowest components can be taken out of the electrophoresis tube. Thus, this type of electrophoretic analysis serves the investigator more as a guide for separation by other procedures than as a separation method in itself. To a certain extent there is thus an analogy to the use of spectral analysis in the purification of metals.

The problem of a real electrophoretic separation "in substance" thus became an important issue. It appeared very tempting to utilize the efficient separation, which can be observed optically in the moving boundary methods, in order to isolate the components for further investigation of their chemical and biochemical properties and eventually also to work out methods suitable on a preparative scale. The overlapping "boundary separation" had thus to be substituted by a "zone separation" in which each component was allowed to form a zone separated from the others by empty regions. This, however, necessitated a radical change in the method. Zones of this kind are gravitationally unstable and would give rise to convections

in the ordinary electrophoresis tube. To avoid this, the tube must be packed with a stabilizing medium, like starch, cellulose, or glass powder, or the experiment must be run in a gel or in a strip of filter paper.

However, it was not only the need for complete separations which gave rise to this development. Several successful early attempts had been made to run electrophoresis experiments in gels or strips of filter paper, for which only a very simple apparatus is needed and very small samples of the material to be investigated are required. The development of paper strip electrophoresis obviously received a great stimulus from the great success of paper chromatography and from the increasing need for a simple and rapid micromethod for electrophoretic analysis of sera in the clinic.

We have in Uppsala suggested the name "zone electrophoresis" for methods of this type, to distinguish them from "boundary electrophoresis" of the kind referred to above. Zone electrophoresis may be performed in stabilizing media of different kinds (or in density gradients) in columns or troughs of various types, and there are also constructions allowing continuous separation of large quantities. Paper electrophoresis is related to these methods very much in the same way as paper chromatography to column chromatography.

Experience has shown that the zone methods can be applied also to low molecular weight substances, like peptides, amino acids, and nucleotides, which cannot easily be studied by boundary electrophoresis. Thus a considerable extension of the use of electrophoretic analysis has taken place, and the method often can compete with chromatography, for example in the fractionation of protein hydrolyzates. It cannot be helped, however, that in introducing carrier systems, necessary for the stabilization of the zones in zone electrophoresis, some of the possibilities of quantitative mobility determinations have to be sacrificed. The stabilizing material is present in an enormous excess over the amount of substances studied, and interactions influencing mobilities in an uncontrollable way can hardly be avoided, even though attempts are being made to find as inert filling materials as possible. Paper electrophoresis experiments are also subject to other complications, e.g. by evaporation from the strip, which is heated by the current passing through. In such cases one has to refer the observed migration to that of known substances in parallel experiments, run simultaneously.

It is only natural that if the purely preparative or separation aspects dominate the purpose of the experiment one is ready to make a sacrifice of some of the quantitative interpretations, especially if very high resolution can be obtained. However, the possibility of identification of new components, visible as boundaries or zones, by their mobilities has always been an attractive feature of the electrophoretic methods. It is therefore

to be hoped that such procedures, where other phenomena interfere—and perhaps even contribute to the separation efficiency—can also be understood in all their details so that a rational prediction of separation efficiency in such systems can be made.

Already in the early history of electrophoresis, attempts were made to construct separation apparatus of considerable capacity. Multicompartment apparatus with membranes have been used with success, and also other types, where the migration is followed by suitable analytical methods. Another type of procedure—electrophoresis convection—has been worked out by Kirkwood and his collaborators and appears to be very efficient for the preparative separation of larger quantities of material.

The microscopic method, where the migration of colloidal particles is observed directly with a microscope, has played an important role in the study of the fundamental physicochemical laws governing electrophoretic phenomena. This method has possibilities also in the characterization of biological materials, and there are some very interesting recent results by this method in the characterization of bacterial surfaces.

This introduction has dealt chiefly with the application of electrophoresis to biochemical substances in aqueous solutions, usually in buffered media of defined pH, and there is no doubt that such work dominates the field today. One should not forget, however, the possibilities of influencing the electrophoretic properties by other components in the medium, for example ions other than H^+ and different complex-forming agents. The separation of sugars and polysaccharides in media containing borate forms a beautiful illustration of the application of this principle.

Electrophoresis in nonaqueous solvents has been explored very little. Naturally the electrical conductivity in such media is governed by laws different from those valid in aqueous solutions, and the conductance often is very low. This, on the other hand, may be compensated for by the possibility of applying high voltage without incurring disturbingly large heating effects as the current will be low. Studies of such systems would no doubt be well worth undertaking.

The development of electrophoretic methods and their application form an unusually beautiful example of the fruitfulness in the application of physical methods to biochemical and biological problems. It is to be hoped that future development—and much remains to be done—will benefit from a similar intimate collaboration between representatives of different fields of science.

References

1. H. Picton and S. E. Linder, *J. Chem. Soc.* **61**, 148 (1892).
2. H. Picton and S. E. Linder, *J. Chem. Soc.* **71**, 568 (1897).

3. W. B. Hardy, *J. Physiol.* (London), **24**, 288 (1899).
4. W. B. Hardy, *Proc. Roy. Soc.* **66**, 110 (1900).
5. W. B. Hardy, *J. Physiol.* (London), **33**, 251 (1905).
6. R. Ellis, *Z. physik. Chem.* (Leipzig) **80**, 597 (1912).
7. F. Powis, *Z. physik. Chem.* (Leipzig) **89**, 186 (1915).
8. L. Michaelis, *Biochem. Z.* **16**, 81 (1909).

CONTENTS

Chapter 1

Electric Potentials in Colloidal Systems

J. TH. G. OVERBEEK and J. LIJKLEMA

I. Introduction

Electrophoresis is the movement of charged particles suspended in a liquid, under the influence of an applied electric field. The usual aims of electrophoresis experiments are the obtaining of information on the electrical double layers surrounding the particles, the analysis of a mixture, or its separation into components.

The first aim requires a theory connecting the electrophoretic velocity with fundamental quantities related to the double layer, such as electric potential, charge, and structure. It is the purpose of this chapter to treat the meaning of the notions charge and potential of the double layer, to

show clearly where confusion with relation to these concepts might arise, and to treat their interrelation with electrophoresis and other electrokinetic phenomena.

The theoretical treatment of the electrical double layer depends on its geometry. The most simple case, *viz.*, the double layer at a flat interface will be treated first. With this example, already many facts connected with double layers can be explained. Later in this chapter attention will be paid to more complicated systems: spherical particles, polyelectrolytes, and proteins.

II. General Considerations on the Structure of the Boundary Layer

The boundary between two phases is not a mathematical plane, but a layer of finite dimensions. The properties of the two adjacent phases change gradually over a certain distance. These changes depend both on the forces between molecules (van der Waals' forces, purely electrostatic ones, hydrogen bonds, etc.) and on geometrical factors (fitting of the two surface layers onto one another). Even in a one-component system the density and orientation of the molecules change gradually when going, for example, from the liquid to the gas phase. This phenomenon has been treated extensively by Bakker (1) in his theory of capillarity. In a multicomponent system the concentrations in the boundary layer are as a rule different from those in bulk, thus leading to what is commonly called *adsorption*.

In most cases the changes near the phase boundary are limited to a very few layers of molecules, but there all properties of the phases are changed, including structure, density, composition, dielectric constant, viscosity, etc. If one or both phases contain ions, the transition layer may be much more extended. In this case it frequently occurs that one type of ion is strongly concentrated at the phase boundary by short-range forces. Ions with a sizable hydrophobic part, like those of detergents, although reasonably soluble in water, will show a tendency to be expelled from the aqueous phase into its boundaries with any less polar phase and so accumulate especially at air-water or oil-water interfaces. Similarly Ba^{++} and SO_4^{--} ions will be more readily adsorbed by $BaSO_4$ crystals than most other ions because they fit into the $BaSO_4$ lattice. Many other examples could be given.

But if ions of one sign are adsorbed at the phase boundary, ions of the opposite sign will be attracted by the resulting electric field and accumulate also near the phase boundary. This accumulation of "counterions" (*Gegenions*), however, will be counteracted by their Brownian motion. The result is the formation of an electrical double layer electrically

neutral as a whole which may extend over a thickness of several hundred Ångströms or even more. The structure of this double layer will be treated more completely in the following sections.

Electrophoresis involves a tangential motion of one phase with respect to the other. It occurs only if the two phases carry free charges of opposite sign. Orientation of dipole molecules near the phase boundary, although creating a potential difference between the two phases, cannot give rise to electrophoresis because the charges of the dipole cannot be separated permanently by the applied electric field. So electrophoresis is intimately connected with the ionic part of the electrical double layer and any theoretical approach to electrophoresis has to start with a study of this electrical double layer.

III. Charge and Potential in the Electrical Double Layer

A. Charge

As mentioned above, the total charge of the electrical double layer is zero, but in it ions and electrons may be accumulated preferentially by adsorption. Analytical measurements allow in principle, although not always in practice, the determination of the amount of ions of any kind adsorbed at the phase boundary. Such measurements, however, do not yield any information on the distribution in space of these charge carriers. But quite often relatively simple theoretical considerations come to our aid. If it is known that, for example, electrons and sodium ions are accumulated at the phase boundary between a metal and a salt solution, it is obvious that the electrons are present in the metal phase and the sodium ions in the aqueous phase. We thus consider the metal to be negatively charged with respect to the water phase, the amount of charge being given by the charge of the excess electrons or by the charge of the sodium ions with sign reversed. Further information on the spatial distribution of the charge carriers demands more refined theories such as will be treated in Section IV, Ionic Double Layer.

B. Potential

The spatial distribution of electric charge is of course connected with an electric potential. The exact nature of electric potentials in and near phase boundaries is, however, a subject with numerous pitfalls. Considering the potential difference between two points in space as defined by the amount of work to be done in transferring a given charge from one point to the other, the question immediately presents itself whether this amount of work is independent of the material carrying this charge. We may expect to find the same amount of work involved in the transport of a