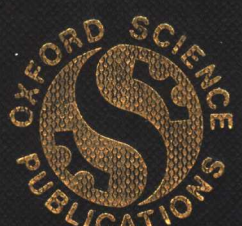

MONOGRAPHS ON PHYSICAL BIOCHEMISTRY

Electrophoresis: theory, techniques, and biochemical and clinical applications

A.T. Andrews

ELECTROPHORESIS:
Theory, Techniques, and Biochemical and Clinical Applications

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PREFACE

THE key step in the great majority of studies on molecules of chemical, biochemical, or biological interest is a separation process. This is true whether the objective is merely to analyse a complex mixture, to describe its composition in terms of what is or is not present, or to separate and isolate a particular constituent for further examination. When the mixture involved is a natural product, including such materials as plant and animal tissues and fluids, the great majority of separation regimes involve at least one of the various forms of electrophoresis. Within the last 20 years or so electrophoresis has rapidly evolved from a generally low resolution method of relatively limited application into a wide variety of analytical and small-scale preparative techniques of unrivalled resolving power and exceptional versatility. These qualities have resulted in a virtual explosion in their use and it is probably no exaggeration to say that well over half and perhaps as many as three-quarters of all research papers in the whole field of biochemistry make some use of electrophoresis.

This tremendous expansion means that methods are being constantly improved and modified, new variations introduced, new equipment built, and yet new areas of exploitation opened up. During the past few years a number of colleagues and students have come to our laboratory either in search of rather general information on the capabilities of electrophoretic methods or for comment on a specific separation problem. It became apparent that there was a gap between a small number of rather elementary treatments of the subject and a rather larger number of highly specialized works. The latter include symposium proceedings or books with individual chapters contributed by leading experts. Excellent although these are, they generally assume some measure of basic knowledge or expertise on the part of the reader. This book is intended to fill the gap.

If I have succeeded, I hope that the student or scientist who has no previous knowledge of electrophoresis may come to understand the principles and capabilities of the various methods which fall within the general definition of electrophoresis and then go on to apply them to specific problems. I have included a wide range of methods in some detail and many of the most recent developments in technique and application which I hope will be of interest and value to the research worker who already uses electrophoretic methods. Inevitably in a book by a single author the choice of what to include and what to leave out, as well as the views expressed, must to some extent reflect his own personal prejudices. I must apologize therefore to those who feel that their own contributions to the

field should have been included or more favourably commented upon, but I hope I have achieved a reasonable balance.

The book places strong emphasis on the various forms of gel electrophoresis, particularly on the methods based on polyacrylamide gel which in recent years have largely superceded earlier techniques. The basic approach to this is described in Chapter 2, which includes a great deal of the methodology common to most electrophoretic measurements. I hope this provides a sound basis from which the reader can go forward with confidence to consider the various aspects covered in later chapters. Each chapter covers the equipment required as well as the actual performance of experiments and ends with a section on biochemical applications which includes reference to many of the most recent uses to which the particular method has been put. Some of these will be to areas of clinical importance but a more general review of clinical applications is reserved until the last chapter. As implied above, I have included extensive experimental details and I hope therefore that this book will find a place as a useful handbook at the laboratory bench and not merely as a reference book sitting on a library shelf.

Shinfield ATA
June 1980

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INTRODUCTION

THE scientist beginning a study of a material of biological origin or of a biological process is often faced with the problem of having to separate and examine the properties of molecules of high molecular weight such as proteins and enzymes, nucleic acids, and complex lipids and carbohydrates. In almost all cases it is also necessary to cause as little damage as possible to the molecules so that their properties are not changed significantly. Thus current separation methods usually lean heavily on physical processes which cause the minimum disturbance to both the physical and chemical properties of the molecules and which result in the maximum retention of any biological activity which the molecule may possess.

Within the fields of protein and nucleic acid chemistry the vast majority of separation methods fall into three broad categories, namely those based on size differences, those based on differences in the electrical charge carried by the molecules, and those based on some specific biological or chemical property of the molecule under investigation. It must be stressed at the outset that it is not always possible or necessary that these categories should be completely distinguished from one another. For example some separations based primarily on charge differences are influenced to some extent by size differences, as will be seen later. However, separation methods which are based mainly on size differences, such as gel filtration, ultrafiltration, and the use of ultracentrifuges, and also methods based on some specific property of the molecules, such as affinity chromatography, immuno-adsorption, precipitation procedures, etc., fall outside the scope of this book and will not be considered further.

The two general areas covered by separations based completely or largely on charge differences between molecules include those using ion-exchange media and those using the various forms of electrophoresis. The former can be used for both analytical and preparative work and may be comparatively readily scaled up to the industrial scale. Although the different methods of electrophoretic separation have their own particular advantages and disadvantages, at present they are all confined to the analytical or small-scale preparative range. At the analytical level electrophoretic methods can be unrivalled for resolution and sensitivity.

1.1. Basic principles of electrophoresis

The term electrophoresis is used to describe the migration of a charged

particle under the influence of an electric field. Under conditions of constant velocity the driving force on the particle is the product of the effective charge on the particle Q and the potential gradient E, and this is balanced by the frictional resistance f of the medium. In free solution this obeys Stokes' law so that

$$f = 6\pi r v \eta,$$

where r is the radius of the particle moving with velocity v through a medium of viscosity η. However, Stokes' law is not obeyed strictly in gels, and f then depends on a number of factors which include gel density and particle size (Maurer 1971).

The electrophoretic mobility m is defined as the distance d travelled in time t by the particle under the influence of the potential gradient E so that

$$m = d/tE \quad \text{or} \quad m = v/E.$$

Thus measured migration distances are proportional to electrophoretic mobilities but direct comparisons between different experiments can only be made if the products tE are equal in all cases. It also follows that, ideally, if all other conditions are equal a second experiment run at double the potential gradient (voltage) for half the time would result in the particle migrating the same distance d as in the first experiment. However, this relation is only approximately true and it is influenced by a number of factors including particularly the effects of the extra heat generated by increasing current. Nevertheless, a quick calculation of volts multiplied by time can be a useful rough practical guide when seeking an idea of how altering either of these two factors will influence the course of an electrophoretic separation.

The potential gradient E also corresponds to the ratio of the current density J to the specific conductivity κ, so the velocity of the charged particles can also be expressed as

$$v = Em = mJ/\kappa.$$

Most of the large molecules with which we shall be concerned possess both anionic and cationic groupings as part of their structure and hence are termed zwitterions. Since the dissociation constants (pK values) of these groups will differ widely, the net charge on such a molecule will depend upon the pH of its environment so that pH will also influence the mobility of the molecule. The ionic strength determines the electrokinetic potential which reduces the net charge to the effective charge and it is found that the mobility of the charged particle is approximately inversely proportional to the square root of the ionic strength. Low ionic strengths permit high rates of migration, while high ionic strengths give slower rates but in practice sharper zones of separation than low ionic strength buffers (Maurer 1971).

Unfortunately, the higher the ionic strength of the buffer the greater the conductivity and the greater the amount of heat generated. Increasing temperature causes an increase in the rates of diffusion of the ions and also

an increase in the ionic mobility amounting to about 2·4 per cent per degree Celsius rise in temperature. At the same time the viscosity of the medium falls with rising temperature. Thus the electrical resistance decreases and at constant voltage the current will rise increasing the heat output still further. The choice of buffer strength then may be seen to be crucial since it effectively determines the amount of electrical power which can be applied to the system. Too high a power input results in excessive heat generation which may lead to an unacceptable rate of evaporation of solvent from the medium, and in free-solution systems can result in convection currents and a mixing of separated zones. In cases which are rather sensitive to heat there may even be a denaturation of proteins or a loss of enzymic activity. In contrast, too low a power input may overcome any heating problem but can also lead to poor separations as a result of the increased amount of diffusion that may occur if the running time is too long.

The removal of heat generated by the passage of the electrical current is one of the major problems for most forms of electrophoresis since cooling inevitably results in the formation of a temperature gradient between those parts of the medium that are better cooled than others. Because of the factors discussed above, any temperature gradient or temperature difference will lead to distortions in the bands of the separated molecules due to variations in the rates of migration through the medium. In the forms of electrophoresis carried out in cyclindrical tubes or in slabs cooling is more effective at the outer edge of the medium than in the middle and curved bands result.

Heating therefore causes variations in both the current and voltage, and in order to minimize these fluctuations it is usual to carry out electrophoresis with power supplies which can be regulated to provide an output at constant voltage or constant current. Although neither of these can provide complete control of heat generation, they are relatively inexpensive and adequate for most purposes. Recently power supplies delivering a constant power output, independent of changes in the electrical resistance, have become commercially available and are probably to be recommended for analytical work of the highest accuracy. These of course only ensure that heat generation is constant throughout the run and in no way overcome the problems associated with heat production in the first place or of attempts to remove it, so it seems likely that the extra expense of such units is not justifiable in most cases. It is clearly best if electrophoresis experiments could be carried out at constant temperature, and as an aid to this when preparative columns are used they can be jacketed and in other cases the experiments can be conducted in a cold room.

Electrophoresis refers simply to the movement of ions through a medium, so that the factors discussed so far which affect this are applicable to all forms of electrophoresis, whether in free solution as in moving-boundary elec-

trophoresis or when a supporting medium such as starch gel, polyacrylamide gel, cellulose acetate, or paper is employed. However, when a supporting medium is used additional factors may also influence mobility and the sharpness of separation. These include adsorption effects on to the support, inhomogeneities within the matrix of the supporting material, ion exchange with charged groups of support molecules, and electro-endosmosis. The first three of these are largely self-explanatory, but electro-endosmosis generally occurs by virtue of charged groups on the supporting medium. For example, paper has a small content of carboxyl groups and agarose possesses sulphonic acid groups. In neutral or basic buffers these will be ionized and will be attracted towards the anode during electrophoresis. In a solid medium movement towards the electrodes is not possible of course, so the effect is compensated for by a migration of H^+ ions (as hydrated protons H_3O^+) towards the cathode which effectively results in a movement of solvent relative to the support medium. Uncharged molecules are then carried towards the cathode in spite of having no ionized groups. The extent of this electro-endosmosis can be measured by studying the movement of suitable uncharged molecules like urea, dextran, sucrose, deoxyribose, or blue dextran (commercially available from Pharmacia Fine Chemicals AB). Blue dextran is especially suitable since being strongly coloured it can readily be observed without further analysis.

We can see then that the basic concept of electrophoresis is a very simple one but that the progress of the charged particle or ion through the medium is influenced by an almost bewilderingly large number of factors. However, it is precisely because of these various influences, that the principle can be turned to good use by the investigator. For example, if size was not a factor there would be no separation between a large particle with a particular charge and a small one with a similar charge. Likewise, a non-homogeneous supporting matrix is deliberately introduced in the technique of gradient gel electrophoresis to aid separation, while an equalization of the charges of cationic and anionic groups to give molecules with no net charge is the basic requirement for isoelectric focusing. The various methods that will be described in subsequent chapters therefore have a fundamental unity, and their apparent diversity is the result of their development to exploit to maximum advantage one or more of these influencing factors in order to achieve the desired goal.

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POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE). HOMOGENEOUS GEL AND BUFFER SYSTEMS

Moving-boundary electrophoresis performed with the components in free solution was historically the first form of electrophoresis to be used widely, but has now been largely superceded by methods employing a supporting medium and especially by electrophoresis on polyacrylamide gels (PAGE). The purpose of a supporting medium is to cut down convection currents and diffusion so that the separated components remain as sharp zones with maximum resolution between them. In most cases it is desirable that this supporting medium should be chemically inert during the separation process. It clearly should be uniform in its properties and be able to be readily and reproducibly prepared. Polyacrylamide gel has been adopted widely for this purpose because it fulfils these requirements very well. It also has the advantage that the composition of the gel can be modified in a controlled way to achieve the best conditions for the problem in hand.

2.1. The formation and structure of polyacrylamide gel

The gel is formed by the vinyl polymerization of acrylamide monomers $CH_2 = CH - CO - NH_2$, into long polyacrylamide chains and cross-linking the chains by the inclusion of an appropriate bifunctional co-monomer, usually N,N′-methylene-bis-acrylamide (or Bis for short) $CH_2 = CH - CO - NH - CH_2 - NH - CO - CH = CH_2$. The polymerization reaction therefore produces random chains of polyacrylamide incorporating a small proportion of Bis molecules, and these can then react with groups in other chains forming cross-links resulting in a three-dimensional network with the general structure shown in Fig. 2.1.

The concentration of acrylamide used determines the average polymer chain length while the Bis concentration determines the extent of cross-link formation. Thus both are important in determining such physical properties of the gel as density, elasticity, mechanical strength, and pore size.

2.2. Pore size effects

In gel media the passage of any particle is hindered by the structure of the matrix, the extent depending upon the relative sizes of the particle and the

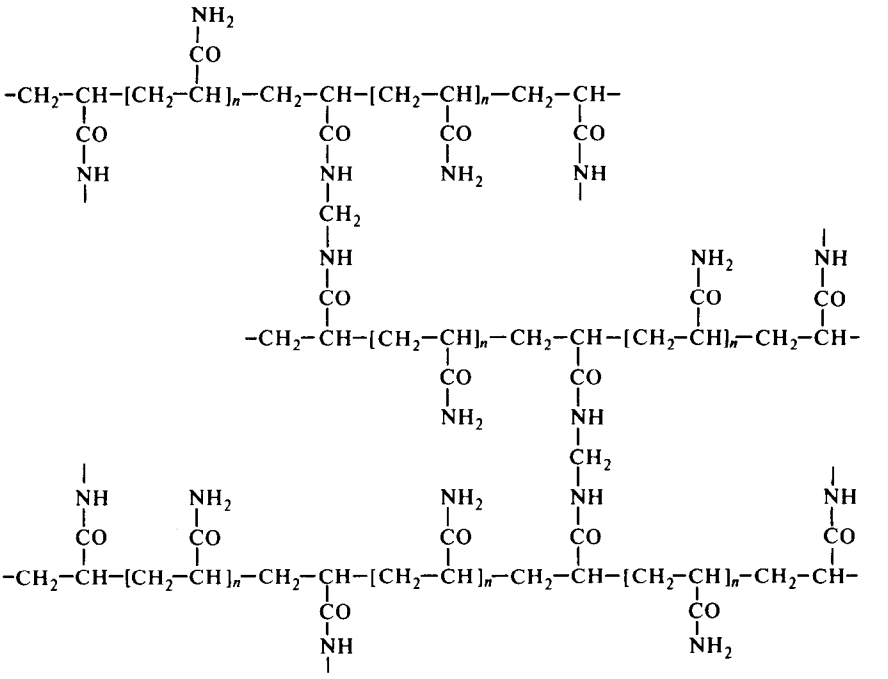

FIG. 2.1. Structure of the polyacrylamide gel matrix formed by copolymerization of acrylamide monomer and N,N'-methylenebisacrylamide cross-linking agent.

pores in the gel network. Therefore both molecular size and charge play a role in the separation process. In agar gels the pore sizes are large, so the influence of size (molecular sieving as it is often termed) is less apparent for most proteins than it is in starch or polyacrylamide gels. The properties of starch gels differ considerably with the type of starch used and from batch to batch so that reproducibility can be a problem, but this difficulty does not exist in the case of polyacrylamide which is made up entirely of synthetic monomers. Polyacrylamide also possesses another major advantage in the relative ease with which the pore size, and hence the degree of molecular sieving, can be altered by simply changing the concentration of acrylamide and/or the proportion of cross-linker.

In any discussion of the properties of polyacrylamide gel the nomenclature introduced by Hjertén (1962) to describe gel composition is very useful. It has been widely adopted and will be used throughout this book. In this nomenclature T represents the total concentration of monomer (acrylamide + Bis) expressed in grams per 100 ml (i.e. weight per volume per cent) and the term C is the percentage (by weight) of total monomer T which is due to the cross-linking agent (Bis).

From gel chromatography experiments Fawcett and Morris (1966) found

that with a fixed proportion of Bis the pore size varied inversely with and approximately linearly to the total monomer concentration T. They also found that at any given value of T the pore diameter showed a minimum when C was about 5 per cent. Experimentally it is found that during PAGE both the absolute and relative mobilities of molecules are influenced by many factors which affect the average chain length of the polyacrylamide molecules. These include not only the acrylamide and Bis concentrations but also the concentration of the catalysts used to initiate the polymerization reaction and the time elapsing between addition of the catalysts and gelation (Kingsbury and Masters 1970).

2.3. Some chemical properties of polyacrylamide gel constituents

The polymerization of acrylamide monomers can be initiated by natural light so both acrylamide and Bis should be stored in brown bottles away from light. A low storage temperature (e.g. 4 °C) may also be beneficial. Solid monomer is quite stable under these conditions, but solutions are less stable and should not be kept for more than 1–2 months. This is especially relevant since it is common practice to prepare concentrated stock solutions. During storage of acrylamide solutions some hydrolysis to acrylic acid occurs. This results in an increase in pH and may cause reduced electrophoretic migrations. N,N,N′,N′–tetramethylethylenediamine (TEMED) is also slightly light sensitive and should be stored in the dark. Ammonium persulphate solid is stable, but solutions should not be stored for more than a few days and it is usually preferable to prepare fresh solutions of this and TEMED shortly before use.

The purity of acrylamide and Bis may vary somewhat but they are generally satisfactory for use without further purification. Both monomers are highly toxic and for this reason it must be emphasized that further purification should not be attempted without good reason. A number of recrystallization procedures have been published (e.g. Maurer 1971) and in a limited number of cases this may be necessary. Monomers of very high purity expressly intended for electrophoresis are commercially available (e.g. British Drug Houses Ltd., BioRad Laboratories, etc.) and as they are relatively inexpensive they are to be recommended.

The monomers exhibit toxicity via skin absorption or by inhalation of particles of acrylamide dust. Reported symptoms caused by even very dilute solutions include skin irritation and disturbances of the central nervous system. The lethal dose (LD_{50}) in mice is 170 mg kg^{-1}. Once polymerization has occurred the resulting gels are relatively non-toxic and can be handled safely, although it is advisable to wear gloves and avoid excessive contact.

2.4. Choosing a suitable gel and buffer system

A mixture of proteins or nucleic acids to be analysed by PAGE almost