Chromatographic & Electrophoretic Techniques

EDITED BY IVOR SMITH

Volume II
Zone Electrophoresis

FOURTH EDITION

CHROMATOGRAPHIC AND ELECTROPHORETIC TECHNIQUES

Edited by

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Volume II

ZONE ELECTROPHORESIS

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PREFACE TO THE FOURTH EDITION

THE newcomer to the subject and literature of electrophoresis is confronted with a great array of commercially available apparatus, often of very similar quality, as well as an almost infinite variety of designs of home-made equipment. However, in practically every case it is very difficult, if not impossible, to find an adequate discussion of the correct techniques or those most suitable for a particular method.

Briefly, therefore, the purpose of this work is threefold: to bring together in as concise a form as possible tried and tested methods of zone electrophoresis; to discuss all those methods which can be applied routinely; and to provide all the essential information to enable the reader to apply these techniques immediately and without recourse to the literature for further details of technique. To this end discussion on the history of electrophoresis and various theories which, though valuable in themselves, provide no further technical information has been omitted as this has been covered elsewhere many times. Similarly, discussion on moving boundary methods is left out as it does not fall within the definition of routine zone methods.

All the work discussed herein has been carried out on commercially available apparatus as this appears to offer many advantages to the majority of laboratories who prefer to buy apparatus rather than to construct it themselves. Nevertheless, details are given as to overall dimensions of apparatus as well as to electrical circuits and requirements so that local construction is possible if desired.

In order that each chapter can be read as a separate unit it has also been necessary to duplicate some of the discussion on the various factors involved in the production of satisfactory results. Although this duplication has been kept to a minimum, it is emphasized that the reader can only gain by a reading of those chapters which may not be of immediate interest to his particular laboratory as the general discussions in each chapter are relevant to the overall technique of zone electrophoresis.

CONTENTS

CHAPTER 1

GENERAL PRINCIPLES OF ZONE ELECTROPHORESIS

Ivor Smith

THE principle of electrophoresis is very simple, namely that a charged ion or group will migrate towards one of the electrodes when placed in an electric field. Hence if two electrodes are inserted, one into each end of a U tube containing a dilute solution of proteins, the proteins will each migrate in one direction only. As the proteins are originally present throughout the whole solution there is little chance of achieving any separation. Zone electrophoresis overcomes this as follows. The mixture of substances to be separated is placed as a narrow zone or band at a suitable distance from each electrode such that, as migration occurs, the different components, which move at different rates, slowly draw away from each other to produce a separation in the direction of migration. A further problem is that of fixing the substances permanently at the positions to which they have migrated after the run is terminated because, being in solution, the possibility of diffusion is present. Thus electrophoresis is rarely carried out in a free solution but, instead, the solution is held in a stabilising medium such as a sheet of paper or a gel. In the former case, the paper is rapidly dried in an oven at the termination of the electrophoresis run. In the latter case the gel is either placed in a fixative which precipitates the substances being examined with the result that the separated substances remain in their correct positions of migration or into some enzyme stain which results in the precipitation of an insoluble reactions product. Subsequently the substances are located as a number of separate, discrete zones or bands. All the techniques discussed in this book therefore are examples of zone electrophoresis in different stabilised media.

The simplest and earliest form of zone electrophoresis is that carried out on paper as the stabilising medium and in Fig. 1.1 is shown an early version of apparatus used. A strip of paper is moistened with buffer, blotted to remove excess liquid and placed horizontally on a glass sheet such that each end dips into a separate beaker containing buffer. The sample is applied at a suitable position on the paper which is then covered with a further plate of glass to prevent evaporation. Current is supplied via a 120 volt battery and carbon electrodes dipping into each beaker. After a suitable time the strip is rapidly removed and dried in an oven at 110°C and then treated with a reagent to locate the separated substances. Although the substances being investigated move towards one electrode only, it must be remembered that the positive and negative buffer ions are also migrating towards one or other electrode as well. In the above case the buffer on the paper and in the beakers is the same. This is referred to as a continuous buffer system and is the usual procedure in paper electrophoresis, because it

was originally thought necessary to maintain conditions as constant as possible for optimum results. Subsequently it was found that advantage did sometime accrue from the use of a more dilute buffer in the beaker and this is used to effect in the cellulose acetate (CA) electrophoresis technique where there is a tendency for sharper bands to result. More recently it was found that a discontinuous buffer system in which the beaker buffer is chemically different and has a different *p*H to that of the stabilising medium results in a great sharpening of the faster moving zones and this is now the standard procedure in starch gel electrophoresis. The mechanism of this sharpening effect is not too

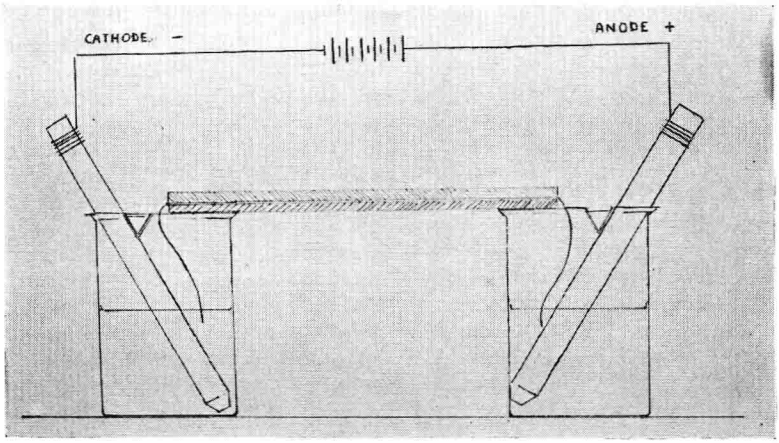

FIG. 1.1. The simplest form of electrophoretic apparatus. Two beakers, containing buffer, support a glass sandwich between the plates of which is laid a length of Whatman 3 MM paper, the ends dipping into the buffer at either end. Carbon pencils form the electrodes and are connected to a 120 V H.T. battery. The sample is applied as a band across the strip.

well understood. Thus it was postulated by the inventors of the acrylamide gel technique, the most recent gel technique in current use, that discontinuous systems were essential to the procedure but it has since been shown that practically identical results can be obtained with a continuous buffer system.

During the passage of current, electrolysis occurs at the electrodes. In order to minimise any effects of this electrolysis the electrode is isolated in a separate compartment from that into which the paper dips and these two compartments are connected by means of a paper wick, glass fibre wick or gel bridge, the former being the most convenient. Hence most apparatus has four electrode chambers, a pair of cathode and a pair of anode chambers, each pair being connected by means of the wick just described.

In the case of paper electrophoresis the ends of the strip dip into the buffer and make direct connection. With CA, agar and starch gel, however, the medium is connected by means of a further paper wick

overlapping the medium by about 1 cm. and whose other end dips into the buffer. In all such cases the medium must be perfectly horizontal and above the buffer level to prevent both syphoning and water-logging.

However, it has been found possible to dispense with the double buffer compartments and the paper wicks in the acrylamide gel techniques without adverse effects. pH changes are overcome by using relatively large buffer volumes, or, by continuously mixing and recirculating the buffer solutions.

Factors Affecting Speed of Migration and Separation

The Stabilising or Supporting Medium. Many supporting media are in current use but they fall into two main classes:

1a. paper, cellulose acetate, glass fibre paper, thin layer materials, agar gel, single cellulose fibres. Analytical and preparative.

1b. pevikon, starch and gypsum blocks, sponge rubber. Preparative only.

2. starch gel, acrylamide gel. Analytical and preparative.

Before discussing the effect of these media on separations, it is worth considering briefly the uses to which the different media are put. Paper, particularly at high voltage but also at low voltage, is used for the separation of small molecules such as the aminoacids, for medium sized molecules such as peptides and nucleotides obtained by enzymic hydrolysis of proteins and nucleic acids, and with low voltage only for large molecules such as proteins, enzymes and nucleic acids. Thin layer materials are used mainly for small and medium sized molecules. Gels were used exclusively for large molecules but by decreasing the pore size of the gel it can also be used for peptides and nucleotides. Blocks are used for preparative purposes.

Supporting media in Group 1 above are relatively inert and have little effect on the compounds being separated (paper has a slight denaturing effect on proteins which results in minor tailing of the bands back towards the origin or point of sample application). Hence separation is dependent on the net effect of charge and mass; thus a large molecule with many charges may move to the same position as a smaller molecule with an equal charge to mass ratio. It is generally thought that separation occurs by migration in and through the liquid held stationary within the stabilising medium.

Supporting media in Group 2 exert a positive influence on the compounds. The gel can be considered as a porous medium in which the pore size is of the same order as the protein molecule with the result that a molecular sieving effect is impressed on the normal protein separation and molecules of similar charge to mass ratio but with different molecule size can be separated. This is well illustrated with serum proteins. Two identical samples are run on paper and one is subsequently stained to locate the proteins when one albumin and four globulin bands are observed. Each globulin band is cut from the unstained wet paper, placed in starch gel, run at the same *p*H and stained as usual when each will be seen to have given rise to a number of bands which must differ only in mass as the electrophoresis conditions were

the same in both runs. With starch gel, a standard procedure is commonly used which results in a standard pore size although it is known that different buffer salts do have minor effects on the pore size. However, acrylamide gels can easily be varied in pore size such that molecules which penetrate a gel with larger pores may be excluded from a tighter gel with smaller pores and this can be particularly useful in the study of a single smaller protein both analytically and preparatively.

Supporting media in Groups 1a and 2 were all originally designed for analytical purposes which require that an optimum volume, namely one which results in the maximum number of sharply defined bands, be applied. Subsequently it was found that larger volumes could be applied and although this resulted in less well-defined separations, this was more than compensated for by the fact that preparative amounts could be recovered. Simultaneously other preparative methods were sought whereby millilitre rather than microlitre quantities could be separated and these are now referred to as the block techniques. Most recently the gel techniques have been found to operate in the preparative range intermediate between the analytical and the block ranges. Blocks, being inert also, yield separations similar to the Group 1a materials so that, frequently, a preliminary separation is carried out on a block and the recovered fraction is then further fractioned on a preparative acrylamide gel.

pH. A substance can only migrate if it carries a charge, i.e. it is ionised. A weak acid will remain at the origin at *p*H's equal to or below its isoelectric point and move to the anode at *p*H's above this point; its rate of migration is proportional to its degree of ionisation and hence the higher the *p*H the faster it travels. The converse is true for weak bases. Most interest is centered around ampholytes such as aminoacids and proteins which migrate to the anode at *p*H's above their iso-electric points and in the reverse direction at *p*H's below this. Indeed one method of determining iso-electric points is based on this effect. The best *p*H for any given mixture is a matter for empirical investigation; one *p*H may be best if it is desired to obtain maximum separation of all the components present whereas another *p*H may be much more suitable if it is desired to isolate a single component.

The Buffer, Chemical Composition, Concentration and Ionic Strength. The value of the buffer was originally thought to be that of controlling the *p*H of the medium at a constant value but this is a gross over-simplification. The buffer does indeed maintain a constant *p*H when used in a continuous system and also ensures that each component will maintain a constant charge throughout the experiment because the ionisation of each compound is stabilised and this is particularly important for the ampholytes. Later it was realised that buffer ions, particularly phosphates, often bind to proteins and this may result in different absolute and relative mobilities of a mixture of compounds when investigated with different buffers of the same *p*H and ionic strength. Likewise borate buffer ions bind to glyco-compounds.

The more concentrated the buffer the slower will the other compounds move because, as current is carried by the ions present, the greater the quantity of buffer ions relative to other ions, the greater the proportion

of current can they carry. Furthermore, the movement of ions surrounded by ions of opposite charge is retarded by the attraction of these ions so that increased buffer concentration has a double effect in reducing migration rates. However, to compensate for this disadvantage, the zones are found to be sharper.

Buffers are available for almost the whole *p*H range and certainly for the range over which proteins remain soluble and apparently undenatured. Thus a protein or other ampholyte mixture can be separated first with an alkaline buffer in which the components are all present as anions, the partially separated fractions can be recovered and separately rerun in an acid buffer in which they exist as cations; this being similar to ion exchange separations on DEAE cellulose followed by CMC.

It is now necessary to consider the question of ionic strength, u, although it must be said that there is some confusion in the literature as to the definition of the term.

$$u = \tfrac{1}{2} \Sigma mc^2$$

where m is defined either as molarity (gram ions/litre) or molality (gram ions/1000 ml. solvent) and c as the charge on the ion. One work defines u on the basis of molality and offers an explanatory calculation based on molarity. In two different works the identical buffer is quoted in one case as 0·05 and in the other as 0·06u. The author has often been tempted to consider what real value the term has in electrophoresis, and no-one has yet had the temerity to calculate ionic strengths for any but the simplest buffers. Two examples of such calculations follow; these being based on molarity: Veronal or barbitone buffer *p*H 8·6: sodium diethylbarbiturate, 10·3 g. (M/20), and barbituric acid, 1·84 g. (M/100), per litre, assuming free acid completely unionised.

$$\mathrm{u} = \frac{1}{2}\left(\sum \frac{1}{20} \times 1^2 + \frac{1}{20} \times 1^2 \right) = 0{\cdot}05$$

Acetate buffer *p*H 4: sodium acetate.$3H_2O$, 13·61 g. (M/10), plus acetic acid 25·59 ml. (0·45M) per litre, assuming acid unionised.

$$\mathrm{u} = \tfrac{1}{2}(\Sigma\, 0{\cdot}1 \times 1^2 + 0{\cdot}1 \times 1^2) = 0{\cdot}1$$

In practice, buffers are made up by weighing solids or measuring volumes of liquid and variations in composition are based on changing these weights or volumes; ionic strengths are calculated subsequently.

It is now worth considering the effect due to the use of different buffer concentrations in the electrode compartment and on the medium. Invariably the electrode buffer is more concentrated than the medium buffer in gel electrophoresis and often of different *p*H. Frequently the buffer used in the electrode reservoir is different from that used to prepare the gel. Briefly the net effect is to produce a voltage discontinuity at the interface of the two buffers and as this interface travels through a protein band, the band compacts to produce a narrower, tighter zone. This effect is discussed in some detail in the chapters on starch and acrylamide electrophoresis.

It has been said that, for electrophoresis to occur, the substances

must exist in an ionic form. However, neutral molecules can be separated if they can easily be converted to carry a charge. Thus sugar molecules are neutral but, as they react with borate to form sugar-borate ions, they can be separated in borate buffers.

Voltage, Current and Heat Effects. An electrophoresis medium, such as a strip of paper moistened with buffer, exerts a resistance to current flow which is a function of the medium, the buffer and its concentration. For a given applied voltage, the resistance of the medium will determine the current passed and, consequently, the heat generated during the experiment. Thus applied voltage, V, across a resistance, R ohms, will result in a current flow of I amps, the consumption of W watts, and the generation of heat as calories, C; these terms being related as follows:

$$V = IR \qquad \text{Ohms Law}$$
$$W = IV$$
$$C = Wt/4{\cdot}18 \quad (t = \text{seconds})$$

It is the ions which carry the current and in order to increase the rate of migration it is obvious that the current must be increased. As the resistance of the medium is fixed it follows that the applied voltage must be raised if it is desired to obtain a higher current.

However, as the experiment proceeds heat is generated and therefore the strip heats up. A number of effects follow from this. First, the resistance of the strip falls and, therefore, the rate of migration increases. Second, distillation from the warmer strip on to the colder walls of the apparatus occurs with the result that the resistance is again altered and is usually decreased. It follows that unless either the current or voltage is stabilized, both will alter with time. It is usual to include either a voltage or current stabilizer in the power pack for these reasons.

Constant Voltage. With a constant applied voltage the current will increase during the experiment because the resistance of the strip is continuously dropping. The heating will also increase with consequent continuous distillation off the strip. Therefore the rate of migration will also increase continuously until an equilibrium is established in the apparatus. With voltages of not more than 100 volts or so (2–3 volts/cm. length of strip) this effect can be ignored and at temperatures around 20°C the current will increase only slightly for a 4 cm. wide strip. With greater voltages, distillation is large and continuous and the rate of migration will vary throughout the experiment.

Constant Current. With a constant applied current the voltage will fall continuously because the resistance of the strip is falling. This results in a lowering of distillation and the rate of migration is more nearly constant for the whole of the experiment.

The above discussion has centered around a single strip but it is now necessary to examine the effect of a number of strips run simultaneously. If two similar strips are run in parallel with the same applied constant voltage as before the resistance of the apparatus is now halved and therefore the current is doubled

$$1/R = 1/r_1 + 1/r_2 \ldots + 1/rn; \quad R = r/n \text{ and } I = nV/R$$

where R is the total resistance of the apparatus, r_1 and r_2 are the resistances of each strip (these will be identical) and n is the number of strips in the apparatus. In order to obtain the same degree and length of separation, therefore, either a constant voltage must be applied irrespective of the number of strips used or the current on the meter must be adjusted by multiplying by n.

The resistance of the strip is proportional to its length and in order to quote figures which can be reproduced elsewhere it is essential to quote not just the voltage or current used for a given separation, which is quite useless, but the voltage drop per centimetre length of strip not dipping into the buffer and the current density per centimetre width of strip as well as the temperature.

Constant Current or Constant Voltage. With paper electrophoresis at low voltage it matters little which choice is made as the heat generated is small and this is easily dissipated. However, with all gels and blocks the thickness may vary from 0·3–1 cm. and heat dissipation becomes a problem particularly when one realises that the heat in the middle of

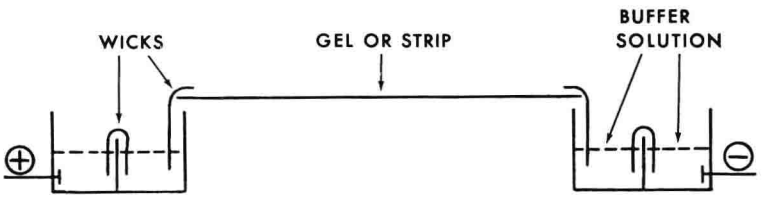

FIG. 1.2. The figure illustrates the fact that current must flow through buffer solutions and various wicks as well as through the electrophoresis medium and that each will, therefore, contribute towards the total resistance of the apparatus. Thus the voltage drop across the medium is only a proportion of that shown on the power pack.

the gel or block must be rapidly removed in order to avoid a temperature gradient within the thickness of the medium. With these latter procedures, constant current tends to be used as this results in less heat.

One final point deserves mention if only because it is so seldom remembered. The resistance of an apparatus, shown diagrammatically in Fig. 1.2, is the sum of the resistance of the individual components. Here the resistances present are due to buffer solution, wicks and supporting medium. The resistance varies with the number of thicknesses of paper wick connecting buffer solutions and the number of thicknesses connecting buffer to medium on both anode and cathode sides. Hence the applied voltage recorded on the power supply is not that across the medium and may, in fact, be up to ten times that which does cross the medium. It is always worthwhile measuring the voltage drop across the medium in quoting experimental details and a correct voltage gradient will refer to this figure, rather than the figure seen on the power pack, divided by the medium length.

Power Supplies. There is no power pack available suitable for all forms of electrophoresis. Indeed this is not even desirable as such an

apparatus would be far too costly for most purposes which require a less sophisticated piece of equipment. The important features a satisfactory apparatus should possess are both constant voltage and

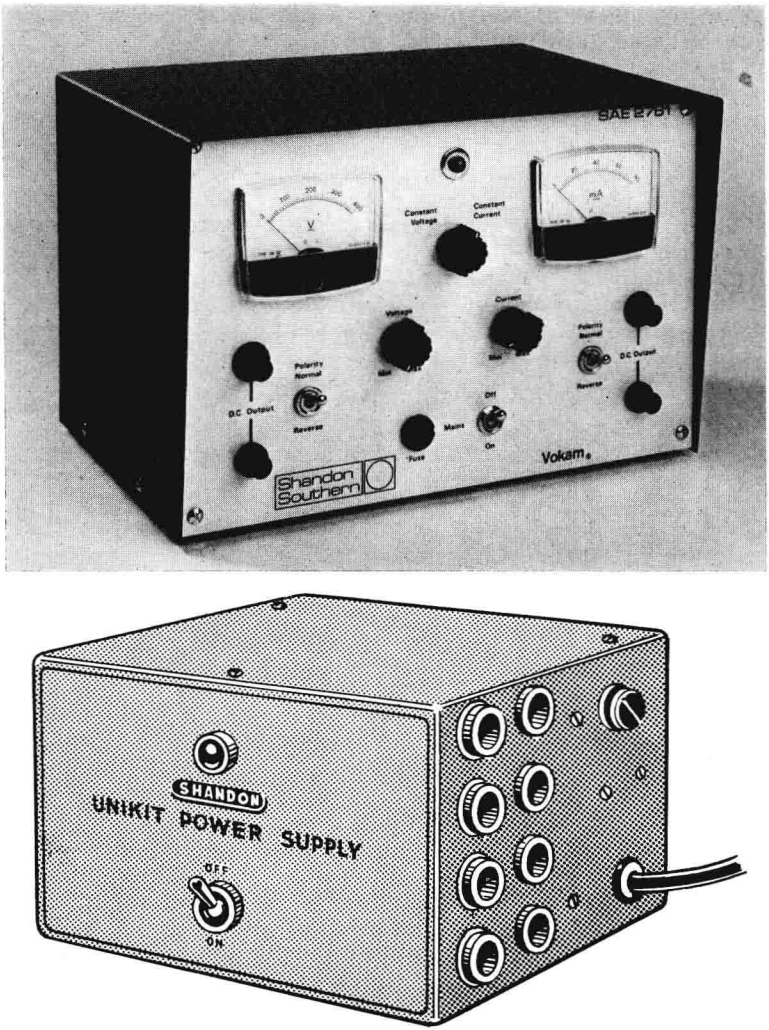

FIG. 1.3. (*a*) The Vokam power pack. (*b*) The Unikit power pack with four independent outlets.

amperage controls (both cannot operate simultaneously) which means that the apparatus should include both a voltmeter and an ammeter covering the desired range.

It has been stressed that the important figures are the voltage and