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WITH 75 ILLUSTRATIONS AND 13 TABLES

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Chapter 1

INTRODUCTION

Progress in modern science depends a great deal on progress in the development of new techniques and improved apparatus. Zone electrophoresis in blocks and columns may be considered as one of these new methods which has come to be a very useful tool for analytical as well as for preparative work in many fields of chemistry and biological sciences.

The overwhelming number of papers dealing with zone electrophoresis on various supporting media indicates the importance of the method and the author of a monograph is compelled to be selective when considering this stream of publications. Naturally, some reports by outstanding workers have also to be neglected. Despite this need for selectivity, ample material remains to enable the interested reader to find valuable pointers which will be helpful if he wishes to apply zone electrophoresis for the solution of his own chemical problem.

There is another trivial difficulty, concerning the definitions of zone electrophoresis in blocks and columns, also known as electrochromatography. We shall arbitrarily consider all electrophoretic experiments carried out in more or less cylindrical tubes as column electrophoresis, if the elution of the separated components can be accomplished without slicing of the supporting medium. Some authors (*e.g.*[101]) employ the term "column electrophoresis" for the technique which is more generally referred to as block electrophoresis. On the other hand we cannot state that block electrophoresis embraces *all* studies carried out using semicylindrical or rectangular troughs followed by sectioning of the packing material. If this were true, preparative paper electrophoresis (at least in thick sheets) would also have to be included. By contrast, if it were to be stated, for example, that the block had to be at least 1 cm thick, it would be necessary to exclude gel electrophoresis.

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An arbitrary choice has therefore been made for the treatment of block electrophoresis. Paper electrophoresis will not be discussed at all, since there are several good books available in which the possibilities of this method are described *in extenso* (*e.g.*[98, 130–132]). Agar gel electrophoresis, which has been very well reviewed by Wieme [133] in this series of Monographs has also been omitted. However, the reader will find in this book an account of continuous electrophoresis in media other than paper sheets and also column electrophoresis in density gradients.

The expression "zone electrophoresis" was introduced by Tiselius[1,2] in order to distinguish this process from moving boundary electrophoresis, which is fundamentally different. In moving boundary or free electrophoresis the charged particles

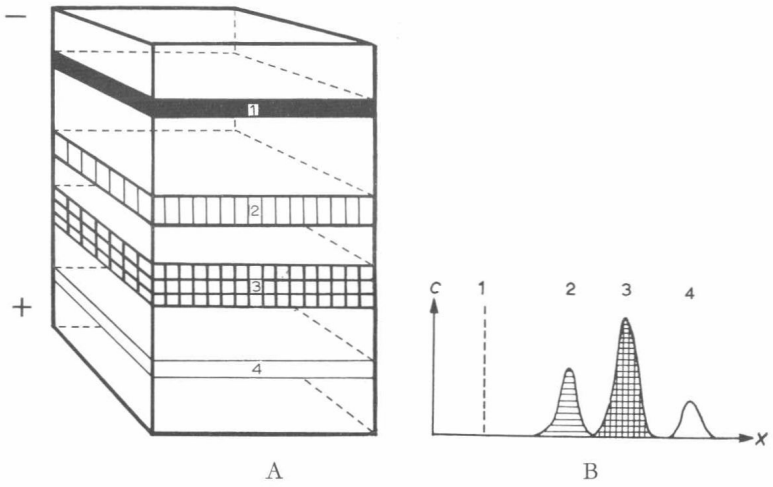

Fig. 1A. Schematic representation of an ideal electrophoretic separation of a mixture consisting of charged components on a supporting medium in a vertical block. The peaks in B are obtained by plotting the concentration C against the distance. X = direction of migration. The rate of migration is a measure of the mobility of each component. 1 = starting zone, 2, 3, 4 = separated components (negatively charged).

migrate in dilute solutions, usually resulting only in partial separation of fractions with differing mobilities. However, the introduction of a solid material as supporting medium, or of a density gradient, permits, in principle, complete separation of the components.

Many substances capable of pulverization may be used as stabilizing media. Generally the best results are obtained with powders which

(I) are insoluble in water and the buffers to be used;
(II) do not adsorb or adsorb only slightly the mixture to be separated, and
(III) form a fairly homogeneous paste with the buffer.

In addition, electro-osmotic flow should not be excessive, although it is possible to minimize this undesirable phenomenon by suitable arrangement of the apparatus [12, 13].

Sponge rubber or foam plastic may also be utilized for the production of stabilized zones and according to Mitchell and Herzenberg [14] exhibit no adsorption effect. A considerable number of gels and liquid density gradients have been found to be useful supporting media in blocks and columns. Density gradient methods have the following advantages:

(I) adsorption cannot occur
(II) the column is transparent
(III) separated fractions may be collected more easily than from packed columns.

For theoretical studies the contributions of Overbeek and Lyklema [10] and Kunkel and Trautman [11] are of great value. The theoretical basis of density gradient electrophoresis has extensively been discussed by Svensson [328].

In Figs. 1A and B, the separation of a mixture of electrically charged components is represented schematically. The sample is introduced in a slit indicated by the black zone. When a potential is applied to the ends of the block or column the ions start to move, each component migrating with its own specific mobility which depends on the magnitude of the charge in a given buffer solution. The sign of net charge of each component determines

its movement towards anode or cathode. The area under each peak is proportional to the concentration and the rate of migration of each zone is a measure of the mobility in a given supporting medium. (Fig. 1. B). In practice, many deviations will occur from the ideal electrophoretic behavior illustrated in Fig. 1. In this connection Peniston *et al.*[3] compared the theoretical and experimental spreading of zones on agar while Boyack and Giddings [4] applied the theory of zone spreading [5, 6] to electrophoretic zones.

Eqn. 1 is an expression for the velocity in a liquid of a charged particle under the influence of an electric field.[7].

$$V = f\frac{\varepsilon \cdot \xi \cdot E}{\pi\eta} \tag{1}$$

where V = electrophoretic velocity
ε = dielectric constant of the solvent
ξ = electrokinetic potential
E = field strength
η = viscosity of the solution

The factor f depends on the radius of the particle and reciprocally upon the thickness of the surrounding double layer. For globular and cylindrical particles its numerical value lies between $\frac{1}{4}$–$\frac{1}{8}$. Effects such as the relaxation time of the double layer [8, 9] and the difference between the field strengths acting on the particles and in the surrounding solution are not considered here. Eqn. 1 is not directly applicable to electrophoretic experiments with macromolecules in stabilized media. The velocity of those particles depends among others upon their net charge, the potential gradient, the ionic strength of the solution, the temperature, the degree of wetness, the electro-osmosis, and the barrier effect interposed by the stabilizing medium. As a rough approximation, however, one may conclude that different particles in stabilized media will possess different mobilities when a potential is applied, provided that their ξ values are not equal.

The distance over which a substance s migrates in a stabilized medium is:

$$Eu_s t \text{ (cm)} \tag{2}$$

E = field strength
u_s = mobility ($cm^2\ volt^{-1}\ sec^{-1}$)
t = time of movement through the block or column.

The electrophoretic velocity may also be defined according to eqn. (3).

$$V = Eu_s \tag{3}$$

As E is the quotient of current density and specific conductivity, eqn. (3) may be replaced by

$$V = \frac{Iu_s}{\kappa} \tag{4}$$

I = current density (Acm^{-2})
κ = specific conductivity ($\Omega^{-1}cm^{-1}$).

Further relations are:

$$\kappa = F \Sigma c_s u_s \tag{5}$$

F = Faraday's constant (96,500 Coulomb equivalent^{-1})
c_s = ionic concentration (equivalent cm^{-3}).

$$u_s = f(c_1, c_2, c_3, c_4, \ldots\ldots T) \tag{6}$$

T = temperature.

It is beyond the scope of this monograph – which is primarily a practical guide – to give a full account of the theoretical background of zone electrophoresis.

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Chapter 2

BLOCK ELECTROPHORESIS

Without any doubt the block technique is the simplest form of zone electrophoresis to be discussed in the following chapters. The construction of the apparatus and the preparation of the supporting medium can be performed without any great difficulty. In contrast with column electrophoresis the surface of the stabilizing medium is accessible, so that contact prints can be taken and staining reactions carried out. This is of the utmost importance for a rapid localization of the separated components of a mixture under investigation.

The various fractions may be isolated by cutting and elution of the segments, while the necessity of re-packing for a new experiment is only a slight disadvantage.

Block electrophoresis is now generally considered a rather gentle purification method for proteins. When there is danger of irreversible denaturation processes leading to loss of protein, enzymatic or hormonal activity, better results may be obtained by using buffers of low ionic strength, varying the dimensions of the box, performing the experiment at lower temperatures, or diminishing the potential gradient. Poor recoveries are often caused by heat development.

Block electrophoresis is convenient for analytical studies as well as for preparative work. Although the method has hitherto been applied principally to proteins, it may also be a useful tool for the separation of other soluble charged macromolecules. It is difficult to predict ideal circumstances which will hold for every experiment and the best conditions can only be found by trial and error. Reproducible results will only be obtained if the working methods are completely standardized.

1. APPARATUS

a. Power supply

Hardly any commercially available power source is supplied with a device which makes measurement of the potential gradient in the block possible. As a consequence, some authors quote only the voltage between the electrodes in their reports. This is not in itself sufficient to render an experiment reproducible since this measured voltage includes the potential losses in buffer vessels and in contact strips.

The power supply of 1500 V constructed in our laboratory [15] has a capacity of 25 mA regulated to maintain a constant voltage. The power supply unit is placed in a steel box 45 × 20 × 20 cm (Fig. 2) and the apparatus has a stabilization circuit which maintains the voltage in the system constant (± 0.5%) in spite of possible variations in the supply voltage (± 10%).

Both the current flowing, and the voltage between the electrodes and the ends of the supporting medium, can be monitored. The design makes it possible to supply two separate stations placed in a cold room (if desired), although for safety and convenience the power supply is placed outside the cold room.

From eqn. (4), page 5, it is apparent that the electrophoretic velocity is controlled by regulation of the current supplied, provided that the temperature and the chemical composition remain unaltered. Voltage control can replace the current control if the resistance outside the block is constant.

In practice, the ratio

$$\frac{u_s}{\kappa}$$

is treated as a constant, within limited temperature ranges. If this is true the only variable in eqn. (4) is the current density. According to Brattsten [16] the temperature dependence of the velocities is never completely eliminated when the current is supplied from a constant voltage source. For preparative purposes however, a power supply with constant voltage is satisfactory.

There are several commercially available power packs which can supply 500 or more volts and which may be very generally used in block and column electrophoresis. For example: LKB.-Produkter Fabriksaktiebolag, Stockholm 12. LKB. – D.C. power supply, max. 60 mA, max. 30 watts, dual range voltmeter 0–600 and 0–1200 V.

Shandon Power Pack. Shandon Scientific Co. Ltd., 6 Cromwell Place, London S.W. 7.

1. D.C. power supply output voltage 0–500 V, 40 mA.
2. Stabilized D.C. power supply 1,000 V at max. 25 mA.

Beckmann Power Pack. Beckmann-Spinco Division, Stanford Industrial Park, Palo Alto, California.

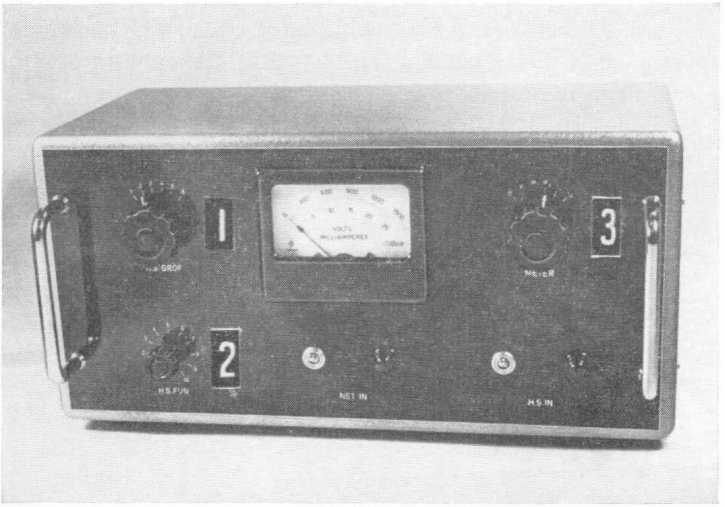

Fig. 2. Power pack for zone electrophoresis (constructed by Mr. W. L. van Es of the Physical Department of the Netherlands Cancer Institute); constant voltage from 0–1,500 V, maximum current 25 mA. 1 = Voltage range switch (coarse), 2 = Voltage range switch (fine), 3 = Switch for monitoring system: O = off, V = output voltage, M_1 = voltage on the column, M_2 = voltage on the 2nd column, I_1 = current, I_2 = current through the 2nd column.

Suitable for constant voltage and constant current operations.
Constant voltage range 500–1.000 V
current 0–100 mA
Constant current range 10–100 mA
voltage 100–1.000 V.

Servonuclear Power Pack. Servonuclear Coorp., 28–21 Astoria Boulevard, Long Island City 2, N.Y.

D.C. power supply 0–2,000 V, 0–150 mA.

CVC-A Power pack. Gerard Pleuger S. A. Wijnegem, Belgium. Suitable for constant current or constant voltage 0–60 mA, 0–1,000 V.

E. C. power supply, E. C. Company, 538 Walnut Lane, Swarthmore (Penn.)
output voltage 0–1,000 V at 200 mA or less.

b. Troughs

Block electrophoresis is carried out either in semi-cylindrical glass troughs or in rectangular plastic boxes which can easily be constructed in a laboratory workshop. Glass troughs can be obtained by cutting Pyrex tubing in half longitudinally[17]. In our laboratory, perspex boxes 40 × 2 × 4 cm, (compare Fig. 3 and Fig. 11) or 80 × 2 × 8 cm proved to be convenient both for analytical and for large scale preparative work. Miller [18] used even longer rectangular troughs, consisting of Pyrex plates 100 × 5.1 × 0.4 cm, for which plexiglass shoulders 100 × 0.9 × 0.3 cm were constructed with the aid of Pliobond 30. The use of a block simply packed with wax paper as originally described by Kunkel and Slater [19–21] is not advisable. When very high voltages are required, a water-jacketed box should be employed [22].

For zone electrophoresis on foam rubber, $\frac{1}{4}$-inch Lucite troughs 40 × 2 × 2 cm are utilized[14]. These are fitted with a cooling jacket at the bottom for circulation of ice-water, although in our experience such non-uniform cooling often results in irregular zones. The effect does, in fact, become visible when hemoglobin migrates in this type of trough. According to Wieland *et al.*[23]

such disturbances occur if the layer on the cooling surface is more than 0.5 cm thick. These authors employed boxes having the dimensions 16 × 30 × 0.6 cm, which permitted the application of field strengths up to 25 V/cm.

In their method, a homogeneous paste of starch or cellulose powder was poured onto the jacketed plate of a high voltage electrophoresis apparatus [24] and its final shape determined by parallel side walls of Vinidur (polyvinyl chloride).

2. BUFFERS

The ionic strength I of a buffer solution [364] is given by:

$$I = \tfrac{1}{2} \sum_{i=1}^{i=n} c_i Z_i^2$$

where c is the molality of a particular ion, and Z its charge. Σ implies a summation over all ions in solution. Thus for a buffer containing 0.1 M KH_2PO_4 and 0.1 M Na_2HPO_4,

$1 = \tfrac{1}{2}\{[K^+]Z^2_{K^+} + [H_2PO_4^-]Z^2_{H_2PO_4^-} + [Na^+]Z^2_{Na^+} + [HPO_4^{2-}]Z^2_{HPO_4^{2-}}\} = \tfrac{1}{2}\{0.1 \times 1 + 0.1 \times 1 + 0.2 \times 1 + 0.1 \times 4\} = 0.4$

Low ionic strength buffers permit fast migration rates and low heat development. High ionic strength promotes the sharpening of zones, but causes high heat production and low migration rates.

Buffers of ionic strength between 0.025 and 0.1 are recommended.

Some examples of typical buffer solutions used in block electrophoresis are given below: (see also Table I)

Veronal pH 8.6, 0.1 μ
Diethylbarbituric acid, 2.8 g
Sodium diethylbarbiturate, 20.6 g
Distilled water to 1 l

Veronal pH 8.6, 0.075 μ
Diethylbarbituric acid, 2.76 g
Sodium diethylbarbiturate, 15.45 g
Distilled water to 1 l