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Note

An electrolyte system for discontinuous electrophoresis based on the use of two counter ions

Some experiments on cellulose polyacetate strips

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The aim of this work was to develop a new type of electrophoretic buffer system that can generate discontinuous electrophoresis in capillaries, strips or gels. This paper describes some experiments on the analysis of serum proteins on cellulose polyacetate strips.

In discontinuous electrophoresis, a change in the effective mobility of one or more ions of the system causes a change from one type of electrophoresis to another. In disc electrophoresis according to Ornstein[1], a so-called stacking stage is followed by an unstacking stage. In the first stage, the sample components are separated and concentrated as in isotachophoresis[2], while in the second stage, the terminating ions have a higher effective mobility than those of the sample components. The terminating ions over-run the sample component zones and subsequently serve as a background electrolyte in which the sample components are separated as in zone electrophoresis. Multi-phase buffer systems in polyacrylamide gel electrophoresis[3] are essentially based on Ornstein's idea, although they are more complex and refined.

However, it may be desirable to perform discontinuous electrophoresis without the need for filling the equipment with a gradient of different buffers, part of them embedded in gels, which have to be polymerized inside the tubes, as this may lead to problems[3,4].

As we did not wish to restrict ourselves to gels, we could not make use of a molecular sieving effect to influence the sample component mobilities, as Ornstein did with his lower gel. The system that we searched for had to be as simple as possible and had to effect a concentration of the sample and also the re-separation of the sample components, in order to make possible usual zone electrophoresis detection (*e.g.*, staining).

These demands are fulfilled by an isotachophoretic system in which the effective mobilities of the sample ions lie initially between those of the leading and the terminating ions, but do not obey this condition after a certain time.

In the system described, after some time the terminating ions become faster than the sample components, as in disc electrophoresis according to Ornstein[1], but the increase in mobility is caused in a different way and makes the system introduced in this work easier to handle.

THEORETICAL

For the sake of simplicity, only the analysis of anions will be considered. As usual, our electrophoresis equipment consists of two electrode compartments, connected by a "separation part" (*e.g.*, strip, gel or capillary). At the start of the analysis, the separation part is filled with the leading electrolyte (LE) and the cathode compartment is filled with the terminator (TE). The anode compartment electrolyte, however, contains another cation different to that which serves as a counter-ion in LE and TE. Previously the same counter-ion has always been used for all buffer phases in multi-phase buffer system (mostly Tris for anion analyses).

When a voltage is put across the separation part, the LE anions will migrate to the anode, being followed by the TE anions. Sample ions, applied at or near the boundary between LE and TE zones, will be concentrated into consecutive zones between LE and TE. The cations of the LE migrate to the cathode in the opposite direction. In our system, they will be followed (or over-run) by the second type of cations from the anode compartment. The type of electrophoretic boundary in the cation system that will result depends, of course, on the effective mobilities of both the LE cations and the anode compartment cations.

Wherever the LE cations are replaced with the anode compartment cations, there will be a change in pH and in the system tested there will be an increase in the pH. Depending on the sharpness of the boundary between the cations, this increase is achieved rapidly or more slowly. For a correctly chosen electrolyte system, the increase in pH in the TE zone will be sufficient to increase the dissociation of the terminating anions such that their effective mobility becomes higher than the sample component mobilities. In this way, a pH change that moves in the opposite direction to the sample ions causes the necessary increase in the mobility of the terminating ions. In multi-phase buffer systems, their mobility will be increased after these ions have passed a stationary concentration boundary.

EXPERIMENTAL AND RESULTS

In order to test this idea, some experiments were performed on cellulose polyacetate strips. As the buffer system chosen defines only the type of electrophoresis that results, it is possible to perform isotachophoresis in strips, as shown by Vestermark and co-workers[5,6].

Although proteins are far from ideal samples, as they are influenced by a wide range of physical and chemical effects, this type of sample was chosen owing to the importance of its analysis.

Materials

The leading electrolyte was Tris–acetate, prepared by adjusting 0.04 *M* acetic acid (Merck, Darmstadt, G.F.R., Cat. No. 90063) to pH 4.5 with Tris (Merck; Cat. No. 8382), and the terminator was Tris–glycine prepared by adjusting 2.5 g/l of Tris to pH 8.6 with glycine (Merck; Cat. No. 4201). The second cation solution was 0.05 *M* piperidine (Merck; Cat. No. 9724). Moni-Trol I human serum, containing 7 g/l of protein, was obtained from Dade Division, American Hospital Supply Corp. (Miami, Fla., U.S.A.). In some experiments, up to 10-fold dilutions of this serum with

saline were used. Sepraphore III cellulose polyacetate strips were obtained from Gelman (Ann Arbor, Mich., U.S.A.) (Cat. No. 51003, Lot No. 80466). The 1 × 6 in. strips were cut into 1/3 × 3½ in. strips.

All solutions were prepared with deionized and distilled water with reagents of the highest purity commercially available. In some experiments triethanolamine (Riedel de Haën, Seelze, G.F.R.; pure grade) was used instead of piperidine, with good results.

Method

After soaking the strips for 10 min in LE and carefully wiping of the excess of liquid, they were placed directly on the cooling bridge of the electrophoresis tank (Fig. 1), their ends being in the electrode compartment electrolytes. Siliconization of the position at which the strip is located[6] was not necessary and the use of a piece of filter-paper for the strip–TE contact only introduced an extra resistance. The length of the strip outside the electrolytes was nearly 3 in.

A constant voltage of 250 V was applied, which resulted in an initial current

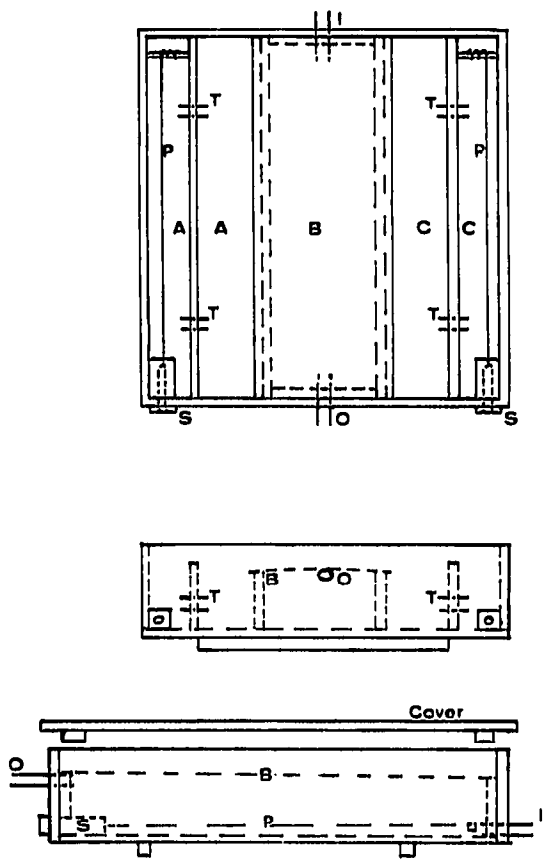
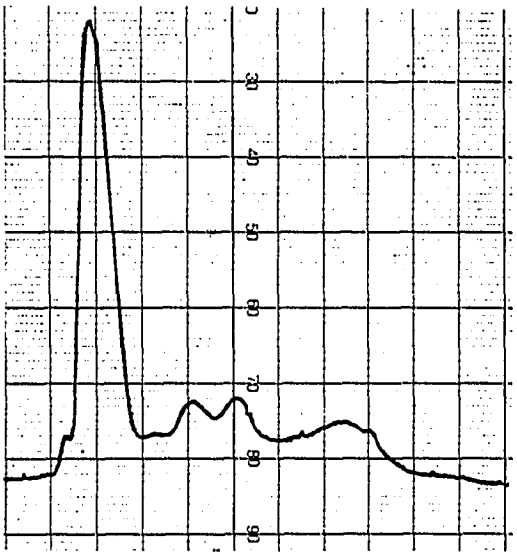


Fig. 1. Electrophoresis tank. Material: Perspex. Scale: 1:4. Cooling: tap water. Thickness of bridge cover: 1.5 mm. A = Anode compartment; B = bridge; C = cathode compartment; I = inlet water; O = outlet water; P = platinum wire; S = socket; T = tube filled with glass-wool.

of about 0.5 mA per strip. This current slowly decreased during the isotachophoretic stage and increased to an approximately constant value during the zone electrophoresis stage. If this current is examined, it gives a good indication of the progress of the analysis.

The optimal sample volume was determined by its protein concentration. A total amount of protein of $4 \cdot 10^{-5}$ g per strip gave good results (*e.g.*, 0.5 μl of normal serum or about 100 μl of normal spinal fluid). Small sample volumes (less than 10 μl) were applied with a cotton thread stretched in a metal fork with non-conducting handle. Larger samples were applied with the aid of a piece of filter-paper placed on the strip. Care must be taken that the sample is applied equally over the full width of the strip.

The samples were applied 5–10 min after the current had been switched on. The best position for the application is on the boundary of LE and TE, but in this system it does not matter if it is applied in the LE or TE zone close to this boundary. This boundary can be made visible by the use of a trace amount of anionic dye, which should have an effective mobility intermediate between those of the LE and TE anions. This dye should be applied on the strip at the edge of the cooling bridge (cathode side) soon after the analysis has been started, and it will be concentrated at the boundary. However, the dye may react with some protein fraction. Another possibility is to use several strips simultaneously, on only one of which is the trace amount of dye applied and this "dye strip" is used to indicate the position of the LE–TE boundary. Strips that have been started simultaneously have boundaries at equal distances from the liquid level in the cathode compartment. Also, a small part of the protein sample can be put on the strip instead of a dye and it will be concentrated



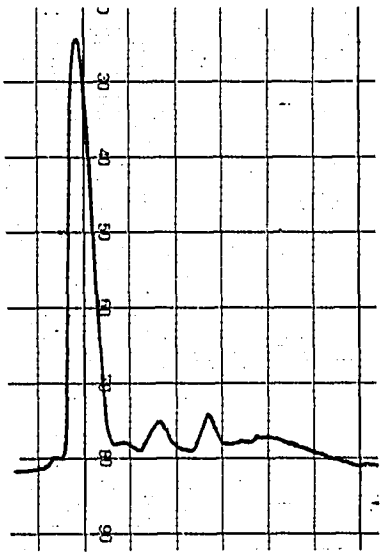


Fig. 2. Densitometer scan of serum analysis. Conditions as described in the text. Densitometer: Kipp & Sons DD 2.

Fig. 3. Densitometer scan of spinal fluid analysis.

into a faintly grey-yellow band between LE and TE. The main part of the sample is applied later, on this band.

About 10–15 min after the start of the second stage, the current was switched off and the proteins were fixed by means of Amido Black 10B or a Ponceau solution. The total analysis time was 30–40 min.

Some experiments were also carried out with spinal fluid. The time available for the concentration stage (about 15 min under the circumstances described) is too short for the application and concentration of the large sample volume that is needed. A preliminary concentration was carried out on the same strip in a separate tank, which contained only LE and TE. The strip was then placed in the discontinuous electrophoresis tank. The total time for these analysis was 2–3 h, depending on the concentration of proteins in the spinal fluid. Samples with low concentrations need more time.

Densitometric scans of serum and spinal fluid, analysed according these principles, showed no differences to normal zone electrophoresis patterns of the same samples. Figs. 2 and 3 show some scans after discontinuous electrophoresis on strips.

DISCUSSION

The reproducibility of the serum analysis on Sepraphore III strips was very good. However, more recent experiments had to be carried out on an improved version of these strips (Lot Nos. 80664 and 80956). These new lots may give better results for zone electrophoresis, but the results of this type of discontinuous electrophoresis on the new strips were less reproducible.

In isotachophoresis, each type of ion will be separated from other ions with the same sign (unless the differences in effective mobilities of these ions are too small). This implies that there will be no other anions in the zones of the proteins when the steady state is reached, which may lead to denaturation and coagulation of the proteins in the strip. In some instances better results were obtained by doping the TE with sodium chloride, for example an amount of 2% (w/w) of the glycine used. Impurities such as Cl^- (or SO_4^{2-}) ions will migrate through the protein zones and in this way the stability of the proteins is increased.

When using strips, water will evaporate owing to the development of Joule heat. Care has to be taken that experiments are performed with low current densities and adequate cooling of the strips. Evaporation of water will change the electrolyte conditions and so it will influence the whole system of zones. Evaporation of water will also cause liquid flow in the strip.

After the writing of this paper, the author found that the same idea had already been published by Jovin[7]. However, Jovin did not indicate any electrolyte system that can generate the desired effects. Our first experiments with the acetate–glycinate/Tris–piperidine system were carried out in 1972[8].

CONCLUSION

It is possible to perform discontinuous electrophoresis based on the use of two counter-ions. Although there is much work still to be carried out, this type of electro-

phoretic system can be used to analyze dilute samples of proteins or other ionogenic compounds. The concentration step characteristic of isotachophoresis has been employed. Detection of the zones can be achieved as in normal zone electrophoresis.

Analysis in gel-filled tubes or in capillaries will probably be even easier to perform.

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