ZONE ELECTROPHORESIS IN STARCH COLUMNS

by

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In the last few years several methods have been developed for the preparative separation of substances by zone electrophoresis (*cf.* TISELIUS AND FLODIN[1]). Paper strip electrophoresis in its usual form is not suitable since only very small quantities can be separated. Electrophoresis in piles of paper (KUNKEL AND TISELIUS[2]) or in troughs packed with silica gel (CONSDEN *et al.*[3]), paper powder or starch (KUNKEL AND SLATER[4]) often gives good results. However, the reproducibility is not quite satisfactory and the separations are not always clear-cut. BUTLER AND STEPHEN[5] and HAGLUND AND TISELIUS[6] used columns packed with asbestos fibres and glass beads, respectively. The arrangement of the last mentioned authors has formed the basis for the apparatus and technique described in the present communication. BOCKEMÜLLER AND REBLING[7] recently published the details of an apparatus in which as much as 200 mg blood serum proteins were separated with paper powder as supporting medium.

Irreversible adsorption of high molecular weight substances is a common cause of difficulties in this type of work and makes the proper choice of a supporting medium a matter of utmost importance. In order to minimize adsorption on glass beads the particles must be relatively large (0.1 mm diameter), which is undesirable, since it makes difficult the control of the displacement of the liquid in the column. Of the media mentioned above, only starch was satisfactory for use with the mixtures analysed by us. In general the adsorption on starch was found to be small, although some positively charged proteins gave the unsymmetrical peaks and trails, characteristic of this phenomenon. In addition, a good quality starch is easily obtained, easily packed and gives low electro-osmotic flow. However, starch is not always the ideal supporting medium and difficulties such as adsorption and strong electro-osmosis may arise in certain systems or with certain buffer media.

EXPERIMENTAL TECHNIQUE

The apparatus. An experiment of the type to be described here is made in three main operations: the application of the zone, the electrophoresis and the elution. All three operations are important if good results are to be obtained, and, therefore, the apparatus should be constructed so that they all can be performed as conveniently and reliably as possible.

The main part of the apparatus, the column, consists of a glass tube 50 × 3 cm (Fig. 1). A coarse porous glass filter, c, at the bottom supports the starch. The porosity and the thickness of the glass filter must be chosen so that the electrical resistance will

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be as small as possible. An ordinary glass filter (#2) from a glass filter funnel is used by us. The ground joint at the lower end permits connection via the limb to one of the electrode vessels. The other electrode vessel is joined to the column by the side tube. The top of the column is closed by a glass stopper during the run. When the electrophoresis is finished, the column is disconnected, whereupon a container for the elution liquid is connected to the system*.

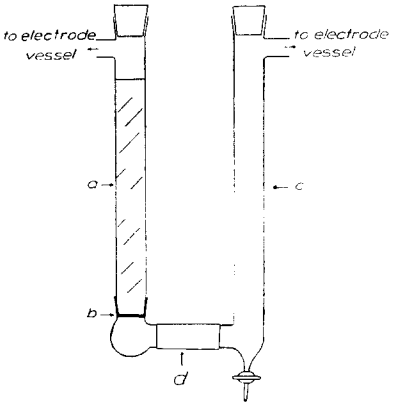

Fig. 1. Schematic drawing of the column and the U-tube arrangement. *a*, glass tube 50 × 3 cm filled with starch; *b*, glass filter; *c*, glass tube; *d*, rubber tubing.

Platinum electrodes have been used occasionally but special precaution must then be taken to diminish the effect of the electrode reactions. The conventional silver-silver chloride electrodes of the type used in the Tiselius apparatus were therefore used in all experiments described here. The electrode vessel volume is about one liter each and the electrodes permit the passage of about 3000 coulomb. Since electro-osmosis and volume changes in the electrode reactions tend to change the buffer levels in the electrode vessels they are connected by a narrow rubber tubing so that no liquid will be forced to move through the column by inequalities in hydrostatic pressure. The system is open at one point to allow for changes in the total volume caused, for instance, by temperature changes.

Packing of the column. Ordinary commercial potato starch was used after repeated washing with buffer solution and decanting four times with the buffer. It is somewhat better to use that fraction of the starch which passes a 300-mesh sieve. Since the starch varies in quality from batch to batch, it is recommended to test the packed column by eluting a suitably coloured zone as described below.

When packing the column, certain precautions must be taken. It is necessary that the glass tube be kept vertical. Air bubbles in the glass filter, which may prevent any passage of current, are avoided by dipping the empty tube into a beaker with buffer solution and letting the buffer penetrate the filter. Filter papers are then placed on the glass filter and a suspension of starch in buffer solution (1:4 wet volume) is poured in and the particles allowed to settle. An indication of good packing is that a boundary can be seen moving upwards. When most of the starch has sedimented, the nearly clear supernatant is removed and another portion is poured in. This procedure is continued until the starch boundary has reached a level about 5 cm below the side tube. If the starch is allowed to settle completely before a new portion is added discontinuities appear, which may disturb zones passing through. After washing with about half a liter of buffer solution, the column is ready for use.

Application of the initial zone. In order to make the initial zone narrow and well-defined it is of importance that the upper starch-buffer interface is horizontal. This can be achieved by stirring up about one cm of the starch column in a few ml of supernatant and letting it sediment. Just when the buffer surface coincides with that of the starch the flow through the column is stopped and the mixture to be analysed added on top of it. In order not to disturb the starch surface, this should be done by careful

* An apparatus of this type is manufactured by AB LKB-produkter, Stockholm 12.

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dropwise addition from a pipette or, better, a syringe. When all the solution has been applied, the flow is started again. At the moment the liquid disappears the flow is stopped and a few ml of buffer is added in the same way as the sample. When the zone has left the boundary region, a plug of cotton is placed on top of the starch whereupon the system can be filled with buffer. To minimize the disturbances due to electrical boundary phenomena it is advantageous to displace the zone about 5–10 cm down the column when the sample only contains components moving in one direction. This is accomplished as described later for the elution of the components. If both positively and negatively charged substances are present, a suitable starting point should be chosen, taking the electro-osmotic flow into account.

Electrophoresis. The U-tube and the electrode vessels are now connected, the whole apparatus immersed in a thermostat and voltage applied. A simple vacuum-tube rectifier limiting variations in the voltage to about one per cent was used. With the Veronal buffer of pH 8.6, μ 0.05, 700 volts were applied giving about 25 mA. The time required for the electrophoretic part of the experiment is 15–20 hours with blood serum.

Elution. At the end of the electrophoresis, the starch column is disconnected and the solution displaced with fresh buffer. The eluate is collected in a fraction collector, which in most cases was a time-regulated one. Too rapid elution will make the zones wide. With the column used by us, a flow rate of about 10 ml/hour gave well-defined peaks. Generally 50 fractions of 2 ml were taken. The protein concentrations were determined according to the modified Folin reaction as described by LOWRY *et al.*[8] but no attempt was made to obtain strictly quantitative results. It should be noted that the regeneration takes place in the same operation as the elution. Thus, a column once packed can be used many times, *e.g.* ten experiments have been performed in one column without any detectable changes in its properties (provided growth of microorganisms is avoided).

PROPERTIES OF THE COLUMN

It was, of course, desirable to investigate some of the more important factors involved in the electrophoresis in starch columns. This is most conveniently done if the movement of a zone during different conditions is followed directly on the column. A zone containing phycoerythrin was introduced for such purposes. This strongly coloured protein is not adsorbed by the starch under the conditions used in the present study. It may be mentioned that phycoerythrin is rather strongly adsorbed in filter paper.

Cross-sectional area etc. To determine the evenness of packing, the cross-sectional area and the hold-up volume, a narrow zone of phycoerythrin dissolved in buffer was displaced through the column. In an ideal column the eluted zone should have the same volume as the one applied. In the best columns with 2-ml starting zone, more than 90% of the coloured material emerged in 6 ml.

When plotting the migration of the zone against the effluent volume, a straight line relationship should be obtained. The slope of this line gives the cross-sectional area which may be used for mobility calculations. It was found to be approximately 1.9 cm^2 in all our experiments. The ratio (starch volume/mobile liquid volume) can now be calculated and was found to be 2/1. KUNKEL AND SLATER[4], weighing the starch dry and wet, found the corresponding ratio to be 1/1. The discrepancy is probably due to the fact that a considerable part of the water is firmly bound to the starch and thus not detectable by the mobile liquid determinations made by us.

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Potential gradient. Since the starch-buffer ratio is initially constant throughout the column, the potential gradient is also constant. However, boundary polarization phenomena at the starch-buffer and glass filter-buffer interfaces will gradually distort the electric field thus giving rise to conductivity gradients in the column, as discussed by TISELIUS AND FLODIN[1]. As in boundary electrophoresis the substances to be separated are liable ultimately to contribute to the conductivity changes. It can thus be expected that a very complex conductivity curve will be obtained when measuring the eluate resistance. A continuous curve of the effluent conductivity was taken and compared with the Folin reagent curve. There was very little correlation between the two curves, which shows that large conductivity changes do occur. This could also be demonstrated by UV measurements since Veronal buffers have high molar extinction at 280 mμ. However, these large variations did not substantially influence the separations obtained, not even when very highly concentrated samples were analysed.

Electro-osmosis. The electro-osmotic flow is most conveniently measured with a substance of zero mobility, for instance dextran or glucose. The liquid transport through the system can be directly measured but the volume changes during the electrode reactions must be taken into consideration. The electro-osmotic flow is largely dependent on the buffer system used. While Veronal buffers gave low flow, as was also found by KUNKEL AND SLATER[4], phosphate buffers in the same pH range gave rise to strong electro-osmosis making it difficult to obtain clear-cut results. Therefore, Veronal and acetate buffers were used exclusively in this investigation.

Cooling. At first, the experiments were made in a cold room with air cooling only. Good separations were obtained but the cooling was inefficient and the current rose considerably. When only 0.075 watt/cm length of the column were applied (calculated from $w = i^2/q$) the current increased 20–30% during an experiment. When the apparatus was immersed in a water thermostat, 0.13 watt/cm gave only small change in the current and not too great a spreading of the zones. Still higher wattages were found to disturb the zones because of temperature gradients inside the column. A cooling device like that of HAGLUND AND TISELIUS[6] allows the use of a higher power and is probably indispensable if columns of a larger diameter are to be used.

Capacity of the column. The zone spreading limits the resolution of a mixture and is determined by several factors such as filtering properties of the medium and convections. The extent to which the loading of the column effects the zone spreading during an electrophoresis run was studied as follows. Two experiments were first performed in which the width of the starting zone was the same (2 ml). In the first one 1.5%, in the second one 0.15% human serum albumin solution was used. A total recovery of the albumin was obtained, between 80 and 90% of which was found in a volume three times as large as that of the solution added. As the spreading of the zone is roughly the same in the two experiments, it is evident that the resolving power cannot be increased appreciably by decreasing the amount of substance.

It is of particular interest to find out to what an extent the column can be loaded. An experiment was therefore performed with a 10% albumin solution. Even with this high concentration the zone does not widen appreciably more than in the experiment with 0.15% albumin solution (see Fig. 2). Thus, the amount of substance (in a very wide range) has a smaller influence on zone spreading than other factors, which obviously is of great interest for preparative purposes.

Adsorption. As seen from the good recoveries of serum albumin, the fact that

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albumin is displaced through the column by an amount of liquid equal to the hold-up volume and the symmetrical shape of the curves obtained, it appears that adsorption does not occurs. Similar results were obtained with all negatively charged substances tried. Electrophoresis of lysozyme, however, gave a much wider zone with tailing. The curve has the same shape as that found by KUNKEL AND SLATER[4] and can be explained by irreversible adsorption of the positively charged protein.

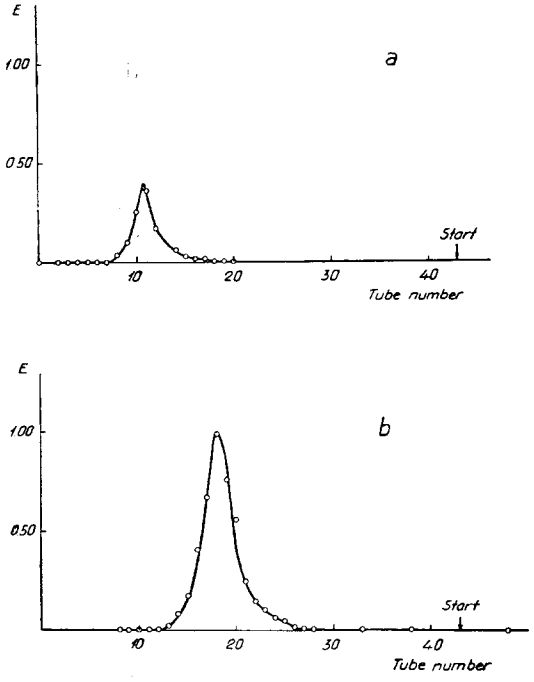

Fig. 2. Distribution curves obtained in two experiments with human serum albumin. Veronal buffer pH 8.6, $\mu = 0.05$. Starting zone 2 ml. Each fraction about 2 ml. Protein concentrations: *a*, 0.15%; *b*, 10%. In *a* the entire fractions, but in *b* 0.1 ml of each fraction were used for the Folin reaction.

Separations performed. Blood serum and plasma have been extensively studied and therefore represent excellent model mixtures for comparative studies. Fig. 3 shows a diagram of human blood plasma mixed with some phycoerythrin as obtained by starch column electrophoresis. The crossed points refer to phycoerythrin measured at 535 mμ. The overlapping of the two large peaks is small, making it possible to recover most of the material in each peak completely free from that in the other one. The diagram agrees very well with the curves obtained by other zone electrophoresis methods. In Table I, some serum and plasma experiments with the present technique are compared to results with paper and boundary electrophoresis. The mobilities relative to those of albumin and γ-globulin as determined by boundary electrophoresis have been calculated. The values obtained vary very little from serum

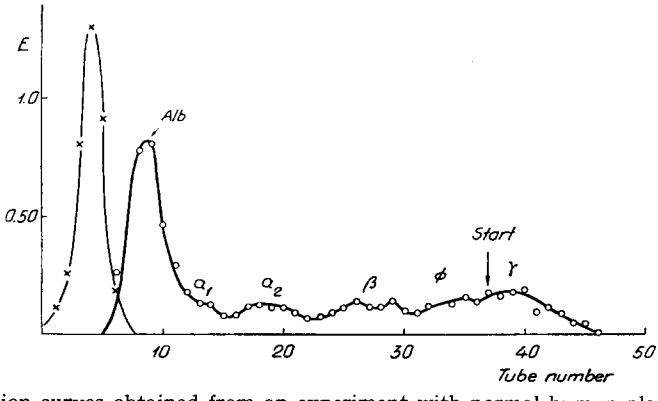

Fig. 3. Distribution curves obtained from an experiment with normal human plasma with phycoerythrin added. Veronal buffer pH 8.6, $\mu = 0.1$, 18 hours with 30 mA. The distribution of the phycoerythrin (X) was measured spectrophotometrically at 535 mμ. The plasma protein was determined by the modified Folin reaction.

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to plasma. It is very interesting that an experiment with high protein concentration gives similar figures (Experiment 4). The relative mobilities are also closer to the values in boundary electrophoresis than are those from paper electrophoresis. Only little work has been carried out on absolute mobilities in this study, but the results indicate that, using the value for the cross-section determined as described above, correct values are obtained.

TABLE I

RELATIVE MOBILITIES OF SOME BLOOD SERUM PROTEIN FRACTIONS. COMPARISON BETWEEN THE RESULTS WITH STARCH COLUMN, PAPER AND BOUNDARY ELECTROPHORESIS. MOBILITY VALUES OF 6.43 AND 1.11 $cm^2\ sec^{-1}\ volt^{-1}$ (OBTAINED BY BOUNDARY ELECTROPHORESIS) USED FOR ALBUMIN AND γ-GLOBULIN, RESPECTIVELY

Experiment		*Globulin*	
	α_1	α_2	β
1 (plasma)	5.82	4.59	3.10
2 (serum)	5.87	4.64	3.03
3 (serum)	5.65	4.60	3.18
4 (serum)*	—	4.52	3.28
KUNKEL-TISELIUS (1951)**	5.05	3.74	2.85
Boundary electrophoresis	5.49	4.51	3.06

* About 300 mg protein in a starting zone of 6 ml
** Average values

An antibiotic mixture containing bacitracin has been found to be an interesting object for separation studies because it contains peptides of about the same molecular weight (1400–1500) and amino acid composition. Fig. 4 shows the material distribution in one experiment. The bulk of activity was concentrated in the largest peak. When the concentrations were raised it was found that the overlapping of the zones became disturbing only at concentrations higher than 20%. Zone electrophoresis is, therefore, very suitable for preliminary separations of these polypeptide mixtures. It should be mentioned, however, that the material in the two main peaks was found to be heterogeneous by other methods (J. PORATH, to be published).

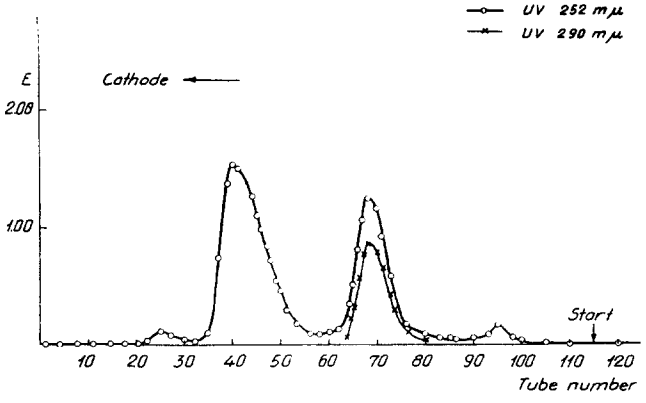

Fig. 4. Distribution of bacitracin polypeptides. Acetate buffer pH 4.5, $\mu = 0.15$; 44 hours, 35 mA. One ml buffer containing 100 mg peptides. The UV absorption was measured at 252 mμ (O) and at 290 mμ (X).

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DISCUSSION

The method described here has some definite advantages compared with other zone electrophoresis methods designed for preparative work. It is inexpensive and simple to use, the experiments are easily reproducible and are quantitative. Since a column is regenerated in the elution process it can be used repeatedly. In this Institute the method has been used to separate serum and plasma proteins, peptide mixtures and the proteins in liquor cerebrospinalis and to purify synthetic cocarboxylase. As a supporting medium, starch seems to offer some advantages over most other materials tried so far.

An important advantage is that relatively high concentrations may be analysed without sacrificing the resolving power. With proteins and peptides, amounts of about half a gram have been separated. It may, however, be possible to separate still larger quantities by increasing the dimensions of the apparatus. For the electrophoresis with these larger columns, it will certainly be necessary to use some internal cooling device and also electrode vessels of larger capacity. Perhaps continuously flowing buffer over the electrodes will prove to be the best solution to the latter problem. It has been estimated that a ten-gram scale can be reached without introducing too great technical problems. Work is in progress to extend the applicability of zone electrophoresis in columns.

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SUMMARY

1. An apparatus for zone electrophoresis in a starch column with subsequent elution into a fraction collector is described.
2. Some properties of the column are discussed.
3. Separations of artificial and natural mixtures as well as the use of the apparatus on preparative scale are described.
4. The advantages of the method described as compared to other zone electrophoresis methods are discussed.

RÉSUMÉ

1. Un appareil pour l'électrophorèse sur colonne d'amidon suivie d'élution sur collecteur de fractions est décrit.
2. Les propriétés de la colonne sont discutées.
3. Des séparations de mélanges naturels et artificiels ainsi que l'emploi de l'appareil à l'échelle préparative sont décrits.
4. Les avantages de la méthode décrite par rapport aux autres méthodes d'électrophorèses sont discutés.

ZUSAMMENFASSUNG

1. Es wird ein Apparat zur Zonenelektrophosphorese in einer Stärkesäule mit darauffolgender Elution in einem Fraktionssammler beschrieben.
2. Einige Eigenschaften dieser Säule werden besprochen.

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3. Es wird die Trennung künstlicher und natürlicher Gemenge sowie die Benützung des Apparates für präparative Zwecke beschrieben.

4. Die Vorteile der beschriebenen Methode verglichen mit anderen zonenelektrophoretischen Methoden werden besprochen.

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