REVIEW

STARCH ELECTROPHORESIS
II. STARCH COLUMN ELECTROPHORESIS

H. BLOEMENDAL

Department of Biochemistry, Antoni van Leeuwenhoek-Huis: The Netherlands Cancer Institute, Amsterdam (The Netherlands)

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In the first part of this survey on starch electrophoresis[63] the starch block technique was discussed and the conclusion was drawn that generally the best separations are realised when the starch block is in a vertical position during the electrophoretic run. In principle similar results are obtained when columns are used. Nevertheless I wish to make a distinction between vertical starch block electrophoresis and starch column electrophoresis, because the analytical operations after the experiment are different in these two methods. In the first technique the block is cut into equal segments to separate the zones, whereas in the second the separated zones are forced out by means of a buffer stream, which makes the use of a fraction collector necessary. Although originally only starch was used as the inert supporting medium[3,4] in column electrophoresis, at present there is a tendency to apply cellulose powders instead of starch[64–69]. Actually more separations are carried out with other media than starch as stationary phase. These experiments will not be discussed in this article. But even cellulose column electrophoresis is not so widely applied as starch block electrophoresis and starch gel electrophoresis. Undoubtedly the reason for this is that in general no better results are obtained with column electrophoresis than with the two last-mentioned techniques, while its procedure is more complicated.

METHODS

1. *Apparatus*

HAGLUND AND TISELIUS[70] described an apparatus for glass powder electrophoresis, which consisted mainly of a U tube and two electrode vessels. This apparatus was improved by FLODIN AND PORATH[4] who replaced the glass powder by potato starch.

A glass tube s (Fig. 7), 50 × 3 cm, with a sintered glass filter f at the bottom is joined to a second tube by means of rubber tubing. The porosity and thickness of the glass filter must be chosen so that the electrical resistance is as small as possible. The filter surface may be covered with a disc of Whatman No. 1 paper. The tube, which is filled with starch, is connected to the cathode and the second tube is connected to the anode, both connections being made with rubber tubing that can be closed by clamps. Reversible silver–silver chloride electrodes as well as platinum wire wound round glass rods may be used as cathode and anode. HAGLUND AND TISELIUS recom-

mend the use of an open system, which allows the electroosmotic flow to develop freely. This is realized by connecting a narrow horizontal glass tube, open at one point, to the electrode vessels. In this way a constant hydrostatic pressure is maintained in the whole system during the electrophoretic run. In some experiments the apparatus is placed in a thermostat.

VANDEGAER, PRÉAUX AND LONTIE[71] found a more simple solution for the cooling problem. The apparatus that these investigators employed was in principle the same

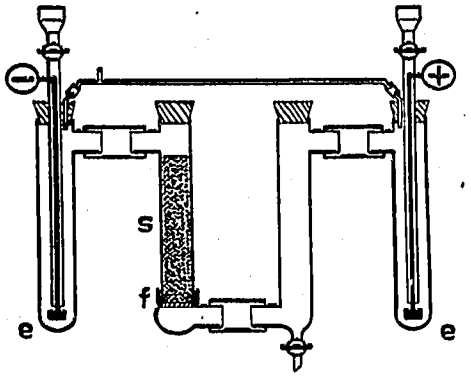

Fig. 7. Apparatus according to HAGLUND AND TISELIUS. e = electrodes; s = tube filled with starch; f = glass filter.

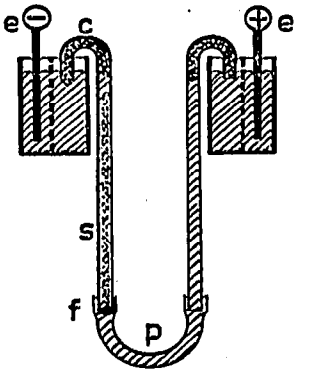

Fig. 8. Apparatus according to KUNKEL. e = electrodes; s = tube filled with starch; f = filter disc; c = cotton wicks; p = plastic tubing.

as that of FLODIN AND PORATH, but they constructed a water-jacketed column. Efficient cooling was obtained by tap water of 2°.

CARLSON[3], keeping to the design of HAGLUND AND TISELIUS, placed a central cooling tube in the starch column.

BOLINGER, VAN DER GELD, WILLEBRANDS AND GROEN[72] improved the original apparatus of FLODIN AND PORATH by applying a device that allowed constant removal of the electrolysis products from the electrode vessels.

KUNKEL[73] described an apparatus that can easily be constructed in any laboratory. Two glass tubes s, 40 × 1 cm, are joined together by rubber or plastic tubing p (Fig. 8). Tube s is fitted with a glass filter f to support the starch. The connections with the electrode vessels consist of cotton wicks c enclosed in bent glass tubes or merely covered with cellophane or parafilm.

SVENSSON[74], in collaboration with FLODIN AND PORATH, designed a commercial apparatus. The glass tube is surrounded by a water jacket, and the connections with the elec ode vessels and the special filling device are supplied with ground glass joints. For preparative work, columns of considerable length may be used. PORATH[69] purified posterior pituitary extracts in externally cooled columns of 300 × 1.5 cm. These experiments were carried out on cellulose powder, but starch columns of comparable or even greater length may be applied.

2. *Preparation of starch*

Homogeneous packing is essential in all chromatographic techniques where powdered materials are used. Also, in starch column electrophoresis irregular filling of the tube

would result in tailing and spreading of the zones. The starch may conveniently be prepared as described in Part I[63]. FLODIN AND PORATH recommend the use of a starch fraction that passes a 300-mesh sieve. Application of the packing method of MOORE AND STEIN[75] also gives good results. In this technique starch is suspended in butanol and ground in a mortar. Distilled water is added to the suspension until the amount is 30 % of the dry starch. The slurry is poured into the column and slight air pressure is applied. The starch is allowed to settle during a period of one hour. Air bubbles can be avoided by dipping the empty column in water so that the filter-disc surface (Fig. 7, f) within the glass tube is covered with the liquid. The butanol above the starch is withdrawn by suction. Afterwards water is poured in very carefully and the column is connected to a Mariotte flask filled with distilled water. The water is allowed to pass through the column until an eluate shows no, or minimal absorption at 260 or 280 mμ against a water control. These washings are repeated with the buffer solution in which the electrophoresis will be carried out. The column must be kept exactly vertical during the electrophoretic run as well as during the elution.

3. *Introduction of the sample*

Before the electrophoretic run is started, the buffer solution above the starch surface is withdrawn with a pipette or driven down by slight pressure so that the upper starch layer is just dry. The sample is introduced dropwise by means of a funnel fitted with a narrow tube or simply with a pipette. The test solution is then slowly forced into the column by slight pressure. If there are components that will migrate towards the cathode, the sample must be transported over a certain distance before electrophoresis is started. In order to determine this distance the most convenient method is to add known amounts of buffer solution. The relation between the required buffer volume and the vertical displacement is easily found by trial using a coloured substance (*e.g.* haemoglobin or albumin stained with bromophenol blue). When the sample is in the desired position, the column is filled with buffer and connected to the electrodes.

4. *Electrophoresis*

As in the case of starch block electrophoresis, there are no standardized conditions for all starch column electrophoresis experiments. The most favourable voltage, current, buffer, and the duration of the electrophoretic run must be found by trial. Much depends on the packing and the length of the column. Though continuous cooling by circulating ice water allows the application of higher voltages than those used without cooling, the heat development even then represents a serious limitation of the method. In many cases asymmetric band formation causes insufficient separation. This effect becomes serious when columns with a large diameter are used. The best results are generally obtained with columns not much longer than 1 m and with a diameter not exceeding 6 cm.

CARLSON[3] observed considerable spreading of the zones when phosphate buffers

were used. Veronal and ammonium buffers gave better results. Increasing the salt concentration had no influence upon the spreading of the protein zones.

According to FLODIN AND KUPKE[67] citrate and phosphate buffers give rise to strong electroosmotic flow in the starch medium.

In Table III the working conditions of a few investigators are summarized.

TABLE III

Material	*Buffer*	*Ionic strength*	*pH*	*V*	*mA*	*h*	*Reference*
Serum lipoproteins	ammonium	0.1	9	350	—	—	3
Serum	veronal	0.05	8.6	700	25	12–20	4
Bacitracine polypeptide	acetate	0.15	4.5	—	35	44	4
Serum	veronal	0.1	8.6	400	5	18	14
Insulin activity in serum	bicarbonate	—	8.0–8.3 6.8–7.0	350	100	24	72
Thiamine and its phosphoric esters	phosphate	0.05	5.44	—	17	12	82
Vitamin B_6 compounds	acetate	0.05	5.1	—	18	14	83
Pancreatic lipase	acetate*	0.025	5.25	8 V/cm	—	48	85

* 0.005 *M* $CaCl_2$ added.

5. *Influence of temperature*

As mentioned earlier the rise of temperature as a result of the application of high voltages represents one of the most serious limitations of starch electrophoresis. In practice the relation between the applied voltage and the rise of temperature during the electrophoretic run is easily determined by measuring the temperature in the column with a thermistor at certain intervals and plotting the temperature against the time (*cf.* Fig. 3 in Part I). Only a little theoretical work has been reported on the problem of temperature distribution in columns[76, 77]. Recently PORATH[78] described a device for temperature measurements in cylindrical electrophoresis columns without central cooling. This author derived a fairly simple equation which allows a rough estimation of the permissible current values in an electrophoresis experiment.

The assumption is made that heat is transported only by conduction. In this case the heat flow at time t at any point of the system is described by the general equation:

$$\frac{\partial T}{\partial t} = \operatorname{div} \frac{\lambda}{c\rho} \operatorname{grad} T + \frac{Q}{c\rho} \qquad (1)$$

in which T = temperature
λ = thermal conductivity coefficient
c = specific heat
ρ = density
Q = heat generated per unit time and volume.

For cylindrical columns packed with powders like starch this equation reduces to:

$$c\rho \frac{\partial T}{\partial t} = \lambda \Delta T + Q \qquad (2)$$

in which Δ = Laplacian operator.

It can be deduced that if the internally and externally cooled column were filled with buffer alone, the heat generated would be:

$$Q = 0.239 \frac{i^2}{\varkappa} \tag{3}$$

in which i = current density
$\varkappa$ = conductivity of the buffer solution
Q = heat generated (expressed in calories per cm^3).

Actually the effective cross section is reduced by the uncharged starch particles so that equation (3) becomes:

$$Q = 0.239\, k \frac{I^2}{q^2 \varkappa} \tag{4}$$

in which k = a constant characteristic for the (starch) medium
I = current
q = cross section of the column.

According to PORATH the water content in starch and cellulose differs considerably (33 and 85 % per volume, respectively). This difference is reflected in the k values.

Furthermore, PORATH evolved an equation which holds when the column is cooled with circulating water of constant temperature without central cooling:

$$T_m - T_r = \frac{Q r^2}{4 \lambda_c} \tag{5}$$

in which T_m = maximum temperature
T_r = temperature at r cm from the axis
λ_c = conductivity coefficient of the column content.

($\lambda_c = 2.2 \cdot 10^{-3}$ cal·degree $C^{-1} \cdot cm^{-1} \cdot sec^{-1}$ for starch packed in a column of 105 cm length and 3.4 cm diameter).

Inserting Q from equations (4) and (5) gives:

$$T_m - T_r = 0.239\, k \frac{I^2 r^2}{4 q^2 \varkappa \lambda_c} \tag{6a}$$

$$\frac{0.239\, k}{4 \pi^2 \lambda_c} = K$$

$$T_m - T_r = \frac{K \cdot I^2 \cdot r^2}{R^4 \varkappa} \tag{6b}$$

in which R = inner radius of the column.

Fig. 9 is the graphic representation of the latter equation, in which $K/\varkappa = 3200$, $I_I = 150$ mA, $I_{II} = 90$ mA.

For the difference between the temperature at the center T_m and the periphery T_0 (6b) simplifies to:

$$T_m - T_0 = \frac{K \cdot I^2}{R^2 \varkappa} \tag{7}$$

PORATH found that h has the value 4.12 in starch columns (determined at 15° for starch–0.1 M pyridinium acetate medium).

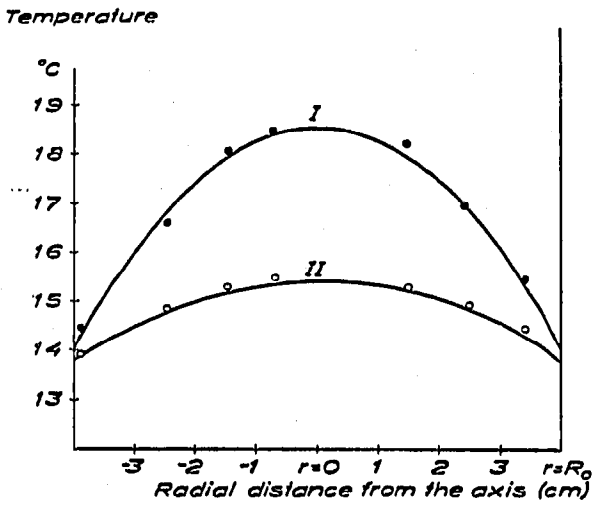

Fig. 9. Radial temperature distribution in a starch column 105 × 3.4 cm, 12 cm below the starch surface; current in I, 150 mA; current in II, 90 mA. (Reprinted with the permission of Dr. J. PORATH.)

6. *Elution and automatic analysis*

After the electrophoretic run the electrode vessels are disconnected and removed, and a Mariotte flask filled with elution liquid is connected to the column by means of rubber or plastic tubing. The Mariotte flask is used in order to obtain a constant hydrostatic pressure during elution. It is possible to collect the separated components in reagent tubes manually, but this is a very time-consuming business, so that an automatic fraction collector becomes really indispensable. Further saving of time is obtained by analysing the eluates in a continuous analyser.

In our laboratory a very simple apparatus has been constructed, which allows

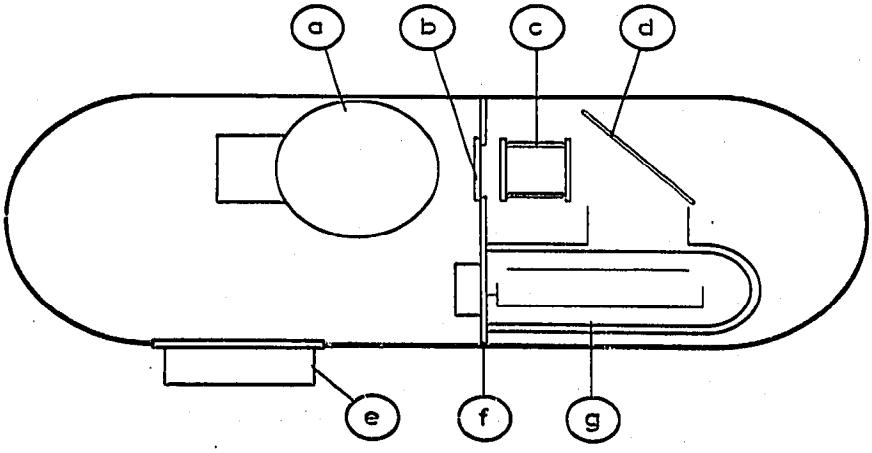

Fig. 10. Schematic diagram of the optical subunit of the fraction collector used in The Netherlands Cancer Institute, Amsterdam. (For explanation see text.)

continuous estimation of nucleotides, polynucleotides, and proteins[70] (Fig. 10). The light source is a low-pressure mercury lamp, a. The lamp compartment is divided into two parts by means of the partition f, in which there is a quartz window, b. The ultraviolet light-beam falls through the glass cuvette c, which is likewise supplied with a quartz window. The cuvette has been constructed in such a way that air bubbles can pass through without adhering to the wall. The transmitted light is not measured directly but via the fluorescent screen, d. The rejected visible light is measured with a common photocell, g. If it is desired to measure at other wave lengths the mercury

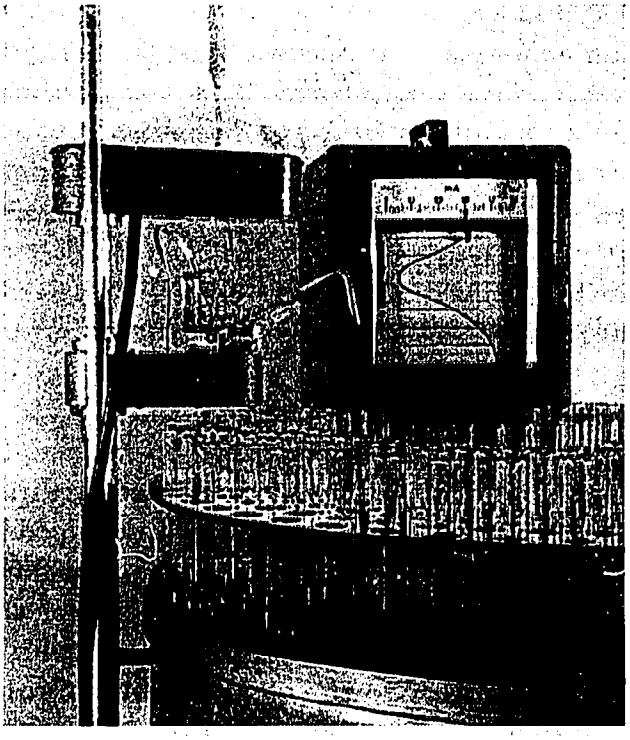

Fig. 11. Automatic fraction collector with recorder.

lamp can be replaced by other suitable light sources, if necessary in combination with filters. The optical subunit is connected to a recorder via a multipole plug, e. The fraction collector (Fig. 11) is furnished with a volume-measuring system. At the very moment that a certain tube has been filled, the connection between the vacuum tube volt meter and recorder is interrupted for a short time. The pen-recorder then falls back to its zero position. In this way, besides the registered curve, straight lines are drawn on the paper indicating the transition from one tube to the following one*.

PORATH and coworkers[87] designed an apparatus which allows continuous removal of the separated zones during electrophoresis. A buffer flow is applied in the opposite direction of the electrophoretic migration. This device permits the use of short columns and seems to counteract the broadening of zones during elution.

* Similar fraction collectors are commercially available (Dr. Gilson, Madison, Wisconsin, U.S.A.).

7. Influence of electroosmosis

As described in part I[63] the electroosmotic flow is detected with the aid of substances such as dextran or glucose that do not migrate in the electric field. However, it must be taken into account that there is some interaction between starch and glucose, so corrections must be made for the resulting retardation effect on the glucose molecules. MARCHIS-MOUREN *et al.*[85] gave examples of the electroosmotic flow under various conditions (Table IV).

TABLE IV

Buffer	*Na acetate 0.025 M + $CaCl_2$ 0.005 M pH 5.25*	*Citrate 0.025 M + $CaCl_2$ M/600 pH 6.15*	*Phosphate 0.025 M pH 6.75*	*Veronal 0.025 M + $CaCl_2$ 0.005 M pH 8.0*	*Veronal 0.05 M + $CaCl_2$ 0.005 M pH 8.0*
Potential gradient (V/cm)	6.15	9.4	8.0	6.7	6.7
Flow (cm/h)	— 0.64	— 1.1	— 0.78	— 0.51	— 0.76

According to FLODIN AND PORATH[4] monovalent buffers give rise to lower electroosmotic flow than do polyvalent buffer solutions.

APPLICATIONS

CARLSON[3] studied the electrophoretic behaviour of serum lipoproteins in nephrosis, diabetes, and essential hyperlipaemia. The recovery of the lipids and protein was about 100 % in monovalent buffers such as veronal. Adsorption was noticed when phosphate buffer was used. When the protein concentration was higher than 2%, spreading and overlapping of zones took place. The migration of albumin could be followed by staining with bromophenol blue prior to the electrophoretic run. The method revealed that there are at least five distinct lipoprotein components in serum.

CARLSON AND OLHAGEN[80] investigated chylomicrons in a case of essential hyperlipaemia. The chylomicron migrated as two distinct fractions. One fraction was associated with α-globulin, the other with β-globulin. These findings were in agreement with the results obtained with free electrophoresis, whereas paper electrophoresis gave only a single fraction[81].

FLODIN AND PORATH[4] separated human serum proteins and obtained patterns that agreed very well with the results obtained with other zone electrophoresis techniques. The same authors submitted an antibiotic mixture containing bacitracin to column electrophoresis on starch. Two main peaks were found. The bulk of the activity appeared to be concentrated in the largest peak. Other methods, however, revealed heterogeneity of this peak.

KUNKEL[73] fractionated serum. In some experiments the sample was made to migrate up the column; about 100 % recovery was obtained.

BOLINGER and coworkers[72] separated normal human serum. The fractions obtained were tested for insulin activity and the main activity was found in the β-globulin

fraction. ^{131}I was detected in the same fraction when it was added to the serum. Furthermore, some minor activity was found in the γ-globulins, indicating that in serum endogenous insulin is linked to different proteins.

SILIPRANDI AND SILIPRANDI[82] separated thiamine and its phosphate esters. Thiamine and its monophosphate moved towards the cathode, whereas diphospothiamine and triphosphothiamine migrated towards the anode. However, cellulose powder as inert supporting medium appeared to be superior. With this medium complete separation of thiamine and its phosphoric esters could be realised and quantitative recovery of each compound was achieved.

SILIPRANDI *et al.*[83] also investigated vitamin B_6 compounds. Resolution of a mixture of pyridoxamine, pyridoxal phosphate and pyridoxine was observed, whereas pyridoxal and pyridoxamine phosphate overlapped each other partially.

MARCHIS-MOUREN *et al.*[85] achieved 135-fold purification of pancreatic lipase with a 20 % overall yield, when column electrophoresis on starch was used as final step in a fractionation scheme including ammonium sulphate and acetone precipitation as well as differential adsorption on tricalcium phosphate and aluminium hydroxide. A higher potential gradient (8 V/cm), longer duration of the electrophoretic run and the use of an acid buffer resulted in higher resolution of components than reported earlier[84].

BOUSSIER[86] obtained a pure compound of serum mucoprotein haptoglobin. Starch column electrophoresis was applied in combination with ammonium sulphate fractionation and starch gel electrophoresis.

The last-mentioned technique, which is of growing importance and may possibly supersede paper electrophoresis, will be discussed in the last part of this review.

CONCLUSION

Starch column electrophoresis is not used so widely as the simpler block method. A disadvantage of the former technique is the necessity of elution by means of a buffer flow after the electrophoretic run, which gives rise to broadening of the zones. According to KUNKEL AND TRAUTMAN[88] as much as a two-fold increase in the width of a haemoglobin band was found when elution over a fairly long distance through the column was required. Therefore, this procedure is not reliable in homogeneity studies, since shape and width of the bands are strongly dependent on the distance traversed during the elution. Furthermore, in some cases elution causes tailing of zones which showed no adsorption on the medium during the electrophoretic migration. From these observations it is clear that components originally separated, the mobilities of which do not differ very much, may overlap each other as a result of the transport through the column. By cutting the starch in the starch block technique, this complication does not occur.

As a rule cellulose is a better medium than starch in column electrophoresis for low molecular weight substances. FLODIN AND KUPKE[67] consider the difference in electrophoretic behaviour of these substances in paper and starch as a disadvantage

of starch column electrophoresis. But this unpredictable behaviour would not really be a disadvantage if the resolving power were greater.

The principal reason why starch column electrophoresis is not so popular as the block and gel techniques is that it is not so easily manipulated as either of the two other methods and does not present appreciable advantages.

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