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EQUIPMENT FOR CONTINUOUS ISOELECTRIC FOCUSING

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SUMMARY

Equipment for continuous-flow isoelectric focusing has been constructed and used to study the separation of model proteins. Separation was first investigated in a sloping thin layer of Sephadex gel, but the separation capacity was low and the equipment was unsuitable for long periods of operation. A second apparatus was then constructed, with a vertical water-cooled separation chamber made of glass. Separation of haemoglobin and albumin was studied at different flow-rates, electric potentials and temperatures. The maximal separation capacity obtained was about 150 mg of protein per hour, and was limited by the amount of heat generated. Optimization of parameters influencing the generation of heat is therefore necessary when constructing equipment for continuous isoelectric focusing with high separation capacity.

INTRODUCTION

Isoelectric focusing has become an important tool in biochemistry, and the introduction of ampholytes suitable for establishing a continuous gradient of pH in an electric field has resulted in very high resolution in the separation of ampholytic substances[1]. The pH gradient is most frequently stabilized through the use of density gradients; however, density gradients are mechanically sensitive, and zones with high electrical resistance occurring during the isoelectric focusing can create local overheating and so cause mixing of the density gradient[2]. These disadvantages can be avoided by using a gel medium for stabilization of the pH gradient, and polyacrylamide gel has therefore been used as stabilization medium in column and in flat-bed electrofocusing[3]. Granular gels, such as Sephadex or Bio-Gel, are particularly advantageous, as they provide good anti-convective stabilization of the pH gradient and high resolution, thereby allowing simple techniques and apparatus to be used[4].

Preparative non-continuous isoelectric focusing using density gradients for stabilization of the pH gradient has been widely used on a small scale[5]. Density gradients, however, can only stabilize relatively small amounts of focused sample. Larger amounts of sample can be separated when granular gels are used for stabilization. In a flat bed containing 700 ml of granular gel, up to 2 g of protein could be separated within 30–40 h (ref. 6); separation of such quantities is sufficient for many purposes, but further scaling-up is often desirable.

Continuous-flow techniques will probably be necessary when separation of large quantities is required. An advantage of such a technique over non-continuous procedures is that the separated components are removed by application of a potential, thereby eliminating disturbance by diffusion. Equipment for free-flow continuous non-focusing electrophoresis, and for focusing electrophoresis, in which the liquid flows through an electric field between two glass plates 0.3–0.5 mm apart, has been described[7,8]. Laminar flow is maintained in order to minimize disturbance from gravitation and convection, and, in order to maintain stabilization, the size of the separation chamber is limited; large-scale application of such equipment, therefore, appears also to be limited.

A more promising approach for large-scale application would be continuous-flow isoelectric focusing in a gel-stabilized bed. Based on equipment for continuous electrophoresis as described by Svensson and Brattsten[9], Fawcett[5] designed a water-cooled separation chamber (made of plastic) with a volume of 200 ml, in which Sephadex G-100 gel was used as stabilizer; the sample-loading capacity was 300–500 mg of protein per day. Longer separation chambers and/or more efficient cooling might have permitted increased separation capacity.

In this work, equipment for continuous-flow isoelectric focusing has been constructed, with efficient cooling, and the continuous separation of some proteins has been studied.

EXPERIMENTAL

Equipment for separation in a thin layer

The gel support was made from a 1.5-mm-thick glass plate (150 × 220 mm) arranged with a slope of 30° (see Fig. 1), and cooling was based on a water-sprinkler system developed by AB Analysteknik (Vallentuna, Sweden)[10]. The gel was applied to the plate in a layer 0.8 mm thick, and the liquid flow was led out through 12 sections at the lower end of the gel, by means of strips of filter paper, to collector tubes.

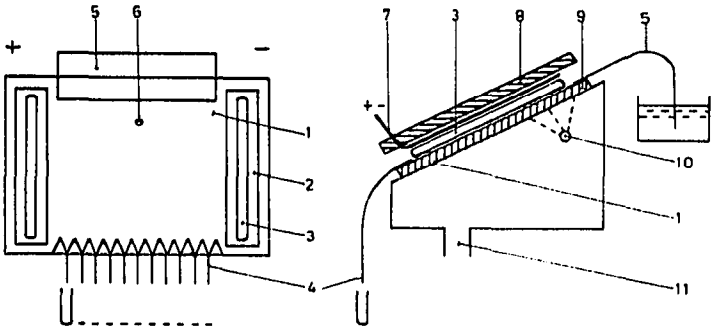

Fig. 1. Schematic diagram of equipment for separation in a sloping thin layer. 1 = Cooled sloping gel support (of glass); 2 = filter paper for electrode support; 3 = dialysis tubing containing electrolyte; 4 = strips of filter paper leading to collector tubes; 5 = filter paper for addition of Ampholine; 6 = site for application of sample; 7 = platinum wire for electrical connection; 8 = Plexiglas lid; 9 = granulated gel; 10 = sprinkler for cooling water; 11 = drainage for cooling water.

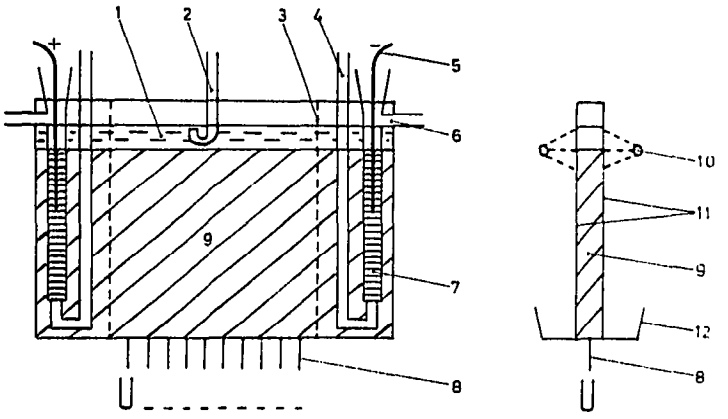

Fig. 2. Schematic diagram of equipment for separation in a gel bed. 1 = Ampholine layer; 2 = flow inlet; 3 = membranes separating electrode compartments from separation chamber; 4 = inlet for electrolytes; 5 = platinum wire for electrical connection; 6 = outlet for electrolyte; 7 = dialysis tubing; 8 = tubing for flow outlet; 9 = gel; 10 = sprinkler for cooling water; 11 = cooled chamber walls (of glass); 12 = drainage for cooling water.

Strips of filter paper were placed along the gel parallel to the direction of flow, and closed lengths of dialysis tubing filled with phosphoric acid and with sodium hydroxide solution, respectively, were laid on the filter paper. Platinum wires for electrical connection were attached to a lid of Plexiglas and were put on the dialysis tubing.

Equipment for separation in a gel bed

A chamber (200 × 250 × 9.5 mm) was constructed from 1.5-mm-thick glass plates (see Fig. 2), and a nylon cloth covered the bottom of the chamber and supported the gel; the liquid flow was led through 36 tubes from the bottom of the chamber to the collector tubes. The level of the outlet from the 36 tubes could be changed in order to regulate the flow through the gel, and the liquid level in the chamber was kept constant by means of an automatic level control.

Two water sprinklers were positioned on each side of the chamber for cooling; drainage for the cooling water was arranged at the bottom of the chamber as shown in Fig. 2.

Stabilizing media and samples

Sephadex G-75 gel swollen in 1% Ampholine solution was the stabilizing medium; 1% Ampholine of pH 3.5–9 was used in all experiments. The electrolytes in the dialysis tubing were 1 *M* phosphoric acid and 1 *M* sodium hydroxide.

Human haemoglobin, albumin and caeruloplasmin were used in solutions containing 50 mg of protein per ml. The albumin was stained with bromphenol blue and then dialyzed to remove excess of colour.

Analysis

The separated fractions were analyzed by isoelectric focusing as described by Vesterberg[11]. The identification and determination of albumin was by electro-immunoassay according to Laurell[12].

RESULTS AND DISCUSSION

Separation in a thin layer

In order to study the separation and isolation of ampholytes by isoelectric focusing, the equipment shown in Fig. 1 was constructed, and the separation of stained albumin and haemoglobin was studied with Sephadex G-75 gel as stabilizing medium in 1% Ampholine of pH 3.5–9.

With a potential of 30 V/cm, a pH gradient was gradually established, which could be followed by measurement of pH at the outlet and at the gel surface. With a gel length of 12 cm, a slope of 30° and a hydrostatic pressure of 10 cm of water, a flow of 5 ml/h was obtained, and a pH gradient developed in the lower part of the gel. The sample was injected at a point on the upper part of the gel, and the flow of sample was about 0.1 ml/h.

The flow through the gel layer was fairly stable and constant. The pH gradient was not developed linearly along the direction of flow, as was indicated by the shape of the bands of the focusing components and confirmed by direct measurements of pH (see Fig. 3). The applied potential was initially 30 V/cm; after some hours, when focusing was achieved, the potential was increased to 50 V/cm. The temperature could be kept below 10–15° over the entire surface (due to the efficient cooling).

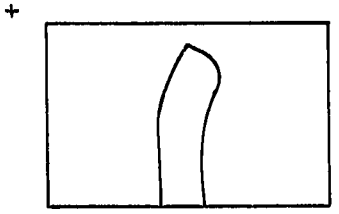

Fig. 3. Separation of albumin (left) and haemoglobin (right) in an established pH gradient.

Good separation was achieved and the fractions obtained were completely separated, as was shown by analytical isoelectric focusing[11] and electro-immunoassay[12]. Haemoglobin and stained albumin were mainly used as model substances, as they have well-separated isoelectric points and can be observed visually; some experiments were also performed with caeruloplasmin, but this protein was precipitated at its isoelectric point, and this distorted the separation.

A stable pH gradient and separation were obtained after a run of 3–4 h, and separation could be continued for up to 10 h. After that time, too much liquid was transferred to the cathode by electro-osmosis, and this liquid disturbed the flow in the gel and washed away gel near the dialysis tubing. It was also found that much of the electrolyte content was consumed during operation of the equipment for 10 h. For long-run operation, a different assembly of electrodes would therefore be necessary. The relatively small separation capacity (in this instance, 5 mg of protein per hour), however, makes it more promising to use a gel bed, which allows a higher flow-rate of sample.

Separation in a gel bed

With the equipment shown in Fig. 2, the electric current was transferred to the

gel via dialysis tubing through which electrolyte was flowing. The electrolytes were at first circulated in a closed system under slight hydrostatic pressure, but transfer of liquid into the cathode compartment occurred due to electro-osmosis. In order to avoid disturbance from electro-osmosis, each piece of dialysis tubing was connected to an overflow at the same level as the liquid in the separation chamber. In the first experiments, cellulose membranes were inserted between the separation chamber and the electrode compartments; these membranes were later omitted, as they sometimes caused electric sparks and became burnt. Separation of the pieces of dialysis tubing from the separation chamber was thought to be necessary in order to permit the electrode compartments to be filled with electrolyte. However, the same separation efficiency could be obtained by placing the dialysis tubing directly in the gel. This simplified the construction, and the flows of electrolyte inside the dialysis tubing and of Ampholine outside the tubing permit very good electrical contact. The separation chamber, as well as the electrodes, was cooled in order to avoid thermal disturbance.

When using this apparatus to study the separation of stained albumin and haemoglobin, the sample was first injected at the top of the separation chamber. With a flow of 200 ml/h and an applied potential of 30 V/cm, the pH gradient was not completely established, but a steady state was reached after about 2 h in which the components were separated and left the chamber without having reached their isoelectric points (see Fig. 4). Focusing was also achieved under these conditions, but was less sharp than that achieved in a wholly developed pH gradient. This may be advantageous for the separation of components that are unstable at their isoelectric points. The flow of sample was 3 ml/h, corresponding to a separation of 150 mg protein/h. With flow of less than 200 ml/h and an applied potential of 50 V/cm, a pH gradient could be established in the separation chamber, and the separated components left the chamber at their isoelectric points parallel to each other and to the direction of flow (see Fig. 3). The flow of sample was about 2 ml/h. The build-up of the pH gradient was somewhat irregular (as it was with the small equipment), but this did not disturb the separation, as shown in Figs. 3 and 5.

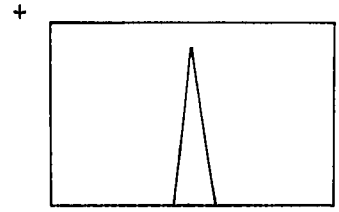

Fig. 4. Separation of albumin (left) and haemoglobin (right) in an undeveloped pH gradient.

In order to study the separation and concentration of more dilute samples, sample proteins were mixed with the Ampholine solution before passage into the separation chamber. The concentration of each protein albumin and haemoglobin was 0.5 mg/ml of Ampholine solution. Electrophoresis was started with a flow of 25 ml/h and an applied potential of 25 V/cm; the potential was then gradually increased so that the effect remained constant at about 100 W or 0.14 W/cm^2. After about 3 h, focusing was achieved in the lower zone of the separation chamber. The line at which focusing

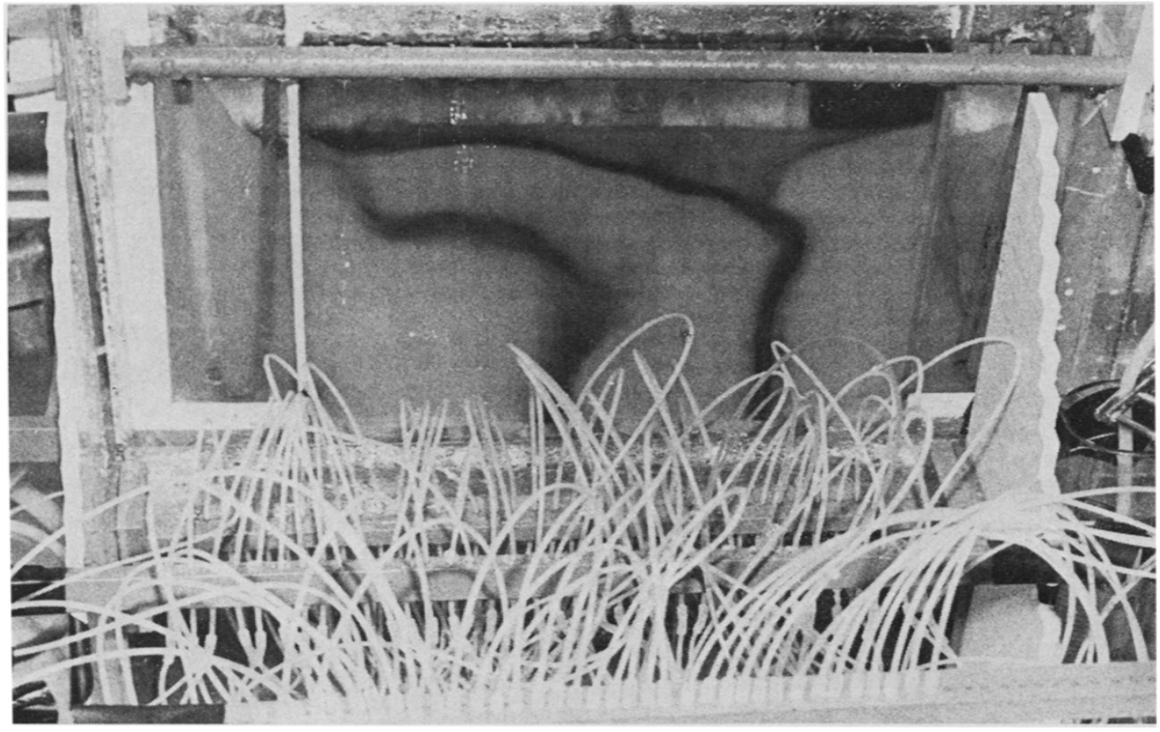

Fig. 5. Separation of albumin (left) and haemoglobin (right) in Ampholine solution with a flow of 25 ml/h.

was obtained then gradually moved upwards and stopped (at equilibrium) about a quarter of the total height from the surface of the Ampholine layer (see Fig. 5). The average residence time at a flow of 25 ml/h with a chamber volume of 345 ml was 14 h. The separation chamber would, however, have been better utilized if the sample had been just separated before leaving the chamber. The flow was therefore increased and, in order to achieve more rapid attainment of the pH gradient, the potential was also increased. Equilibrium was obtained at a flow of 125 ml/h with an applied potential of 50 V/cm, giving an effect of 0.25 W/cm^2. The focusing zone was thus established in the lower part of the separation chamber, and the system appeared to be stable (see Fig. 6). The amount of protein separated in these circumstances was about 150 mg/h or 3.6 g/day. The addition of sample directly into the Ampholine solution before passage into the electrophoresis chamber would probably often be advantageous, as it makes possible the isolation of concentrated fractions from large volumes of very dilute solutions, *e.g.*, the isolation of ampholytes from urine.

Heat generation

Generation of heat in the chamber was most uneven. In the upper part of the chamber, where the Ampholines had not reached their isoelectric points, the electric current and the generation of heat were several times greater than further down in the gel where the pH gradient had been formed. At an applied potential of 64 V/cm

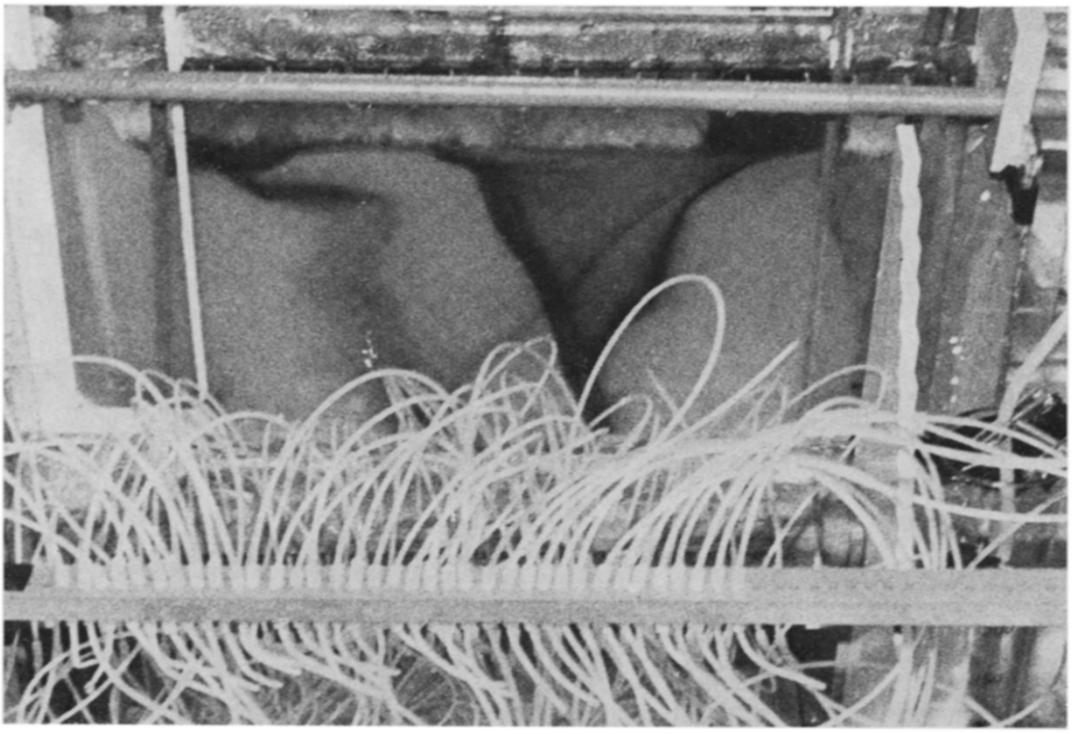

Fig. 6. Separation of albumin (left) and haemoglobin (right) in Ampholine solution with a flow of 125 ml/h.

and a flow of 180 ml/h (and with the cooling water at 5°), the temperature at the liquid surface above the gel was 60°, that at the beginning of the gel was 50° and that 10 cm below the surface of the gel was 10°. The heat generated was 430 W and the total cooling surface was 720 cm^2. The average effect was thus 0.60 W/cm^2, but the effect in the upper part of the chamber was several times greater than this value, although further down in the gel the effect was considerably lower. At 50 V/cm and 180 W and a flow of 125 ml/h, the cooling was efficient enough to keep the temperature at the liquid surface above the gel to 10–15°.

However, even with a high temperature at the top of the chamber, separation might be achieved if the sample was injected into the pH gradient established further down in the gel. In order to avoid the high heat generation in the upper part of the chamber, a potential gradient could be applied. This might be achieved by increasing the distance between the electrodes from the bottom to the top of the chamber.

One of the factors limiting dissipation of heat is the thermal conductivity of the glass. Under the conditions we have used, a heat effect of 0.08 W/cm^2 per degree per mm can be transferred through the glass (this can be calculated from the thermal conductivity of the glass and was confirmed by measurements on the equipment). The temperature gradient through the glass can be as high as the sample to be separated will allow, but the gradient within the gel should be kept as low as possible in order to avoid disturbance due to convection. In equipment for continuous isoelectric

focusing, efficient cooling is particularly important if a high flow of sample through the chamber is to be achieved. The temperature to which the sample can be exposed will determine the separation capacity. If the sample can be exposed to a temperature of 20° instead of 10°, then the temperature gradient can be increased by 10°, resulting in a threefold increase in the heat-dissipation capacity if the cooling water is at 5°.

Flow-rates

The maximal flow-rates that can be used at potentials sufficient to produce focusing are determined by the properties of the granulated gel. For Sephadex G-75, the maximal flow-rate recommended by the manufacturer is about 20 ml per cm^2/h. A total flow-rate of 125 ml/h in the equipment used corresponds to a flow-rate of 6 ml per cm^2/h, and the flow-rate was therefore limited not by the gel properties but by the limits for the applied potential. Different granulated samples of Sephadex and Bio-Gel have been investigated in batchwise isoelectric focusing, and the different gel porosities and fractionating ranges did not affect the isoelectric points of marker proteins[13]. The flow-rate that can be maintained in most of these gels was about the same as for Sephadex G-75.

Scaling-up

The most important parameter when scaling-up equipment for continuous isoelectric focusing is therefore the maximal heat dissipation from the gel to the coolant. The gel thickness, the length and width of the chamber and appropriate potential gradients are the parameters that must be investigated in order to optimize the separation capacity.

For recovery of Ampholines simple dialysis can be used; for long-term operation, continuous dialysis can be applied. The separation capacity achieved in our work seems to be sufficient for separation of Ampholines, thus permitting narrower pH ranges to be separated from a standard Ampholine solution.

The maximal separation capacity obtained in our equipment was about 150 mg of protein/h (3.6 g of protein/day), which might be of use in several applications, but this capacity could probably be considerably increased by optimization of the equipment parameters.

REFERENCES

1 O. Vesterberg, *Acta Chem. Scand.*, 23 (1969) 2653.
2 O. Vesterberg, in H. Peeters (Editor), *Protides of Biological Fluids*, Pergamon Press, New York, 1970, p. 383.
3 N. Catsimpoolas, *Separ. Sci.*, 5 (1970) 523.
4 B. J. Radola, *Biochim. Biophys. Acta*, 194 (1969) 335.
5 J. S. Fawcett, *Ann. N.Y. Acad. Sci.*, 209 (1973) 112.
6 B. J. Radola, *Ann. N.Y. Acad. Sci.*, 209 (1973) 127.
7 K. Hannig, *Z. Anal. Chem.*, 181 (1961) 244.
8 N. Seiler, J. Thobe and G. Werner, *Hoppe-Seyler's Z. Physiol. Chem.*, 351 (1970) 865.
9 H. Svensson and I. Brattsten, *Ark. Kemi*, 1 (1949) 401.
10 B. Granstrand, Swedish Patent No. 225029 (1962).
11 O. Vesterberg, *Biochim. Biophys. Acta*, 257 (1972) 11.
12 C. B. Laurell, *Anal. Biochem.*, 15 (1966) 45.
13 B. J. Radola, *Biochim. Biophys. Acta*, 295 (1973) 412.