PREPARATIVE BIOCHEMISTRY, 10(4), 445-462 (1980)

# PREPARATIVE GEL ELECTROPHORESIS AT HIGH SAMPLE LOAD. THE EFFECT OF SOME EXPERIMENTAL VARIABLES ON SEPARATION PERFORMANCE

S. A. Saeed and T.R.C. Boyde
Department of Clinical Biochemistry
University of Newcastle-upon-Tyne[1]

## ABSTRACT

The separation of model protein pairs (hemoglobin/albumin, trypsin/chymotrypsin, hemoglobin A/hemoglobin F) was studied in an apparatus for preparative gel electrophoresis at loads up to 40 $mg/cm^2$ of the cross-sectional area of the gel bed. Separation was favored by higher ionic strength and by longer migration path. Under the conditions used and within the load range studied, increasing total protein load had no adverse effect but increased voltage gradient, temperature, or gel strength were all unfavorable.

## INTRODUCTION

Preparative electrophoresis has been the subject of numerous publications but studies upon how variables such as

voltage gradient, temperature, ionic strength and path length affect the separations actually achieved have mostly been on a small scale or even on analytical rather than preparative apparatus. Our results indicate that extrapolation from such experiments to predict behaviour at high loads is not always appropriate.

## PREPARATIVE APPARATUS

The original form of an apparatus for single-stage batchwise preparative electrophoresis was described by Boyde[2] and the version used in this work by Saeed[3]. It holds a horizontal slab of polyacrylamide gel 1.0 cm deep and 5.9 cm wide, whose length, measured in the direction of the electrophoretic field, was either 5.9 or 19.5 cm. Sample was applied in a slot made at the time of casting the gel by means of a solid former attached to the lid of the tray used for this purpose. This slot was generally 0.9 cm deep, 5.0 wide and 0.3 cm long (in the electrophoretic axis) and could be placed wherever convenient along the length of the gel to give various lengths of migration path - in the present work 4.0 or 15.0 cm, measured from the centre of the slot.

The ends of the gel are in direct contact with buffer; migrating species emerge from the ends of the gel into collection chambers which are emptied and refilled at intervals of 15 min. Each chamber is 0.6 cm long in the electrophoretic direction and is bounded at one end by the gel slab, at the other by a sheet

of 'cuprophane' dialysis membrane through which electrical continuity is maintained with buffer in an electrode tank. Thus the apparatus provides for collection of species migrating in both directions from the point of origin, a feature which can be useful at times, although not used in these experiments.

Cooling is provided and pH changes minimised, by circulation of mixed buffer from both electrode tanks through a coil immersed in a cold bath and then through 'perspex' boxes, applied to the upper and lower surfaces of the gel, before returning to the electrode tanks. These 'cooling boxes' are fitted with appropriate baffles. The lower box forms part of the structure of the gel tray, the upper is mobile, connected to the lower only by flexible tubing and can thus be raised when necessary to give access to the gel tray. The upper cooling box is the only cover necessary for the gel during electrophoresis.

Apart from flexible tubing and platinum electrodes, the whole of this 'core' of the apparatus is built of perspex. A diagrammatic view is given in Fig. 1. The tray is viewed so that only one end of the separation medium (polyacrylamide gel) is visible. The 'pressing plate' secures the dialysis membrane in place (stretched taut) with the aid of an appropriate gasket of sheet rubber, and is itself held in place by three props spanning the electrode compartment and made of plastic or rubber tubing covering a piece of glass rod. The collection chamber lies between the end of the gel slab and the dialysis membrane: it is cut into a 'V' shape below to facilitate emptying, and the

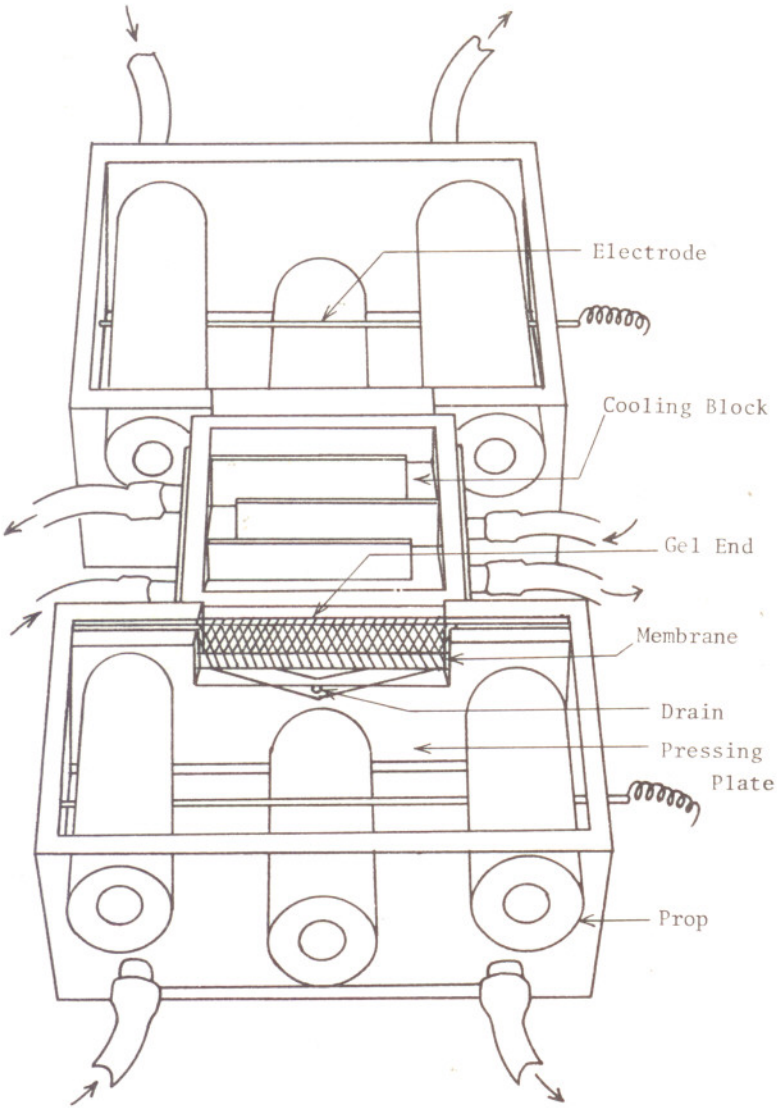


FIGURE 1

Perspective view of preparative electrophoresis tray complete with collection chambers and electrode compartments.

drainage hole proper is at the apex of the V. Small arrows indicate buffer flows in and out of electrode tanks and cooling boxes.

It will be observed that the electrophoretic path maintains approximately the same cross-sectional dimensions for some distance beyond both ends of the gel. The channel for buffer in and out of the collection chamber is via a hole at the bottom. The chamber empties by gravity and refills by equalisation of levels with an independent reservoir. Ancillary equipment, not illustrated, includes a simple electric clock (synchronous motor), a fraction collector capable of accepting samples simultaneously from both collection chambers, and a slave motor which operates appropriate two-way stopcocks, several switches, and the fraction collector escapement.

## MATERIALS AND METHODS

Acrylamide and N,N'-methylene-bis-acrylamide were purchased from Koch-Light Laboratories and N,N,N',N'-tetramethylethylenediamine (TEMED) from Eastman Kodak. Agarose was from Hughes & Hughes, Enzymes, Ltd., or prepared by the method of Hjertén[4]. Naphthalene Black (Amido Black 10B) was purchased from G.T. Gurr, N-benzoyl-L-tyrosine ethyl ester hydrochloride from Calbiochem, p-tosyl-L-arginine methyl ester from Sigma, bovine plasma albumin from Armour Pharmaceutical Co., trypsin and chymotrypsin from Boehringer. Other reagents were of analytical reagent grade, purchased from BDH.

Hemoglobin solutions were prepared from packed erythrocytes by shaking with 1.5 vols. water and 0.4 vols. toluene, then centrifuging and pipetting off the supernatant. Blood for this purpose was obtained from normal adults by venepuncture, or as cord blood specimens.

Polyacrylamide gels for preparative purposes were cast in trays of appropriate dimensions, then transferred to larger perspex trays so that small molecules could be removed and the electrophoresis buffer introduced by dialysis against several changes of the latter before finally placing the gel in the electrophoresis tray. Sample was introduced to the origin slot in the form of a warm solution containing 0.4% agarose, the volume being such as to leave the upper 0.1 cm of the slot unfilled. When the sample-containing solution had set, the slot was 'capped' with a small volume of sample-free agarose solution. In this way we ensured that sample did not approach any surface of the gel closer than about 0.1 cm. The concentration of gels is described in terms of %T (g of total monomer per 100 ml of solution). All were 5% crosslinked - i.e. 5 g N,N'-methylene-bis-acrylamide per 100 g total monomers - and were 5%T unless otherwise stated. Initiators used were (final concentrations) ammonium persulphate 1 g/l, freshly dissolved before use, and TEMED, 1 ml/l added immediately before filling the casting tray. No other buffer was used at the casting stage; the initiators are sufficient for the purpose, giving approximately pH 7.

Electrophoresis buffers used were as follows:- Albumin and hemoglobin - borate/sodium (pH 8.4, 25 mmol/l unless otherwise stated). Trypsin and chymotrypsin - citrate/sodium (pH 4.5, 50 mmol/l unless otherwise stated). Hemoglobins A and F - borate/sodium or according to ref. 8. Voltage gradient was usually 4.0 V/cm as measured by electrodes applied to the gel surface and a valve voltmeter. Samples always contained equal amounts by weight of the two components and the total amount was usually as follows:- albumin/hemoglobin, 45 mg, trypsin/chymotrypsin 16.5 mg, HbA/HbF, 50 mg. Gel temperature was usually 5°C.

Hemoglobin was determined by absorbance at 413 nm and albumin by absorbance at 280 nm (after correction for any hemoglobin present). Hemoglobin F was determined by a kinetic alkali denaturation method based on absorbance at 413 nm, and closely resembling that of Jonxis and Visser[5]. Trypsin and chymotrypsin were determined by the method of Hummel[6] with minor modifications.

We have had no difficulty arising from the use of dialysis membrane to limit the collection chamber, partly because of using a rather broader chamber than is usual (0.6 cm) and partly because in this apparatus proteins emerging from the separative gel forms a relatively dense solution and sink to the bottom of the chamber, essentially clear of the electric field, as can easily be seen when working with colored proteins. The effect is analogous to "electrodecantation" and here is advantageous, but must be taken into account when designing the buffer pathway from collection slot to fraction collector[2].

## RESULTS

There are considerable problems in expressing briefly the degree of separation achieved in any separation experiment, but particularly where the behaviour does not follow a theoretical ideal[7]. In a case such as this, the raw observations are in the form of distribution curves for each component of the original mixture along a time or volume axis. If the distributions were Gaussian it would be appropriate to use the "resolution", $R_s$, defined as peak interval $\div$ mean peak width, where width means the distance between the baseline intercepts of tangents drawn to the points of inflexion on each limb of the distribution curve. But if the curves are non-ideal, the definition loses meaning and $R_s$ can then only be used in a crude and limited sense. If the curves are sufficiently distorted, as sometimes in our experiments, it is impossible even to determine a value of $R_s$.

These limitations do not apply to the purity ratio and related parameters, but a local definition is required of the "component of greatest interest" and the boundaries of the zone to be collected for this component[7]. We have chosen 90% recovery of the leading component, beginning with its first appearance. For the purposes of this paper, Z is used as the symbol for purity ratio, and we may write, simply,

$$Z = \frac{\text{percentage of total A in zone A (i.e., 90\%)}}{\text{percentage of total B in zone A}}$$

The full symbolic representation would be $({}^{e}_{x}Z)_{0\text{-}90\%A,}$ since the local definition given implies an equalised, experimental parameter.

If none of component B is detectable in Zone A experimental values of Z become infinite. To deal with this situation we have first determined the values of $R_s$ (which fortunately it was possible to do) and converted these into Z values, distinguished as $Z_R$, on the assumption of perfect Gaussian curves and using published tables of erfc; again on the basis of 90% recovery of the leading component. From what has been said above it will be appreciated that $Z_R$ values are to be regarded with reserve.

With all three model pairs, an increase in buffer concentration improved separation and so also did a longer migration path - though not generally in ratio to the increase in length. Samples of greater total weight were separated with undiminished efficiency. High temperature, high gel concentration and high voltage gradients all affected separation adversely.

TABLE I

Effect of buffer on separation of Hemoglobins A and F. For experimental details see text

| Buffer | Borate/sodium | | As ref. |
|---|---|---|---|
| | pH 9<br>25 mmol/l | pH 8.4<br>125 mmol/l | 8 |
| log Z | 0.05 | 0.08 | 0.04* |

*Voltage gradient 5.4 V/cm

TABLE II

Effect of buffer concentration on separation. c.f. Fig. 2. Results are log $Z_R$ except where indicated by an asterisk (log Z). For nature of buffers used and other experimental details, see text

| Model Pair | Buffer concentration (mmol/l) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 10 | 25 | 50 | 80 | 100 | 125 | 250 |
| Albumin/ hemoglobin | 1.8 | 4.3 | | 4.7 | | 8.9 | 15.8 |
| Trypsin/ chymotrypsin | | 1.3* | 1.6* | | 3.6 | | |

TABLE III

Effect of sample load on separation of albumin and hemoglobin. See Fig. 3 for experiments with trypsin and chymotrypsin. For experimental details see text

| Total Sample load (mg) | 30 | 45 | 100 | 205 | 250 |
|---|---|---|---|---|---|
| log $Z_R$ | 2.8 | 4.3 | 2.5 | 4.2 | 3.9 |

TABLE IV

Effect of electrophoresis path length on separation. c.f. Fig. 4. Results are log Z for HbA and HbF, and log $Z_R$ for albumin and hemoglobin. The voltage gradient (average) is given in brackets where it differed from 4.0 V/cm. For other experimental details, see text

| Model Pair | Path Length (cm) | Buffer | | | |
|---|---|---|---|---|---|
| | | Borate/sodium pH 8.4 (concentration, mmol/l) | | | Discontinuous (ref. 8) |
| | | 10 | 25 | 125 | |
| Albumin & Hemoglobin | 4.0 | 1.8 | 4.3 | | |
| | 15.0 | 3.5 | 6.5 | | |
| Hemoglobins A and F | 4.0 | 0.10(1.3) | | 0.08 | 0.04(5.4) |
| | 15.0 | 0.09(6.5) | | 0.2 | 0.08 (6.0) |

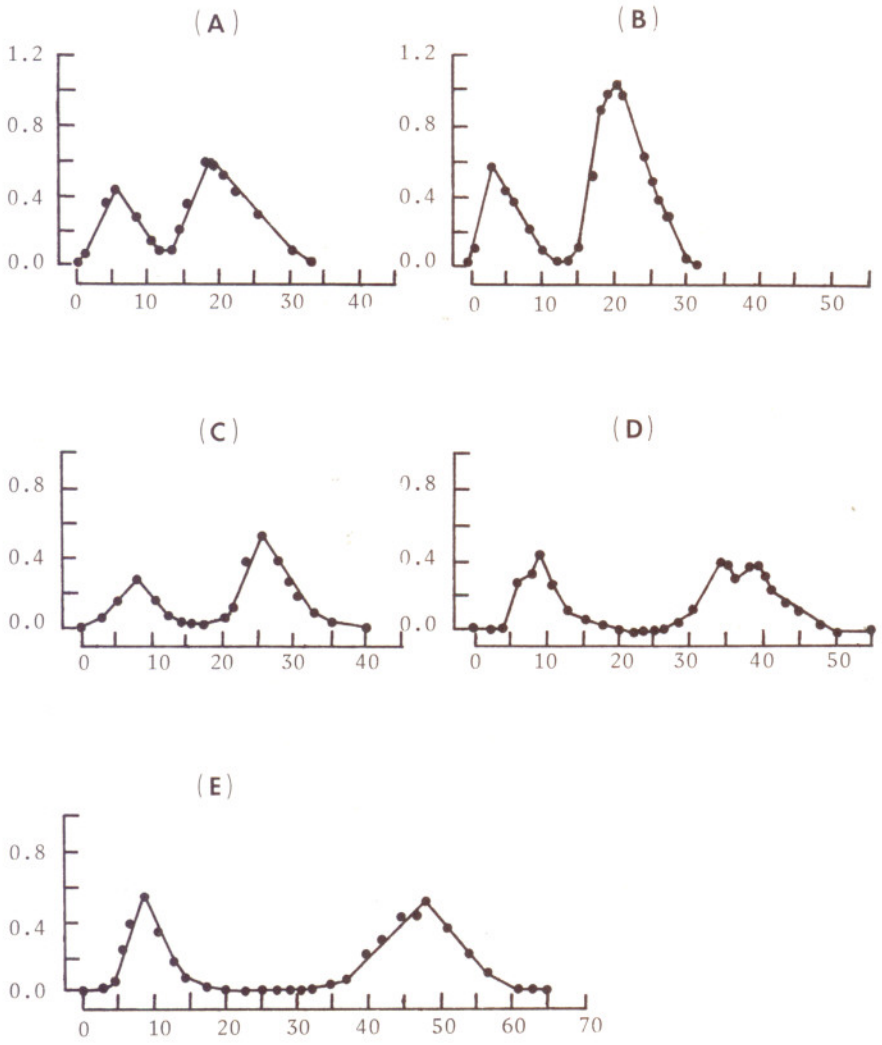


FIGURE 2

Separation of albumin and hemoglobin; effect of buffer concentration. c.f. Table II. Ordinate shows absorbance at 280 nm, abscissa shows fraction number. The leading peak is albumin. Buffer concentrations were (mmol/l) a) 10; b) 25; c) 80; d) 125; e) 250. For other experimental details see text.

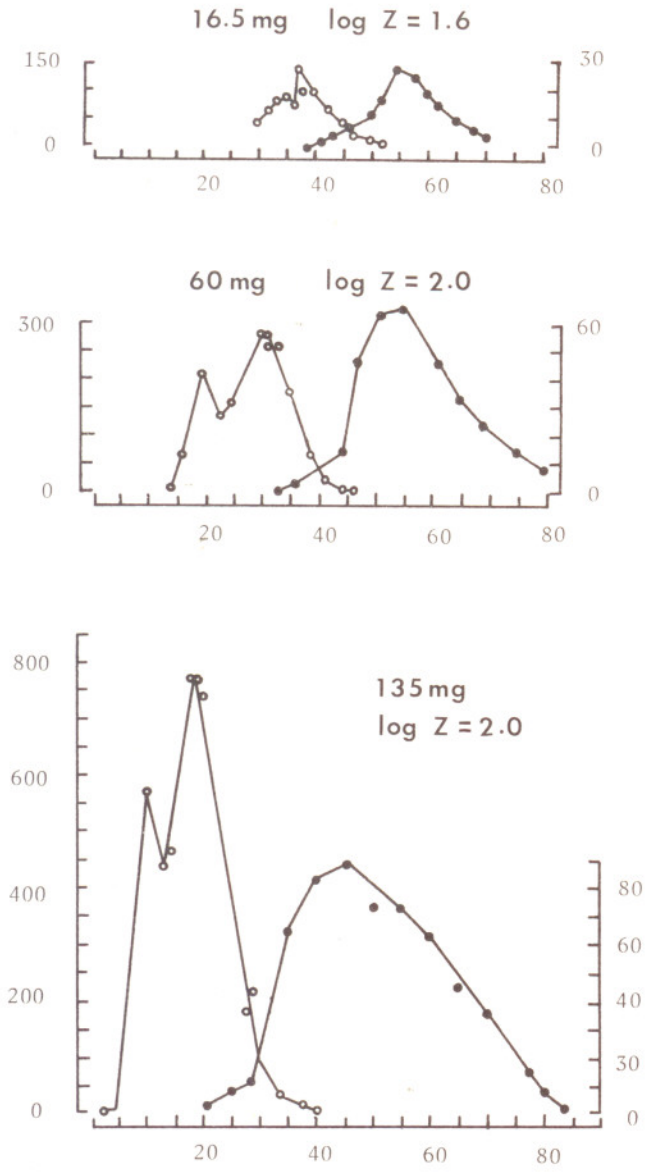


FIGURE 3

Separation of trypsin and chymotrypsin; effect of sample load. For experimental details see text. o and scale at left - trypsin IU per fraction. ● and scale at right chymotrypsin IU per fraction. Total sample load and result (log Z) are shown in the figure.

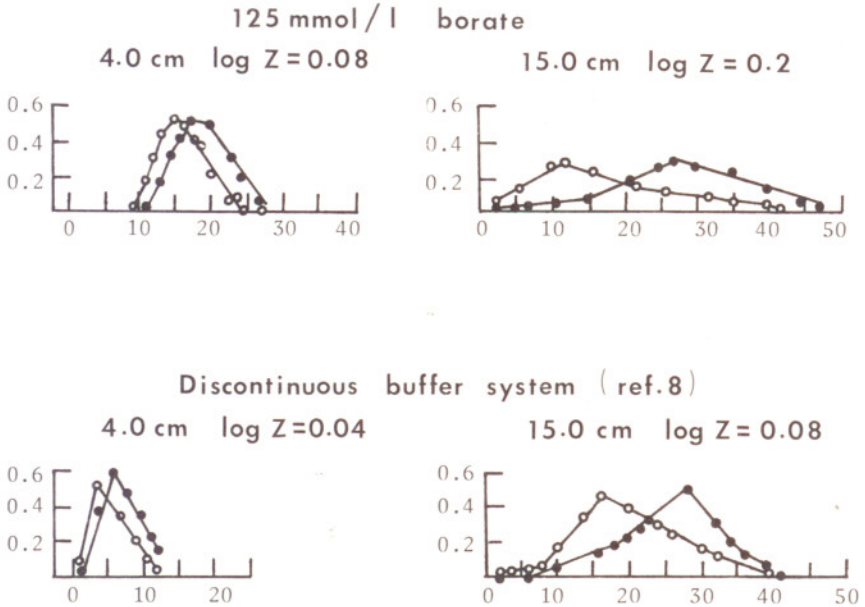


FIGURE 4

Separation of hemoglobins A and F; effect of gel length. c.f. Table IV. For other experimental details, see text. Buffers used were either borate/sodium pH, 8.4, 125 mmol/l or the discontinuous system of ref. 8. Path length and result (log Z) are shown in the figure. Content of fractions is expressed in terms of absorbance at 413 nm : o = HbA, ● = absorbance due to HbF.

TABLE V

Effect of voltage gradient on separation. For other experimental details, see text

| | Model Pair | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Albumin/ hemoglobin | | | | | | | Trypsin/ chymotrypsin | |
| Buffer concentration (mmol/l) | 25 | | | 50 | 125 | | | 50 | |
| Voltage gradient (V/cm) | 1.6 | 4 | 10 | 1.6 | 1.6 | 4 | 10 | 1.3 | 4 |
| log $Z_R$ | 4.1 | 4.3 | 0.8 | 5.0 | 4.8 | 8.9 | 2.8 | 2.2 | 1.6 |

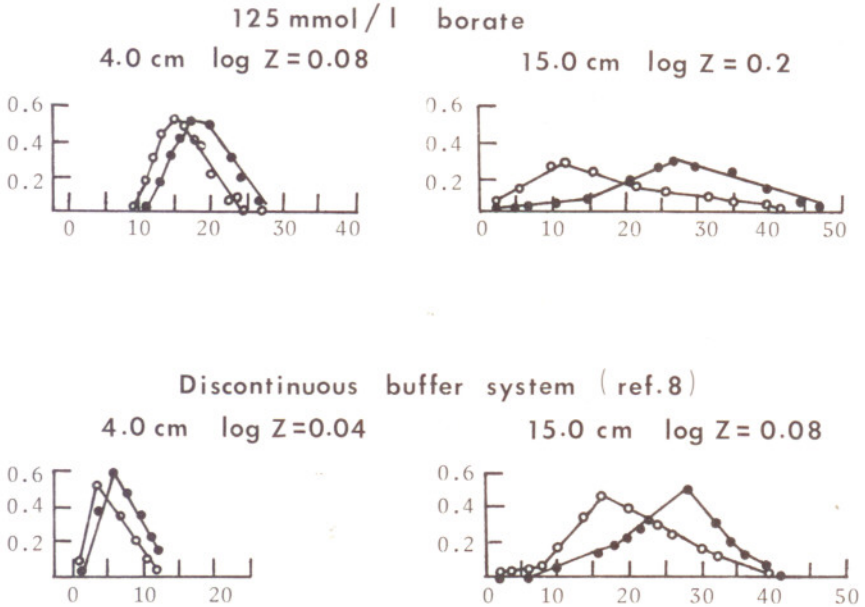


FIGURE 4

Separation of hemoglobins A and F; effect of gel length. c.f. Table IV. For other experimental details, see text. Buffers used were either borate/sodium pH, 8.4, 125 mmol/l or the discontinuous system of ref. 8. Path length and result (log Z) are shown in the figure. Content of fractions is expressed in terms of absorbance at 413 nm : o = HbA, ● = absorbance due to HbF.

TABLE V

Effect of voltage gradient on separation. For other experimental details, see text

| | Model Pair | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Albumin/ hemoglobin | | | | | | | Trypsin/ chymotrypsin | |
| Buffer concentration (mmol/l) | 25 | | | 50 | 125 | | | 50 | |
| Voltage gradient (V/cm) | 1.6 | 4 | 10 | 1.6 | 1.6 | 4 | 10 | 1.3 | 4 |
| log $Z_R$ | 4.1 | 4.3 | 0.8 | 5.0 | 4.8 | 8.9 | 2.8 | 2.2 | 1.6 |

TABLE VI

Effect of gel concentration (%T) on separation of albumin and hemoglobin (log $Z_R$). "N.D." means not determinable because zones were too widely spread. For experimental details see text

| % T | Buffer concentration (mmol/l) | |
|---|---|---|
| | 25 | 125 |
| 5 | 4.3 | 8.9 |
| 10 | 1.6 | 3.9 |
| 12.5 | N.D. | N.D.* |

*T = 15%

TABLE VII

Effect of gel temperature (°C) on separation of albumin and hemoglobin (log $Z_R$). For experimental details, see text

| Temperature (°C) | Buffer concentration (mmol/l) | |
|---|---|---|
| | 25 | 125 |
| 5 | 4.3 | 8.9 |
| 27 | 2.3 | 3.1* |

*Temperature = 29°C

There is no evidence that the "discontinuous" buffer system of Schilling and Klein[8] has any advantage over simple borate buffers in separating hemoglobins A and F.

## DISCUSSION

The word 'preparative' has been used in reference to microgram-scale separations and quite properly since only tiny amounts of the products were required. However, it should not be used of any experiment unless the products are obtained free of the separative medium and in a form suitable for further use. The work described here was preparative in this sense and was

also at loads such that modest scaling up would bring it into the range required for present-day bulk purification of many proteins. The largest load employed was 250 mg (equally divided between two components) applied to a gel bed of only 6 $cm^2$ cross-sectional area. When thinking in terms of future increases in scale, it seems at least reasonable to work in terms of load per unit of area. On this basis our loads were some 1,000-fold those employed by Richards and Lecanidou[9], for example. When making comparisons of performance, load intensities should be taken fully into account.

Most unexpected of our results is the absence of any deleterious effect of sample load on separation achieved. In accordance with every day observation and, for example, the theoretical analysis of Richards and Lecanidou[9], zones became wider as load was increased, but this apparently did not lead to a greater region of overlap of adjacent zones (Fig. 3). It follows that migrations were not independent of each other and it seems probable that field non-uniformities are responsible, as in 'isotachophoresis'. However explained, the phenomenon is plainly advantageous and would not have been predicted from existing theory or low-load experiments.

Two further points should be made here:

1) The zone-widening with increased load commonly observed on stained gels or membranes is partly illusory, since to the eye a more deeply stained zone must always appear wider whether it really is so or not.

2) The phenomenon doubtless reflects in part our use of a model system containing equal amounts of two substantially pure proteins: it may not be fully transferable to the more complex mixtures encountered in practice.

As expected, enhanced buffer concentration gave slower migration but relatively more compact zones and better separation. There are practical limits to the improvements which can be obtained in this way, however, because of the unavoidably increased current (and heating effect) and much prolonged separation times. Greater gel length was favorable, but to a surprisingly small degree and we found little or no loss of separation at diminished voltage gradients (1.6 V/cm against 4 V/cm standard), perhaps because the rate of generation of heat was diminished and temperature gradients correspondingly less.

Our results have plain implications for the design of preparative electrophoresis apparatus and procedures. Among the unfavorable influences to be avoided we would lay particular stress on the effects of unduly high voltage gradients and unduly high gel concentrations.

The particular objective here was to study model separations where the components were of similar molecular dimensions. Not surprisingly, however, the most striking successes have been in separating molecules of different sizes (ferritin oligomers from each other, ferritin-labelled antibody from other products

of the coupling reaction), making use of the selective properties of polyacrylamide gels and far exceeding the performance of gel chromatography[3].

ACKNOWLEDGEMENTS

The authors thank Prof. A.L. Latner for help and facilities and the University of Newcastle-upon-Tyne for the award to S.A.S. of a Luccock Research Studentship. Part of this work was incorporated in the Ph.D. thesis of S.A.S.

REFERENCES AND NOTES

1. Present addresses: S.A.S., Miles Laboratories Ltd., Stoke Court, Stoke Poges, Slough, Bucks, U.K.
T.R.C.B., Department of Biochemistry, University of Hong Kong, Hong Kong.

2. T.R.C. Boyde, Occasional paper No.1 from the Dept. of Biochemistry, Makerere University, Kampala, (1971).

3. S.A. Saeed, Ph.D. thesis, University of Newcastle-upon-Tyne, (1968).

4. S. Hjertén, Biochim. Biophys. Acta, 62, 445-449, (1962).

5. J.H.P. Jonxis and H.K.A. Visser, Amer. J. Dis. Child., 92, 558-591 (1956).

6. B.C.W. Hummel, Can. J. Biochem. Physiol. 37, 1393-1399, (1959).

7. T.R.C. Boyde, Separation Science and Technology, 14, 79-106, (1979).

8. R.W. Schilling and C.C. Klein, Amer. J. Clin. Pathol., 44, 662-664, (1965).

9. E.G. Richards and R. Lecanidou, Anal. Biochem. 40, 43-71, (1971).