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CHROMATOFOCUSING: ISOELECTRIC FOCUSING ON ION-EXCHANGE COLUMNS

II. EXPERIMENTAL VERIFICATION*

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SUMMARY

The focusing effects in the ion-exchange chromatography of proteins in a pH gradient predicted in Part I have been confirmed experimentally. Each protein seeks its own position in the pH gradient. The separations obtained are similar to those in the preparative electrophoretic method.

Donnan potentials, under conditions approximating to those in ion-exchange columns, were determined by employing $^{24}Na^{+}$ as a probe. Utilizing these values, the observed phenomena could be understood in terms of the equations calculated for the width of the protein bands and for the pH of the emerging protein solutions.

INTRODUCTION

In Part I, theoretical considerations indicated the possibility of producing focusing effects in ion-exchange chromatography of proteins in a pH gradient, similar to the effects observed in the well known method of electrofocusing[2]. In this paper, experimental evidence is reported to demonstrate the factors involved and to compare some of the separations obtained by chromatography with those of electrophoresis.

EXPERIMENTAL

Materials

The ion exchangers DEAE-Bio-Gel A and CM-Bio-Gel A were purchased from Bio-Rad Labs. (Richmond, Calif., U.S.A.). The beads contain 4% agarose. The capac-

* The experiments in Figs. 4, 5 and 7a and the principles involved have been recorded on a 16-mm colour film with optical sound of 10-min running time, entitled "Chromatofocusing". Copies can either be obtained on loan for short periods or purchased. All enquiries should be adressed to Dr. C. G. Sluijter, Philips Research Laboratories, Eindhoven, The Netherlands.

ities of the packed gels were stated to be 10 and 50 μequiv./ml, respectively. Sperm whale myoglobin and horse cytochrome c were commercial preparations obtained from Serva (Heidelberg, G.F.R.), bovine serum albumin from Povite (Amsterdam, The Netherlands). Crude papaya latex extract was prepared by utilizing steps I and II of the method of Kimmel and Smith[3] and was dialyzed in a Diaflo cell (Amicon, Lexington, Mass., U.S.A.). During the dialysis some precipitate developed, which was removed by centrifugation. Papain was prepared using the complete procedure of Kimmel and Smith[3], further purified on the mercurial-agarose column[4] and converted into mixed disulphide with mercaptoethanol[5].

Ampholine buffers were obtained from LKB (Bromma, Sweden) and 3-(N-morpholino)propanesulphonic acid (MOPS) from Sigma (St. Louis, Mo., U.S.A.).

Equipment

The dimensions of the chromatographic tubes were 20 × 1.0 cm (Whatman, Maidstone, Great Britain), 40 × 1.6 cm (Type K 16/40) and 45 × 2.5 cm (Type K 25/45, Pharmacia, Uppsala, Sweden) and they were provided at the bottom with flow adaptors. Porous plastic discs in the smallest flow adaptors often caused inhomogeneous flow and proved to be inferior to adaptors with discs of textile fabric.

Flow-rates were adjusted with Perpex pumps (LKB, Bromma, Sweden). The UV absorption at 280 nm was monitored with Uvicord II and Uvicord III instruments (LKB) and later with an SP6-400 UV spectrophotometer (Pye-Unicam, Cambridge, Great Britain), provided with an accessory for liquid chromatography, and with an optical path of 10 mm.

Fractions were collected in a Type 7000 collector (LKB). The pH of the eluent was measured initially in the fractions collected under a current of nitrogen at basic pH. Later the pH was measured continuously in a flow cell, as described by Strongin *et al.*[6]. In both instances a Philips digital pH meter was used.

A vertical 110-ml column (Type 8101, LKB) served for preparative electrofocusing in a density gradient. Gel electrophoresis was carried out in 80 × 80 × 3 mm gel slabs of 7% polyacrylamide in Type GE 4 equipment (Pharmacia).

Determination of Donnan potentials

Columns of 5 ml of packed ion exchanger were equilibrated with the desired buffers. The beads were removed from the columns and re-suspended in a roughly equal amount of the buffers concerned. Trace amounts of ^{24}NaCl (total sodium chloride concentration 0.1–0.15 m*M*) were added and the mixture was stirred for 20 min. The two phases were separated by centrifugal filtration for 1 min at 30 *g* in a Filterfuge tube (International-Equipment Co., Needham Heights, Mass., U.S.A.). The separate phases were placed in vials, weighed, diluted with 0.2 *M* non-radioactive sodium chloride to an equal volume and counted in a scintillation counter provided with a sodium iodide crystal (3 × 3 in.) doped with thallium as a detector. The Donnan potentials were determined by means of the equation[7]

$$\psi = \frac{RT}{nF} \cdot \ln \frac{[Na^+]_s}{[Na^+]_m}$$

or

$$\varphi = \ln \frac{[Na^+]_s}{[Na^+]_m}$$

where the subscripts s and m denote the stationary and mobile phase, respectively. The determinations were carried out in triplicate and were accurate to ±8%.

Chromatographic procedure

A batch of DEAE-Bio-Gel A was equilibrated with 3.5 m*M* glycine–ammonia buffer of pH 10.5 and poured into the tube in a slurry of about one part of packed gel to one part of buffer. After settling, the column packing was forced into mechanical stability by passing it through glycine–ammonia buffer of pH 10.5 at twice the rate to be utilized during the separation. The buffer on top of the bed was replaced with a layer of the elution buffer. The best separations were obtained when the protein samples, containing 1.5% of sucrose, were layered on top of the column below the layer of the elution buffer. The separations were carried out at room temperature (20–23°). Further details are given in the captions to the figures.

After separation, the anion exchanger was cleaned with 1 *M* ammonia solution, 0.1 *M* acetic acid and water and stored in 5 m*M* Tris buffer (pH 8.6) as recommended by the manufacturer. A column was used not more than three times before being recast. In practice, it proved to be most convenient and also to give the most reliable results when the column was used once only and the combined batches of a number of separations were subjected to the cleaning procedure. The cation exchanger was cleaned and cast into a column with 0.1 *M* acetic acid. The column was adjusted to pH 4.8 with 0.1 *M* acetate buffer (pH 4.8) before the elution buffer and the protein were applied.

Other procedures

For the preparative electrofocusing, the procedure recommended by LKB was followed. The gel electrophoresis was run for 5 h at 100 V and 23° in 0.02 *M* acetate buffer (pH 6.0). The proteins were fixed in 12% trichloroacetic acid and stained with Coomassie Blue G-250.

The esterolytic acitivity of proteases was determined with 10 m*M* benzoylarginine ethyl ester at pH 6.0 and 25° in a pH-stat in the presence of 1 m*M* EDTA and 2.5 m*M* dithiothreitol.

RESULTS

Some properties of the ion exchangers

Stirred suspensions of the exchangers were titrated with 0.1 *M* hydrochloric acid and 0.2 *M* sodium hydroxide solution in a total volume (30 ml) of three times the volume of the packed exchangers (10 ml) in the absence of added salt. The results, corrected for the titration of 30 ml of water, are shown in Fig. 1. The titration curves of the ampholine buffers utilized in the separations are also shown in the pH ranges concerned.

The titration curve of DEAE-Bio-Gel A shows a constant buffering capacity from pH 7.5 to 10.0, which diminishes at both ends. The base capacity of the exchanger up to pH 11 is 19.0 mequiv./l, which corresponds to a separately measured chloride

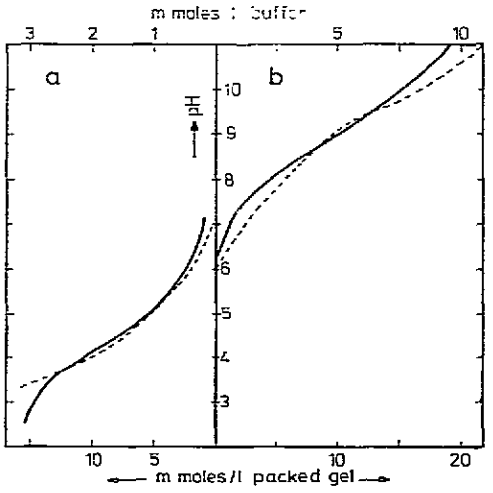

Fig. 1. (a) Solid curve, titration of CM-Bio-Gel A; broken curve, titration of 0.075% Ampholine, pH range 3–10. (b) Solid curve, titration of DEAE-Bio-Gel A; broken curve, titration of 0.15% Ampholine, pH range 8–9.5.

binding capacity of 19.3 mequiv./l. Nevertheless, the DEAE exchanger carries a small number of negative groups. This is revealed by the behaviour of the dyes thymol blue (a negatively charged dye) and methyl green (a positively charged dye) dissolved (50 mg/l) in ammonia solutions of pH 10.0, 10.5 and 11.0. Aliquots of dye solution were applied to small columns of the anion exchanger and eluted with the same ammonia solution.

At pH 11.0 the positive dye was adsorbed and the negative dye was eluted, at pH 10.0 the negative dye was adsorbed and the positive dye was eluted, and at pH 10.5 neither dye was adsorbed, demonstrating the neutrality of the exchanger at this pH. Therefore, pH 10.5 was chosen as the pH at which to pour the columns of the anion exchanger.

The titration curve of CM-Bio-Gel A exhibits only a reasonably constant buffering capacity in the limited range of pH 3.5–4.5. From the titration curve an exchange capacity of 15 mequiv./l is calculated. The binding capacity of K^+ ions proved to be 16.5 mequiv./l.

Thus, it was found that the capacity of the anion exchanger was twice as high and that of the cation exchanger one third as high as those indicated by the manufacturer (see *Materials*).

In calculating the buffering capacity of the stationary phase from the data in Fig. 1, one has to bear in mind that the gel volume occupies two thirds of the total volume of the gel bed (*i.e.*, $r = 2$), as determined on a column of Bio-Gel A with the aid of Blue Dextran 2000 (Pharmacia).

The Donnan potentials of the exchangers in an excess of buffers were determined with the aid of the radioisotope $^{24}Na^+$ as a probe (see Experimental), and the results are shown in Fig. 2 and Table I. As expected, the potential increases with decreasing buffer concentration, *i.e.*, with decreasing ionic strength. As no excess of buffer had yet passed the exchanger at the beginning of a separation and as part of the

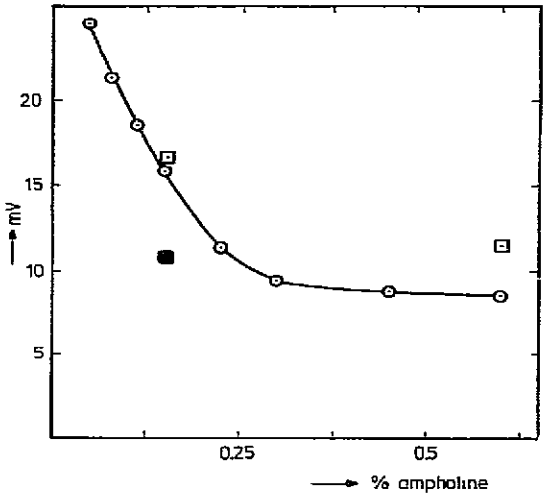

Fig. 2. Mean Donnan potentials of DEAE-Bio-Gel A. ○, Ampholine, pH range 8–9.5, adjusted to pH 7.5 with acetic acid; □, Ampholine, pH range 8–9.5, at pH 8.8; ■, Ampholine, pH range 8–9.5, at pH 8.8 plus 5 m*M* NaCl.

buffer components will be retarded by the exchanger, the potentials at the initial part of the separation are probably higher than indicated in Fig. 2. During a run, the pH in the column changes; with an anion exchanger both the charge of the exchanger and the ionic strength of the buffer increase with decreasing pH. These two effects will partially compensate each other (except at the highest pH > 10). As can be seen in Fig. 2, the potentials at pH 7.5 and pH 8.8 are indeed fairly equal.

Demonstration of the focusing action

The focusing effect can be demonstrated most conveniently by the method of internally generating the pH gradient because the complete pH gradient is present in

TABLE I

SUMMARY OF p*I* VALUES OBTAINED FROM CHROMATOGRAPHIC AND ELECTROPHORETIC SEPARATIONS

Protein	pI_{app}	*pI*	ΔpI	r_c	φ	$-\frac{dZ}{dpH}$	ΔpI_{calc}
Sperm whale myoglobin	9.1*	8.3	+0.8	2.1	+0.65	1.8 (ref.9)	+1.1
Horse cytochrome c	10.2	10.1(ref. 10)	+0.1	—	—	6.5(ref. 10)	—
Papain	9.75	9.6(ref.11)	+0.15	1.2	+0.65	5.0**	+0.2
Bovine serum albumin	5.6 6.2	4.7 5.3	+0.9	4.7	—1.7***	7.1(ref.12)	+0.2

* The same value was found in 35% sucrose.

** S. H. de Bruin, University of Nijmegen, personal communication.

*** Determined in 0.075% Ampholine of pH range 3–10 at pH 7.0.

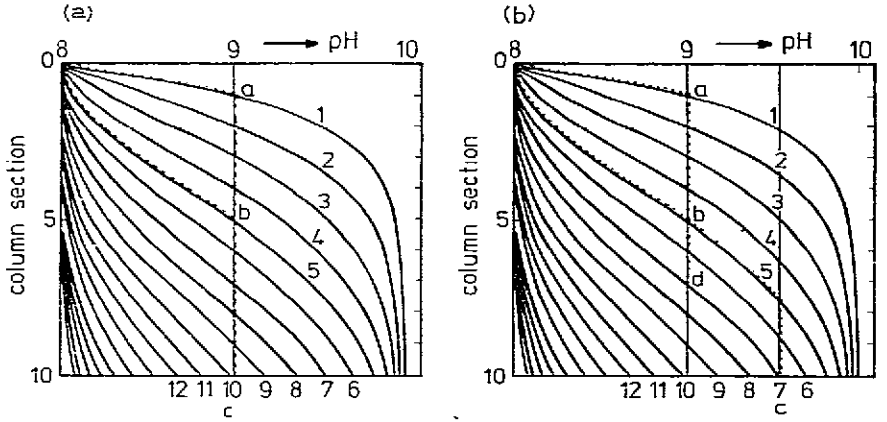

Fig. 3. Principle of the demonstration of focusing action. (a) A sample of protein caught up by a second sample of the same protein applied later on the column; (b) a sample of protein passed by a sample of another protein of higher p*I* value applied later on the column.

the column at the moment that the first aliquot of buffer reaches the bottom of the column (see Fig. 3 in Part I[1]). The principle involved can be derived from the diagram in Fig. 3 here. This diagram, which was explained in detail in Part I, indicates the pH of buffer aliquots 1–18 as they pass the ten sections of the column. In order to demonstrate the focusing action, a sample of protein is applied to the column in aliquot 1. The sample is carried along, quickly at first by aliquot 1, up to point a in Fig. 3a, and then more slowly by successive aliquots as their pH approaches 9. When this sample has travelled a certain distance, a second sample of the same protein is applied, for example in aliquot 5. When the elution is continued, the second sample is carried down more quickly by aliquot 5 than the first sample by successive preceding aliquots. They join each other at point b in Fig. 3a, travel further down as a single band and emerge at point c. This has been verified experimentally with sperm whale myoglobin, purified by the present method (*cf.*, Fig. 7a). The results are shown in Fig. 4; they confirm the predictions and prove the occurrence of a focusing effect.

Another experiment is illustrated in Figs. 3b and 5. A sample of one protein is applied to the column in aliquot 1 and made to travel a certain distance. In aliquot 5 another protein, with a higher p*I* than that of the first protein, is applied and the flow of buffer is continued. The second protein travels at a higher rate than the first; they meet temporarily at point b, but the second protein continues at a higher rate in aliquot 5, by-passes the first protein and emerges first from the column. This has been verified with myoglobin as the first protein and horse cytochrome c as the second (Fig. 5). It is evident that cytochrome c by-passes myoglobin and focuses into its own pH (found to be pH 10.2) in the column.

Distribution volume

It has been argued[1] that a protein can be applied to the column distributed over a certain volume of buffer (the distribution volume) and still emerge as a single band. The distribution volume should be equal to the elution volume minus the accessible

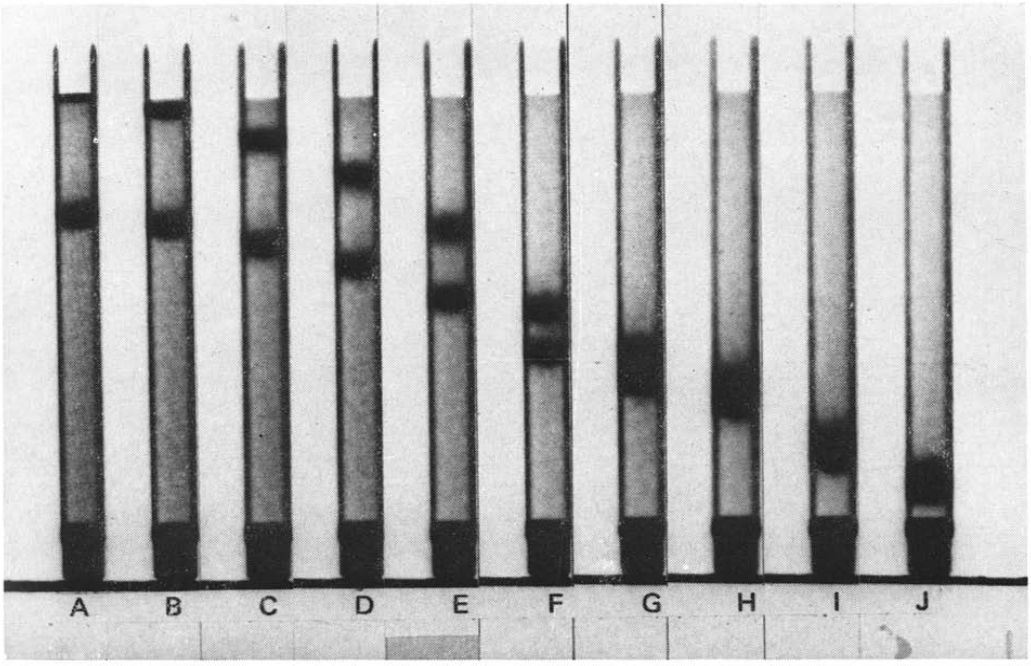

Fig. 4. A sample of myoglobin (4 mg in 0.4 ml) caught up by a second sample of myoglobin. Column bed, 20 × 1.5 cm of DEAE-Bio-Gel A, adjusted to pH 10.5 (see Experimental); buffer, 0.4 % Ampholine, pH range 8–9.5, adjusted to pH 7.5; flow rate, 20 ml/h. Images A–J show the positions of the bands 0, 2, 9, 18, 32, 55, 69, 77, 98 and 113 min, respectively, after application of the second band.

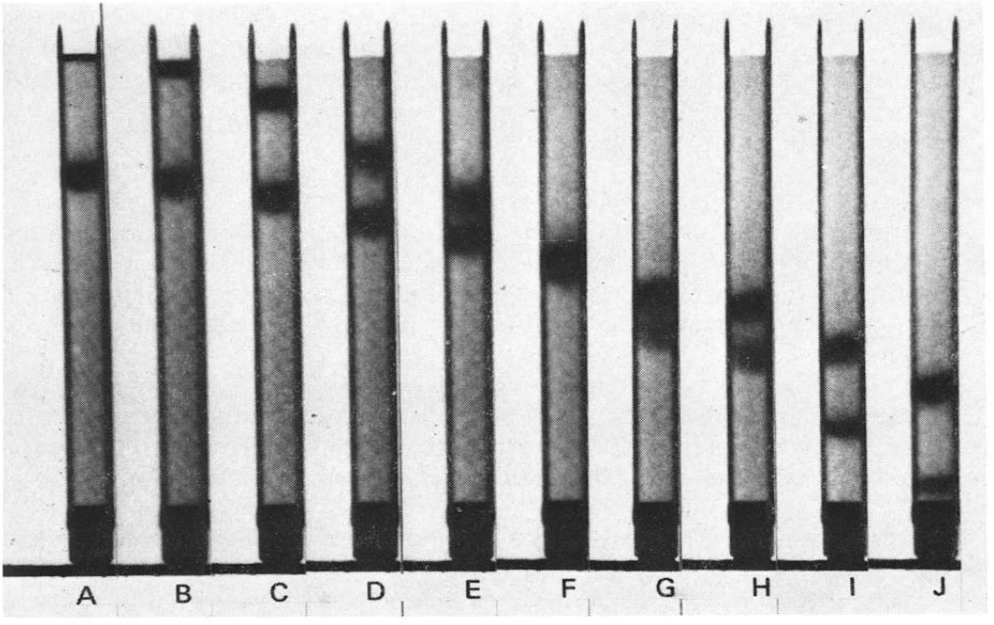

Fig. 5. A sample of myoglobin (4 mg) passed by a sample of cytochrome c (4 mg) applied later. The conditions were identical with those in Fig. 4. Images A–J show the positions of the bands 0, 1.5, 7, 17, 25, 35, 48, 54, 71 and 88 min, respectively, after application of the cytochrome.

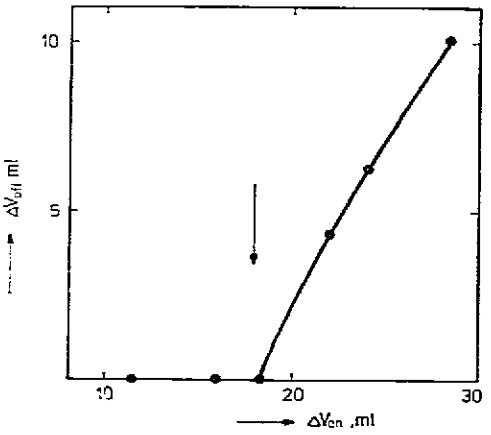

Fig. 6. Difference in elution volume, ΔV_{off} between the two emerging peaks of two separate applications of myoglobin, as a function of ΔV_{on}, the elution volume passed through the column between the two applications of the proteins. The arrow indicates the theoretical value of the distribution volume. Bed volume, 46 ml; 0.4% ampholine, pH 8–9.5; flow-rate, 21 ml/h; elution volume of first band, 63 ml.

volume* of the column[1]. This was verified with a number of experiments of the type illustrated in Figs. 3a and 4, by varying the distance between the first and the second sample of myoglobin and measuring the difference in elution volume between the two emerging peaks. In Fig. 6 the difference in elution volume is plotted against the initial distance between the two peaks, in terms of buffer volume flowing through the column between the two applications of protein. When the latter volume is smaller than the distribution volume, the two samples emerge as a single band and the difference in elution volume is zero; when it exceeds the distribution volume, two peaks emerge and the difference in elution volume can be measured. The intersection of the ascending curve of Fig. 6 with the abscissa yields the distribution volume, which turns out to be equal to the calculated value, indicated by the arrow.

Separations on anion and cation exchangers

A commercial preparation of sperm whale myoglobin, run on an anion exchanger, exhibited one main band and four minor bands. A similar picture was obtained with the conventional electrophoretic method in a sucrose gradient (Fig. 7). The minor component nearest to the main band was best separated in the chromatographic system.

Samples of bovine serum albumin were run on a column of a cation exchanger and in the electrophoretic equipment. In both instances two peaks were observed (Fig. 8). Two, or even more, peaks of serum albumin have also been observed in moving boundary electrophoresis. They proved to be affected by the composition of the buffer[13,14].

These two examples show that both anion and cation exchangers can be utilized

* This volume is equal to the total volume of the column minus the volume occupied by the agarose polymer. Assuming that the specific gravity of agarose is the same as that of sucrose (1.6), the agarose polymer occupies about 2 of the total volume.

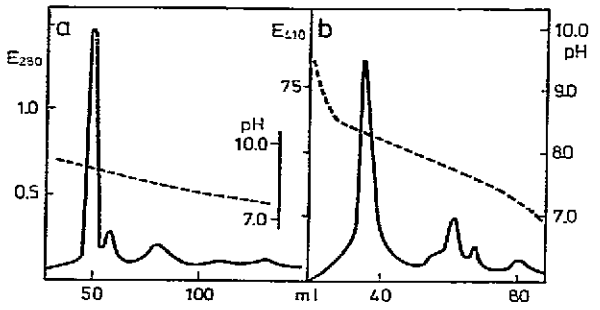

Fig. 7. (a) Elution pattern of 12 mg of commercial sperm whale myoglobin on a column bed of 12 × 1.6 cm of DEAE-Bio-Gel A, adjusted to pH 10.5. Elution buffer, 0.15% Ampholine, pH 8–9.5, adjusted to pH 7.5 with acetic acid. Flow-rate, 20 ml/h. (b) Pattern of 15 mg of commercial sperm whale myoglobin on electrofocusing. Buffer, 0.75% Ampholine, pH 8–9.5; 3 days, 400–1000 V, 25°.

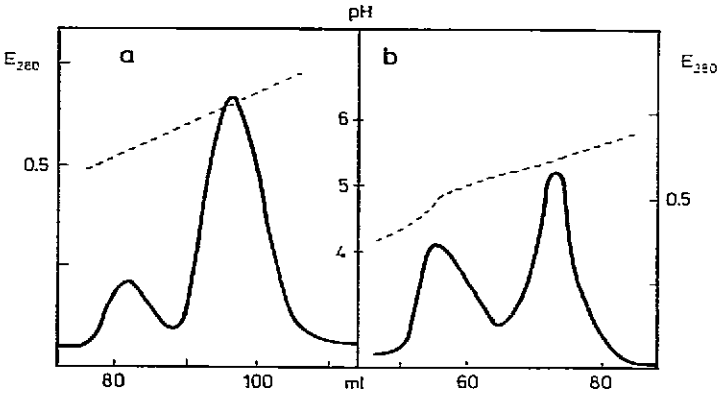

Fig. 8. (a) Elution pattern of 9 mg of bovine serum albumin on a column bed of 10 × 1 cm of CM-Bio-Gel A. The column was adjusted to pH 4.8 with 0.1 *M* acetate buffer before application of the protein. Elution with 0.075% Ampholine, pH 3–10, adjusted to pH 7.0 with ammonia. Flow-rate, 4 ml/h. (b) Pattern of 15 mg of bovine serum albumin on electrofocusing. Buffer, 0.6 % Ampholine, pH 3.7; 1 day, 400 V, 25°.

to effect separations that are similar to those obtained by the conventional electrophoretic method. The main difference between the two methods is the value of the isoelectric points, which will be discussed below.

Factors that affect the separation

According to the theoretical study (eqn. 29 in Part I[1]), the theoretical half-width of a protein band is, in a first approach, given by

$$(\Delta\mathrm{pH})^2 = \frac{1 + rK_0}{rK_0(1 + r)^2}\left[\frac{D}{\frac{\mathrm{d}V}{\mathrm{d}t}} + (1 + r)^2 q\right]\frac{\frac{\mathrm{dpH}}{\mathrm{d}V}}{\varphi \cdot \frac{\mathrm{dpH}}{\mathrm{d}Z}} \qquad (1)$$

when axial diffusion and axial dispersion by non-uniformity of flow are taken into

account. In this equation r denotes a_s/a_m, the ratio of the cross-sectional areas of the stationary phase s and the mobile phase m, K_0 the partition coefficient (C_s/C_m) of the protein when in focus, D the diffusion constant of the protein, (dV/dt) the flow-rate per square centimetre of column diameter, q a constant related to the packing quality, (dpH/dV) the pH gradient and (dZ/dpH) the slope of the titration curve of the protein around its isoelectric point.

For myoglobin, the bandwidth caused by axial diffusion only, *i.e.*, in eqn. 1 $q = 0$, can be estimated by inserting into eqn. 1 the values $D = 1.1 \cdot 10^{-6}$ (ref. 15), (dpH/dV) = 0.042 (Fig. 7a), (dV/dt) = 10 ml/h, φ = 16.6 mV, (dZ/dpH) = 1.8 (ref. 9), $r = 2$ and $K_0 = 1$. This yields $\Delta pH = \pm 1.5 \cdot 10^{-3}$. The experimental value of the main component proved to be $\pm 4 \cdot 10^{-2}$ (Fig. 7a), *i.e.*, an order of magnitude larger. In eqn. 1, the term containing D apparently can be neglected, which reduces eqn. 1 to

$$(\Delta pH)^2 = \frac{1 + rK_0}{rK_0} \cdot q \cdot \frac{\frac{dpH}{dV}}{\varphi \cdot \frac{dZ}{dpH}} \qquad (2)$$

According to eqn. 2, the quality of the separation is governed by q and is independent of the flow-rate. It was indeed found that the pattern of Fig. 7a did not change appreciably when the flow-rate was varied from 2 to 20 ml/h per unit cross-sectional area, which is the workable range of flow-rates.

According to eqn. 2, the resolution will decrease when the buffer concentration is increased four-fold, because (dpH/dV) will increase (from 0.042 in Fig. 7a to 0.15 in Fig. 8a) and φ will decrease from 16.6 to 11.4 mV (Fig. 1). Insertion of these values into eqn. 2 yields a ratio of 2.3 for the bandwidths for the main bands of Fig. 9a and Fig. 7a. Actually, the half-widths (at 60% of the maximum height) of the bands are 0.20 and 0.04, respectively, a ratio of 2.5, which is close enough to the calculated value.

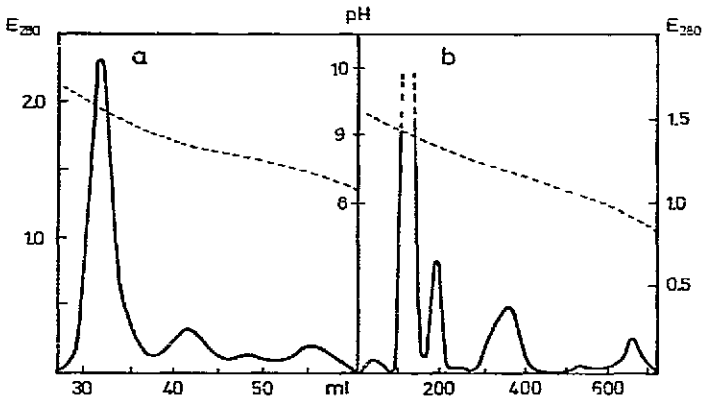

Fig. 9. (a) Elution pattern of 6 mg of commercial myoglobin. Conditions as in Fig. 7a, except for the concentration of the elution buffer (0.6% Ampholine). Flow-rate, 5 ml/h. (b) Elution pattern of 225 mg of commercial myoglobin on a column bed of 27 × 2.5 cm of DEAE-Bio-Gel A. Buffer, 0.15% Ampholine, pH 8–9.5, adjusted to pH 7.5. Flow-rate, 40 ml/h.

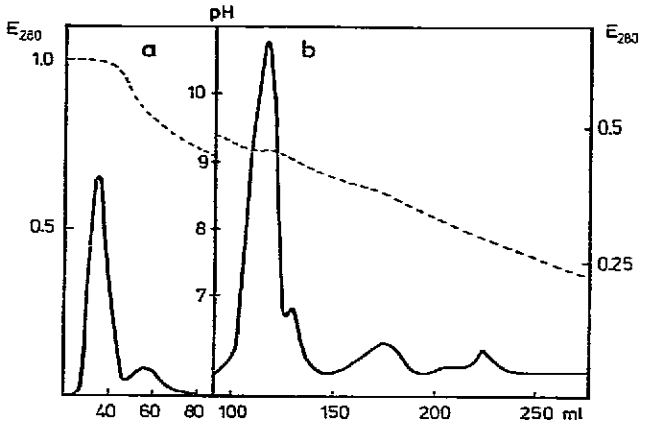

Fig. 10. (a) Elution pattern of commercial myoglobin. Conditions as in Fig. 7a, except for the addition of 5 m*M* NaCl to the ammonia buffer and the elution buffer. (b) Elution pattern of 12 mg of commercial myoglobin on a 15 × 1.6 cm column bed of DEAE-Bio-Gel A of pH 10.5. Elution with 3.5 m*M* glycine and 3.5 m*M* diethanolamine, pH 7.5, linearly diluted with 1 m*M* MOPS and 3.5 m*M* Tris, pH 6.8, in a mixing chamber, in order to effect a more constant gradient. Flow-rate, 22 ml/h.

The poorer resolution in 0.6% ampholine is also evident from the absence of the small peak next to the main band in Fig. 9a, which can be distinguished in Fig. 7a. Making the column longer will decrease the pH gradient (Fig. 2 in Part I[1]) and will improve the separation. As shown in Fig. 9a, the separation is in fact better than that in the shorter column (Fig. 7a).

A decrease in φ will be disadvantageous for separation according to eqn. 2. This was verified by adding 5 m*M* sodium chloride to the 0.15% ampholine buffer. The resolution was very poor indeed (Fig. 10a), in fact even poorer than expected, because the decrease in φ was not very large (Fig. 2). This may be due to some direct competition effect.

A protein that is "out of focus" carries a positive charge and is therefore partitioned in favour of the mobile phase, *i.e.*, $K_0 < 1$. According to eqn. 2, $K_0 < 1$ will cause an increase in bandwidth, which is demonstrated in Figs. 4 and 5. The second sample of myoglobin in Fig. 4, image E, not yet in focus, is wider than the first sample which is in focus. Similarly, cytochrome c in Fig. 5, images D–H, is out of focus and is somewhat wider than the myoglobin band. Once the cytochrome c is in focus (images I and J) it narrows considerably to a band even narrower than the myoglobin band, owing to the fact that dZ/dpH for cytochrome c is larger than that for myoglobin (Table I).

Although most separations were carried out in ampholine buffers for the sake of convenience, these buffers are not essential and can be replaced with appropriate mixtures of ordinary buffers (Fig. 10b). The separation in Fig. 10b is almost as good as that in Fig. 7a.

Factors that affect the apparent pI

According to Part I[1], the pH of the emerging protein solution may differ from the p*I* determined by electrophoresis. This was borne out experimentally. In a first

approach, the difference has been calculated (eqn. 34 in Part I[1]) to be

$$pI_{obs} - pI = -\frac{1}{4.6} \cdot \varphi - \frac{1}{\varphi \cdot \frac{dZ}{dpH}} \ln (1 + r_c + \frac{r_c}{r}) \qquad (3)$$

where r_c is the ratio of the buffer capacities of the exchanger and the buffer. This ratio can be calculated from the titration data in Fig. 1. The data concerned are given in Table I.

The calculated and observed pI values of the main band of myoglobin proved to be of the same order of magnitude. Further, cytochrome c and papain, with higher values of dZ/dpH, exhibit smaller values of pI than myoglobin, in accordance with eqn. 3. Serum albumin, on the other hand, does not fit the present picture. This is not surprising, as the electrophoretic behaviour of serum albumin is known to be sensitive to buffer composition, etc.[13,14], and the buffer composition in the two methods of separation is not the same; in the electrophoretic method the buffer components are separated according to their pI values, whereas in the chromatographic method they emerge from the column in increasing number as the separation proceeds, the complete mixture emerging finally.

Applications

A crude extract of papaya latex was subjected to chromatographic and electrophoretic focusing. The results are shown in Fig. 11. The separating power is slightly greater in the chromatographic procedure, even though the separation of the first components that emerged occurred at the limit of the pH range in which the anion exchanger is positively charged. All fractions proved to exhibit activity towards benzoyl-arginine ethyl ester.

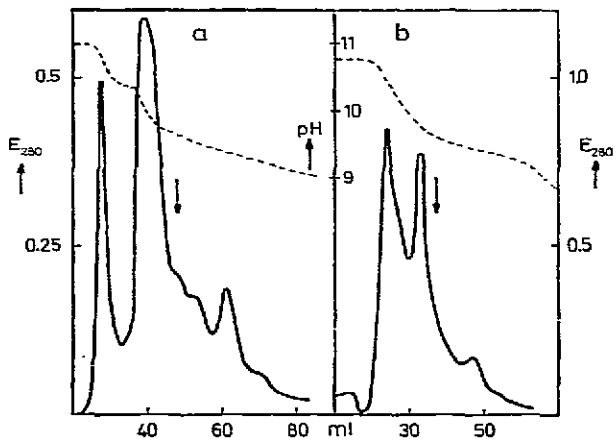

Fig. 11. (a). Fractionation of 2 ml of crude papaya latex extract. $E_{280} = 20$ on a DEAE-Bio-Gel A column, bed volume 15 × 1.6 cm; initial pH, 11. Elution buffer, 0.15% Ampholine, pH 8–9.5, adjusted to pH 7.5 containing 5% DMSO. Flow-rate, 20 ml/h. (b) Fractionation of 0.8 ml of crude papaya latex extract. $E_{280} = 60$ in the electrophoretic focusing column. Buffer, 0.25% Ampholine, pH 9–11, containing 5% DMSO; 66 h, 450 V, 25°. The arrows indicate the expected position of papain.

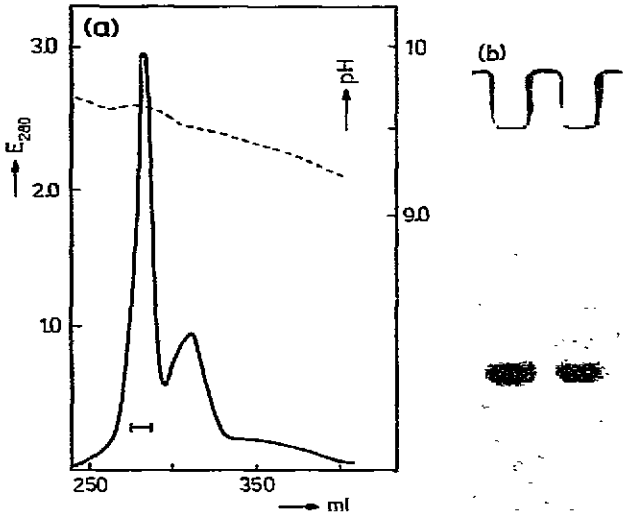

Fig. 12. (a) Purification of 50 mg of papain (mixed disulphide with mercaptoethanol) on a column bed of 30 × 2.5 cm DEAE-Bio-Gel A, pH 10.5, applied in 8 ml of elution buffer, 0.15% Ampholine, pH 8–9.5, 5% DMSO pre-adjusted to pH 8.0 with acetic acid. Flow-rate, 44 ml/h. (b) Gel electrophoresis of papain at pH 6.0. Left, initial preparation; right, purified preparation [corresponding to the fractions in (a), indicated by the horizontal bar].

Papain prepared according to the method of Kimmel and Smith[3] and purified further on the mercurial-agarose column still contained two or three minor impurities of lower electrophoretic mobility, differing by one, two and three charge units from the main component (Fig. 12b). These charge differences were assessed by the method of partial acetylation[16]. The impurities, also evident on chromatofocusing, could virtually be removed (Fig. 12). It might be worth investigating the properties of these minor components.

DISCUSSION

The separations that were obtained in chromatofocusing proved to be similar to, although not necessarily identical with, those obtained in the electrophoretic method. The half-widths of the protein bands in the chromatographic procedure, mostly ± 0.05 pH unit, are similar to those reported for the preparative electrophoretic method, both in this paper and in recent literature on the purification of proteins.

Compared with the electrophoretic method, the chromatographic method has the following advantages. Ordinary chromatographic equipment can be utilized. The size of the column can be freely chosen; even large columns can be employed, because there is no cooling problem. Ampholine buffers, although convenient for obtaining smooth pH gradients, in some instances at least can be replaced with mixtures of ordinary buffers, which are cheaper and can be separated more easily from the proteins afterwards.

The pI_{app} values only approximate to the pI values observed in the electrophoretic method. Nevertheless, the pI_{app} values, as far as can be judged from the limited number of examples in this paper, are adequate for the purpose of deciding in

which pH range the pI values can be determined more accurately with the aid of electrofocusing in gel slabs.

An essential feature of the procedure described in these two papers is the use of low buffer concentrations in order to permit high Donnan potentials, which are favourable towards resolution. Some of the effects reported in this paper may have occurred, without the investigators realizing it, in instances where the buffer concentration was low compared with the capacity of the exchanger[17].

The main advantage of the internal generation of the pH gradient, as pointed out in Part I[1], is that the proteins are not subjected to more extreme pH values than correspond to their pI_{app} values. It is therefore useful to try to adjust φ and r_c according to eqn. 3 in such a way as to keep pK_{app} and pI as close together as possible. On the other hand, a separation might be effected on the basis of eqn. 3 between proteins with equal pI but of different values of dZ/dpH. In this way, chromatofocusing provides an additional possibility of separation. The difference in the relative positions of the first minor component of commercial myoglobin in Figs. 7a and 7b could be due to this factor.

The experiments described here were designed more to verify the general principle than to find optimal conditions. So far, commercially available ion exchangers have been utilized. A future development could be the preparation of exchangers specially designed in order to extend the pH range of applicability and, if possible, to increase the resolution and to speed up the separations.

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