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# CHROMATOFOCUSING

## III. THE PROPERTIES OF A DEAE-AGAROSE ANION EXCHANGER AND ITS SUITABILITY FOR PROTEIN SEPARATIONS

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## SUMMARY

Columns of DEAE-agarose beads of high maximum capacity (0.18 *M* inside the gel) were examined for their suitability for protein separation in the system of chromatofocusing. It was concluded that: (a) large sudden changes of ionic strength should be avoided; (b) when an anion exchanger is used, the components of the pre-equilibration buffer and the elution buffer should be of the cationic type, whereas the counter ions should not be subject to association–dissociation equilibria in the pH range of operation. Furthermore slow equilibration between ion exchanger and surrounding medium caused small maxima in the pH gradient. These complications were mainly circumvented while maintaining the advantages of chromatofocusing by using the following buffer system: pre-equilibration of the column with 10 m*M* ammonia, pH 9.2, and elution with a linear gradient from 10 m*M* ammonia, pH 7.5, to 10 m*M* triethanolamine, pH 7.5, produced in a gradient mixer. In the approximately linear pH gradient which results a commercial preparation of myoglobin could be separated into several components. The resolution was twice as good as in previous experiments, corresponding to theoretical calculations. However, the sluggishness of the exchanger has to be taken into account when designing new ion exchangers.

## INTRODUCTION

In two recent papers[1,2] it was shown theoretically and experimentally that proteins travelling down an ion-exchange column in a pH gradient are subjected to focusing and emerge from the column at pH values approximately equal to their isoelectric points. Especially favourable conditions, similar to those of electrophoretic focusing, are obtained when the pH gradient is produced inside the column. This can be achieved as follows. The anion-exchange column is first adjusted to the highest pH to be utilized. Next the protein sample is dissolved in a buffer at the lowest pH to be used, dialysed against the same buffer and applied to the column. Elution is continued with the buffer. The acid–base exchange between the elution buffer and ion exchanger produces a pH gradient within the limits of the two initial pH values,

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which moves slowly down the column. Details of this process were given in our first paper[1].

The width of a protein band in a moving pH gradient was calculated to be proportional to

$$\Delta \mathrm{pH} \approx \pm \sqrt{\frac{\mathrm{dpH}}{\mathrm{d}V} \Big/ \varphi \frac{\mathrm{d}Z}{\mathrm{dpH}}} \qquad (1)$$

where:

$$\varphi = F\psi/RT$$

$\psi$ denotes the Donnan potential, $\mathrm{dpH}/\mathrm{d}V$ the slope of the pH gradient per unit cross-sectional area and $\mathrm{d}Z/\mathrm{dpH}$ the slope of the titration curve of a protein near its isoelectric point. Eqn. 1 was verified experimentally.

Most of the experiments described in our second paper[2] were carried out on columns of DEAE-agarose beads of low maximum capacity (19 $\mu$moles per ml of packed gel) and with low buffer concentration (0.15% ampholine or 3.5 m*M* conventional buffer components, *cf.*, Fig. 1). The simplest way of improving the separation would be to lower the buffer concentration, as this would both decrease $\mathrm{dpH}/\mathrm{d}V$ and increase $\psi$. However this would lead to inacceptably low buffer concentrations.

Another commercially available anion exchanger of much higher capacity was therefore tested, which permitted the use of less dilute buffers. In this paper, the complications which arose and the methods employed to circumvent these are reported.

## EXPERIMENTAL

The anion exchanger DEAE-Sepharose CL was purchased from Pharmacia (Uppsala, Sweden). Sperm-whale myoglobin was a commercial preparation (Serva, Heidelberg, G.F.R.). Ampholine buffers were obtained from LKB (Stockholm, Sweden). Stock solutions of the buffer components and of the acids for pH adjustment were 1 *M*. The dilute buffer solutions were prepared by deaerating the required amount of distilled water *in vacuo*, adding the desired portions of buffer stock solutions and adjusting the pH. During the pH adjustment the solutions were stirred slowly with a magnetic stirrer. Since the stirring somewhat affected the apparent pH of these dilute solutions, the final pH readings were made in the stationary solution.

The chromatographic equipment and the general procedures were as described previously[2]. The eluent was monitored initially at 410 nm with an SPG-400 UV spectrophotometer (Pye-Unicam, Cambridge, Great Britain), then at 280 nm with a Pharmacia Type UV-2 UV monitor. Absorption and pH were recorded on Philips Type PM 8221 recorders.

Dialyses were carried out by the ultrafiltration technique in Diaflo cells (Amicon, Lexington, MA, U.S.A.) utilizing Type PM 10 membranes.

## RESULTS

The maximum capacity of the anion exchanger tested, DEAE-Sepharose, was 120 $\mu$moles per ml packed gel, six times higher than that of the DEAE-Bio-Gel

(19 μmoles/ml) used previously. The buffering capacity of the exchanger increased to the same extent. If the concentration of the elution buffer had been the same as before, the elution time would have been prolonged six-fold. If the buffer concentration were increased six-fold, in proportion to the capacity of the exchanger, no decrease in $dpH/dV$ and no increase in Donnan potential and therefore no increased resolution would be expected. A middle course was taken, the buffer concentration being increased from 3.5 m*M* to 10 m*M*.

As previously[2], commercial myoglobin which contains some impurities was used as test material. The results were disappointing. Poor separations were obtained, and the pH gradients were freakish, with sudden changes, plateaus and even maxima. An example is given in Fig. 1a. Even at a higher concentration the buffer mixture that was previously successful produced an unusable pH gradient (Fig. 1b). A large number of experiments were conducted in an attempt to determine the reasons for these irregularities and to improve the shape of the pH gradients. Only the most informative of these will be reported.

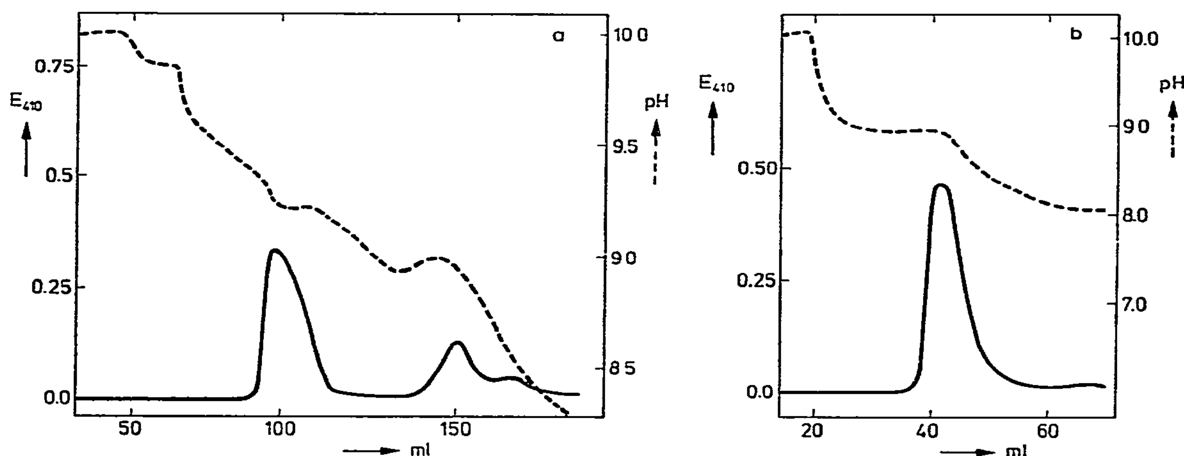



Fig. 1. Examples of irregular pH gradients and of protein elution patterns from DEAE-Sepharose columns. a, Column bed (13.5 × 1.6 cm) pretreated with 250 ml buffer (50 ml/h) containing 10 m*M* Tris and 10 m*M* trimethylamine, adjusted to pH 10.1 with formic acid. Elution buffer identical, adjusted to pH 8.00. Sample: 14 mg of myoglobin applied to the column in 2.5 ml of elution buffer containing 2% saccharose. Flow-rate 13.4 ml/h. b, Column bed (6.0 × 1.0 cm) pretreated with ammonia, pH 10.5. Elution buffer: 17.5 m*M* glycine and 17.5 m*M* diethanolamine, pH 7.5, diluted with 5 m*M* morpholinopropanesulphonic acid (MOPS) and 17.5 m*M* Tris, pH 6.0. Sample: 6 mg of myoglobin applied to the column in 1.0 ml of the first elution buffer. Flow-rate 10 ml/h.

Even under favourable conditions irregularities were observed. The conditions were: (a) low pH, *i.e.*, pH 4–5, where the ion exchanger has only a low buffering capacity (Fig. 5, see below); (b) equal pH of the pre-equilibration buffer and the elution buffer.

When the column of anion exchanger was equilibrated with 5 m*M* sodium acetate and acetic acid at pH 4.7 and then with 1 m*M* sodium acetate and acetic acid at pH 4.7, there was a temporary decrease in pH (Fig. 2a). When the original buffer was replaced a temporary increase in pH was observed. These phenomena can be

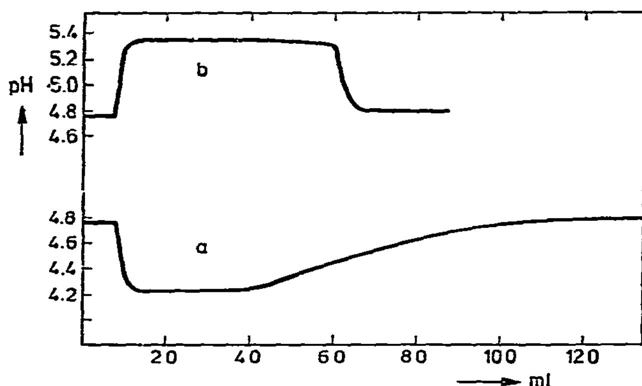
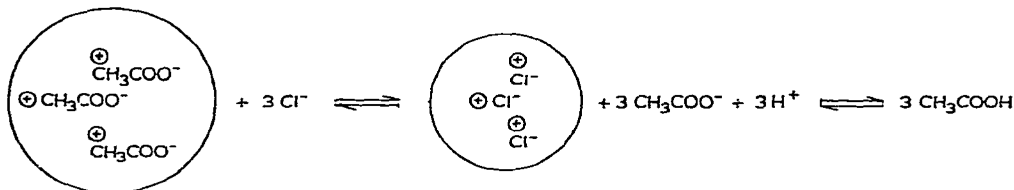



Fig. 2. The effect of change of buffers of equal pH and of different concentration on DEAE-Sepharose (7 × 1 cm). Pre-equilibration buffer: 5 m*M* sodium acetate and acetic acid down to pH 4.76. a, 1 m*M* sodium acetate and acetic acid down to pH 4.76; b, replacement by the first buffer. Flow-rate 10 ml/h.

qualitatively understood as follows. A change of buffer concentration results in a change in the Donnan potential, which is accompanied by a change in pH difference between the two phases. Decreasing the buffer concentration increases the pH difference and effects the release of protons from the exchanger into the buffer until a new equilibrium is established. When the buffer concentration is increased, the opposite occurs.

These experiments were then repeated, keeping the ionic strength constant with sodium chloride. On changing the buffer from 5 m*M* sodium acetate plus acetic acid, pH 4.71, to 1 m*M* sodium acetate + 4 m*M* sodium chloride plus acetic acid, pH 4.71, a temporary increase in pH of more than one unit was observed (Fig. 3a). When the original buffer was replaced a temporary decrease of pH was observed (not shown). The increase in pH shown in Fig. 3a can be explained in the following way. After equilibration in the first buffer the fixed positive charges of the ion exchanger are surrounded by an equal number of acetate counter ions. When the second buffer is introduced four fifths of the acetate counter ions are gradually replaced by chloride ions. Some of the exchanged acetate ions, being subject to an acid dissociation equilibrium, are protonated and the pH is thus raised:




Only slight effects, of 0.2–0.3 pH units, over very short periods were observed when a similar experiment was carried out with aniline as buffer (Fig. 3b). These effects are interpreted as follows. The pre-equilibration buffer contained 5 m*M* anilinium chloride. Because the pH, 4.58, was approximately equal to the p$K_a$ value of

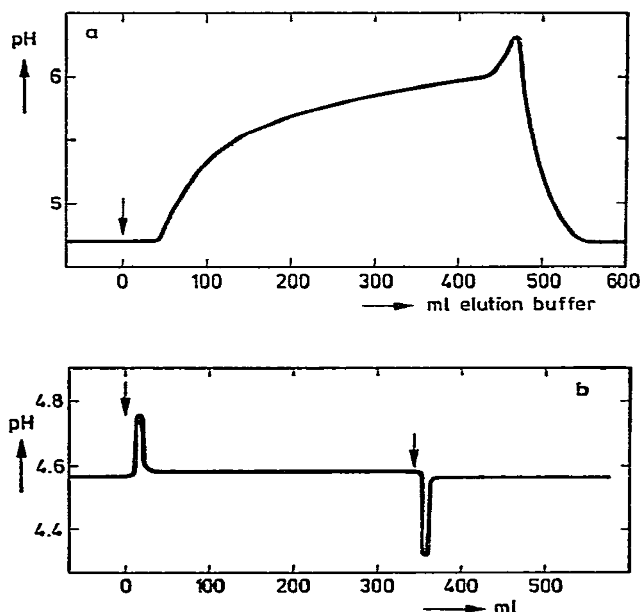



Fig. 3. The effect of buffer concentration at constant ionic strength and equal pH. a, Column bed: 10 × 1.6 cm. Equilibration buffer: 5 m*M* sodium acetate and acetic acid down to pH 4.71. Second buffer: 1 m*M* sodium acetate, 4 m*M* NaCl and acetic acid down to pH 4.71. Flow-rate 40 ml/h. b, Column bed: 13 × 1.6 cm. Equilibration buffer and third buffer: 5 m*M* HCl and aniline up to pH 4.58. Second buffer: 1 m*M* HCl, 5 m*M* NaCl and aniline up to pH 4.59. Flow-rate 50 ml/h. The arrows indicate change of buffer.

aniline, 4.6, the concentration of unprotonated aniline was therefore also 5 m*M*. At equilibrium the concentration of the unprotonated species is the same inside and outside the beads of ion exchanger. In contrast, the concentration of the anilinium ions inside the beads is much lower than the concentration outside, owing to the Donnan potential, and results in a higher pH inside the beads than outside. When the pre-equilibration buffer is replaced by the second buffer containing 1 m*M* anilinium ions, 1 m*M* unprotonated aniline and 4 m*M* NaCl, four fifths of the unprotonated aniline in the beads, originating from the first buffer, diffuse into the mobile phase, taking up protons owing to the acid–base equilibrium and raising the pH. This rise is not compensated by the much smaller simultaneous outward diffusion of anilinium ions into the mobile phase. When the second buffer is replaced by the first buffer the opposite phenomena occur.

The main reason why the perturbation with the aniline buffer was much smaller than with the acetate buffer is that, after pre-equilibration, with the former buffer the concentration of the buffering species, aniline, inside the beads is only 1–5 m*M*, *i.e.*, equal to the amount dissolved in the buffer. With the acetate buffer, on the other hand, the content of acetate ions inside the beads is 180 m*M*, *i.e.*, equal to the concentration of fixed positive charges.

It may be concluded that when using simple buffer systems on an anion exchanger, cationic buffering components such as amines should be used with ions like chloride ions as counter ions. A buffer meeting these conditions is ammonium formate–

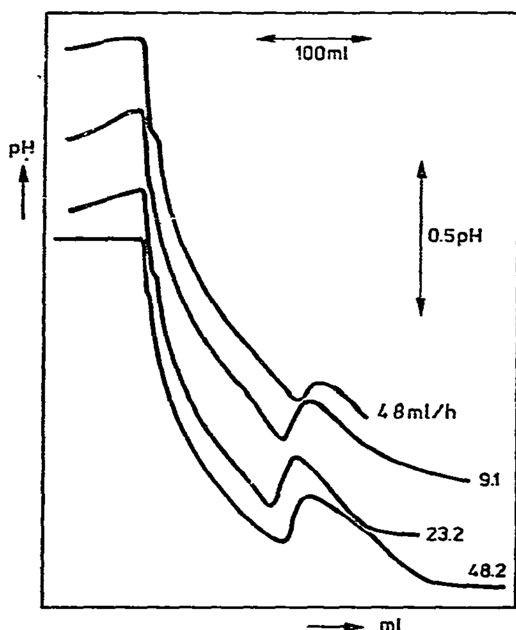



Fig. 4. The effect of flow-rate on the shape of the pH gradient. Column bed: 12.5 × 1.6 cm. Equilibration buffer: 11 m*M* ammonia and formic acid down to pH 9.85. Second buffer: 11 m*M* ammonia and formic acid down to pH 8.61. Flow-rate as indicated.

ammonia. However, gradients from pH 9.85 to pH 8.61 produced with this buffer still exhibited maxima (Fig. 4). These maxima increased with increasing flow-rate (Fig. 4 and Table I), with the exception of the highest flow-rate. It is suggested that the maxima are due to "sluggishness" of the ion exchanger. Some sluggishness was also observed when a suspension of the exchanger was subjected to acid–base titration (Fig. 5). Curve a was obtained by addition of titrant and taking pH readings every minute, whereas for curve b time was allowed for approximate equilibration to be achieved, which took a few minutes in the range below pH 6 and above 9 and about 10 min in the intermediate pH range. Under these conditions there were differences of up to 0.8 pH units between these curves. The maxima in Fig. 4 can be explained as follows. At the beginning of the elution the pH rapidly decreases, but the ion exchanger cannot quite keep pace with the decrease. Towards the end of the elution the pH gradient levels off and the ion exchanger can "catch up" with the equilibrium,

TABLE I

pH DIFFERENCE BETWEEN MINIMUM AND MAXIMUM AS A FUNCTION OF FLOW-RATE (*cf.*, FIG. 4)

| *Flow-rate (ml/h)* | *Δ pH* |
|---|---|
| 4.8 | 0.06 |
| 9.1 | 0.12 |
| 23.2 | 0.16 |
| 48.2 | 0.15 |

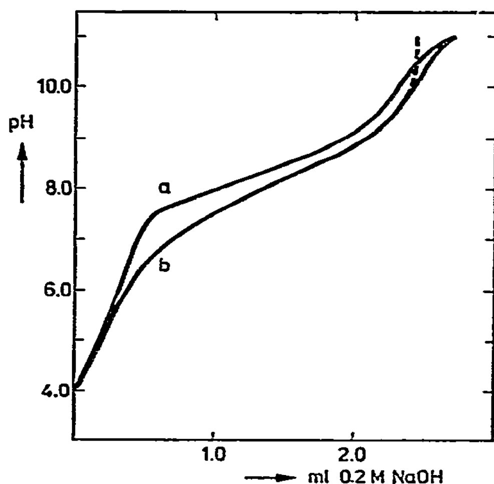



Fig. 5. Titration of 4.0 ml packed DEAE-Sepharose, suspended in 30 ml water, with 0.2 *M* NaOH. a, Addition of titrant and reading of pH every 1 min; b, as a but readings every 5–10 min. Broken line: curve corrected for titration of pure water.

take up protons from the buffer and temporarily increases the pH of the latter. At the highest flow-rate (Table I), the flow-rate is too high to permit complete equilibration even when the gradient levels off.

Even if there had been no maximum, the shape of the curve would not be ideal, because the gradient is high at the beginning and low at the end. A more linear pH gradient would be more favourable for separations and would diminish the possibility of pH maxima occurring. At the same time it is desirable to retain the advantages of elution with a buffer of low pH[1,2]. These objectives were fulfilled by the following system. A column of DEAE-Sepharose was equilibrated with 10 m*M* ammonia adjusted with dilute formic acid to pH 9.24. The pH gradient was produced by mixing two buffers, A and B, of equal pH in a gradient mixer. Buffer A comprised 10 m*M* ammonia, adjusted to about pH 7.5 with dilute formic acid; buffer B comprised triethanolamine, adjusted to pH 7.49. The mixer was set so as to produce a linear gradient from buffer A to buffer B in 24 h. The pH gradient emerging from the column is shown in Fig. 6. It proved to be fairly linear, without maxima or minima.

The reasons for choosing this buffer system were as follows. Ammonia, p*K* 9.2, exhibits its maximum buffering action at pH 9.2 and has hardly any buffering action at pH 7.5. The initial ammonia buffer of pH 7.5 is therefore unable to decrease the pH rapidly. Triethanolamine having a p*K* of 7.8, exerts its maximum buffering action around pH 7.8. Buffer B, increasingly predominating towards the end of a run, is therefore able to decrease the pH in the later part of the run. The overall effect is the almost linear pH gradient shown in Fig. 6. As there is no high pH gradient at the beginning and a low one later on, the sluggishness of the anion exchanger does not produce a maximum in the gradient.

This gradient was used to separate the various components of commercial myoglobin (Fig. 7). The separation obtained was better than that observed previously[2] on DEAE-agarose gel of low capacity with a simple buffer (Fig. 7, inset).

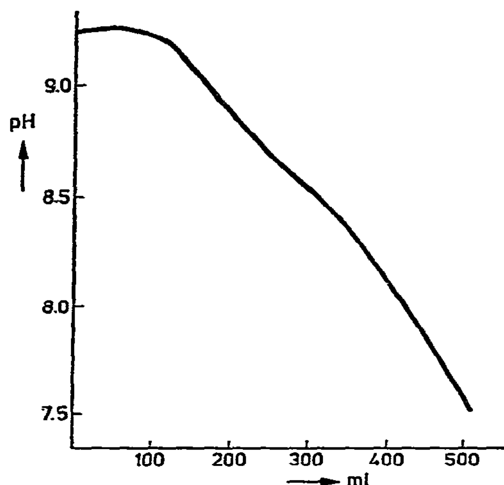



Fig. 6. pH gradient produced when two buffers of equal pH were mixed and passed through the column (15 × 1.6 cm), first equilibrated with 10 m*M* ammonia and formic acid down to pH 9.4, at 50 ml/h for 7 h. During the run a linear gradient from buffer A to buffer B (see text) was produced in 24 h and passed through the column. Flow-rate 18 ml/h.

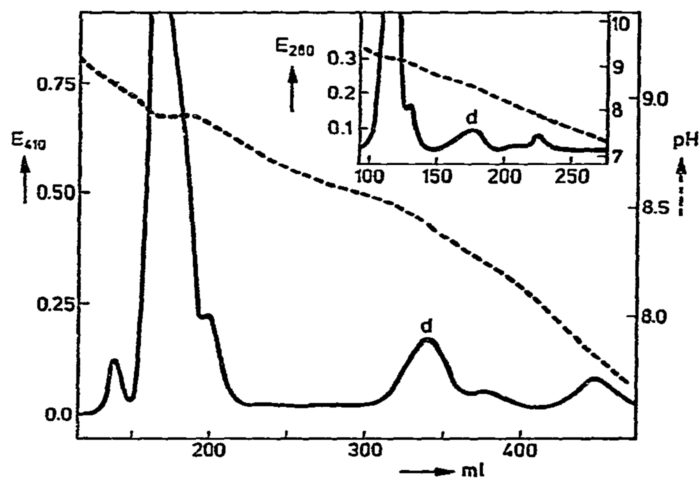



Fig. 7. Separation of the components of commercial myoglobin. Conditions as in Fig. 6. After pre-equilibration of the column, 5.0 mg of myoglobin were applied, dissolved in 2.5 ml of buffer A, and dialyzed against the same buffer with 3% of saccharose. Inset: buffer system like that of Fig. 1b, five-fold diluted (*cf.*, Fig. 10b of ref. 2).

The present method, however, still has one disadvantage. The pH gradient exhibits a slight maximum near the main band of Fig. 7, which adversely affects the separation. This effect is more evident when the amount of protein applied to the column is increased from 5 mg (Fig. 7) to 23 mg (Fig. 8a). The main band is broadened and tends to show an artificial second band. The small peak, evident just behind the main band in Fig. 7, can be distinguished only as a very slight shoulder in Fig. 8a. The pH

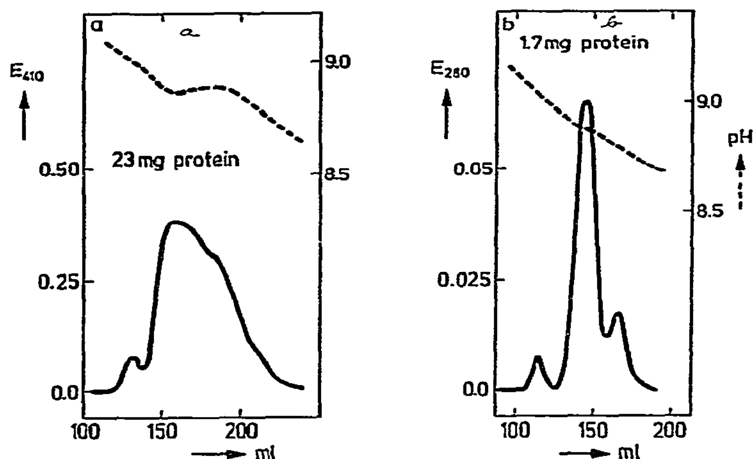



Fig. 8. First part of the elution of commercial myoglobin. Conditions as in Figs. 6 and 7. a, 23 mg of protein; b, 1.7 mg of protein.

maximum was absent when the amount of protein was reduced to 1.7 mg and the separation was improved (Fig. 8b). Similar results (not shown) were obtained with 10 m*M* ethanolamine–formic acid buffer, pH 9.60, for pre-equilibration, 10 m*M* ethanolamine–formic acid as buffer A and 10 m*M* Tris–formic acid, pH 7.9, as buffer B.

Apparently a high protein concentration tends to cause the pH gradient to level off. The sluggishness of the ion exchanger then results in a slight maximum. When the second elution buffer, B, was replaced by a dilute ampholine buffer the pH gradient was not affected by 5 mg of myoglobin and only partly by 14 mg of the protein.

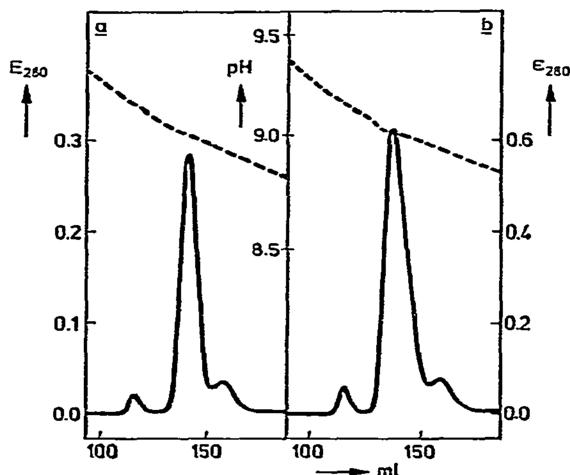



Fig. 9. Elution of the main band of commercial myoglobin. Column bed: 15 × 1.6 cm. Pre-equilibration with 10 m*M* ethanolamine–formic acid, pH 9.50. First elution buffer: 10 m*M* ethanolamine–formic acid, pH 7.90. Second elution buffer: 200 ml water plus 0.80 ml of Ampholine solution pH range 7–9, pH 7.89. Gradient mixer adjusted to linear mixing from first to second elution buffer in 24 h. Flow-rate 18 ml/h. a, 5.0 mg of myoglobin; b, 14 mg of myoglobin. Both samples were dialyzed against the first elution buffer.

## DISCUSSION

Eqn. 1 can be used to compare the resolution on the present ion exchanger with that on the previous one. The bandwidth for a protein is proportional to:

$$\Delta\text{pH} \approx \pm \sqrt{\frac{\text{dpH}}{\text{d}V} / \varphi} \qquad \text{(1A)}$$

The Donnan potential is calculated for the case of two compartments of equal volume, one of which contains a polyelectrolyte, separated by a semipermeable membrane. The present case in which beads of polyelectrolyte gel are equilibrated with a larger volume of buffer can be compared with a limited volume of polyelectrolyte in equilibrium with an excess of electrolyte (Fig. 10). Under these conditions the outside concentration of electrolyte is unchanged by equilibration.

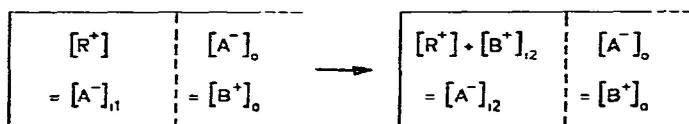



Fig. 10. Adjustment of the Donnan equilibrium. $[R^+]$, $[A^-]$ and $[B^+]$ denote the concentrations of fixed charges, anions and cations respectively. Subscripts: i and o = internal and external phases; 1 and 2 = before and after equilibration respectively.

Since in the present experiments the concentration of fixed charges inside the ion exchanger is much higher than the buffer concentration in the mobile phase:

$$[R^+] \gg [B^+] > [B^+]_{i2} \qquad (2)$$

Hence $[A^-]_{i2} \approx [R^+]$. The Donnan potential for monovalent ions is given[3] by:

$$\psi = (RT/F) \ln [A^-]_{i2}/[A^-]_o \qquad (3)$$

Insertion of eqn. 2 into eqn. 3 yields:

$$\psi F/RT = \varphi = \ln [R^+]/[A^-]_o \qquad (4)$$

Owing to this logarithmic relationship, $\varphi$ is less sensitive to the ratio of the exchange capacity and buffer concentration than is dpH/d$V$, which is inversely proportional to it.

Eqns. 1A and 4 can be used to compare the width of band d in Fig. 7 and in the inset of Fig. 7. The relevant data are presented in Table II. The concentration of counter ions, $[A^-]_o$, requires some comment. The ion concentration when band d emerges from the column of DEAE-Sepharose can be calculated from the data of Fig. 7. Band d emerges at 340 ml and since the flow-rate was 18 ml/h, this occurred after 19 h. Since the mixer was adjusted to produce a linear gradient from buffer A to buffer B in 24 h, the buffer at that moment contained (5/24)·10 m$M$ ammonia and (19/24)·10 m$M$ triethanolamine. Also, since the pH was 8.4, and the p$K$ values of

these compounds are 9.2 and 7.8 respectively, their degrees of ionization were 0.9 and 0.2 respectively. This yields 3.5 m$M$ as the sum of their ion concentrations. A similar calculation for the buffer system of Fig. 7 (inset) gives an ion concentration of 2.1 m$M$. There is a reasonable correspondence between calculated and observed ratios of the bandwidth of bands d in Fig. 7 and in Fig. 7 (inset) (Table II).

TABLE II

CALCULATION OF BANDWIDTHS

| | *dpH/dV* | *pH* | *[R⁺] (mM)* | *[A⁻]₀*** (mM)* | $\varphi$ | $\sqrt{\frac{dpH}{dV}\cdot\frac{1}{\varphi}}$ | *Ratio calc.* | *ΔpH* | *Ratio obs.* |
|---|---|---|---|---|---|---|---|---|---|
| Fig. 7 | 0.0092 | 8.4 | 60* | 3.6 | 2.8 | 0.057 | 0.52 | ±0.06 | 0.6 |
| Inset | 0.025 | 8.6 | 15** | 2.1 | 2.0 | 0.11 | | ±0.10 | |

* Calculated from curve b of Fig. 5, taking into account, that one third of the packed beads is void volume.

** Calculated from Fig. 1b of ref. 2, taking pH 10.5 at the end-point.

*** See text.

A similar calculation could not be carried out for the main band of myoglobin, because the width of this band cannot be measured with confidence, owing to the small plateau in the pH gradient at the site of the main band in Fig. 7 (inset). Nevertheless, a two-fold improvement is observed for the main band, the bandwidth being $\Delta$pH = ±0.02 in Figs. 8b and 9a, compared with a value of $\Delta$pH = ±0.04 reported previously (elution with ampholine buffer).

It has been shown previously that a protein emerges at a pH, p$I_{obs}$, which differs from the p$I$ value determined by electrofucusing according to eqn. 5:

$$pI_{obs} - pI = -\frac{\varphi}{4.6} - \left(\frac{1}{\varphi\frac{dZ}{dpH}}\right)\ln\frac{R_c}{r} \qquad (5)$$

$R_c$ denotes the ratio of the buffer capacity of the stationary phase, $a_s$, to that of the mobile phase, $a_m$, respectively, $r$ the ratio of the cross-sectional area of the two phases, equal to 2.0, and d$Z$/dpH the slope of the titration curve of the protein near its isoelectric point, equal to −1.8 for myoglobin. The main band of myoglobin emerges at pH 8.9 (Fig. 8b), whereas p$I$ = 8.3 (Table I of ref. 2). The buffer composition at the point of emergence was calculated from the elution volume as 6.5 m$M$ ammonia (p$K$ 9.25) and 3.5 m$M$ triethanolamine (p$K$ 7.76). The counter-ion concentration and $a_m$ were then calculated to be 4.73 m$M$ and 3.74/(1 + $r$) = 1.25 m$M$/pH. From curve b of Fig. 5, one obtains pH 8.9 [R⁺] = 21 m$M$ and $a_s$ = 30 m$M$/pH. Insertion of these values into eqns. 4 and 5 yields p$I_{obs}$ − p$I$ = 0.6, in excellent agreement with the observed difference of 0.6.

It may be concluded that DEAE-Sepharose permits improved resolution. There is, however, a drawback; the sluggishness of the exchanger causes complications at high protein concentrations. When designing new ion exchangers it is, therefore,

recommended that first their rate of equilibration should be checked. The experiments shown in Fig. 9 suggest that the utilization of ampholite buffers may overcome this complication. For a large-scale separation, however, these buffers are expensive. The titration curves of Fig. 5 show lag times of several minutes; the "tail" of these effects may last several hours and may be responsible for the maxima shown in Fig. 4. Although these effects are only small (Table I) they are inconvenient, especially in separations of high resolution. It will therefore be profitable to design ion exchangers which show no time lag. An example of such an exchanger will be reported in the following paper[4].

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