Journal of Chromatography, 470 (1989) 317–326
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1577

TEN YEARS OF CHROMATOFOCUSING: A DISCUSSION

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SUMMARY

The theory of chromatofocusing has been clarified, in a qualitative manner, in terms of the pH gradient, Donnan potential, non-uniformity of flow, retardation factor and the titration curve of the protein. The main equations were refined to include the effect of the moving frame. A number of representative experimental results from the literature are given to illustrate the utility and limitations of the method. Some of these data were used to substantiate the theory. It is shown that no displacement by ampholyte components can occur under the conditions of chromatofocusing.

INTRODUCTION

As the present conference was held 10 years since the first two papers on chromatofocusing (CF) appeared in this journal[1,2], it seemed appropriate to review and discuss that method. By way of introduction, a simple qualitative explanation of the principles and factors involved is in order.

THE PRINCIPLES AND THEIR VERIFICATION

The objective of CF is to elute proteins from a column in the order of their pI values, *i.e.*, to mimic isoelectric focusing (IEF). For that purpose an ion exchanger is used, usually an anion exchanger, which exhibits a certain buffering capacity, owing to the fact that it carries ionizing groups having a wide range of pK values. The column is first equilibrated to the highest pH value desired for the separation. A pH gradient is then produced inside the column by passing through it an ampholyte solution that had been adjusted to the lower limit of the desired pH range, the protein sample having been applied in the first few ml of this solution. All protein components immediately start moving down the column in the order of their pI values at the same rate as the pH gradient. The focusing effect can be understood from Fig. 1. Owing to the buffering effect of the column, the pH gradient moves at a much slower rate than does the mobile liquid. In the moving frame of the protein the liquid flows at a rate equal to its rate outside the frame minus the rate of the frame, the latter being equal to the rate of the pH gradient.

If in the middle of the protein band the pH is equal to its pI value, the protein is

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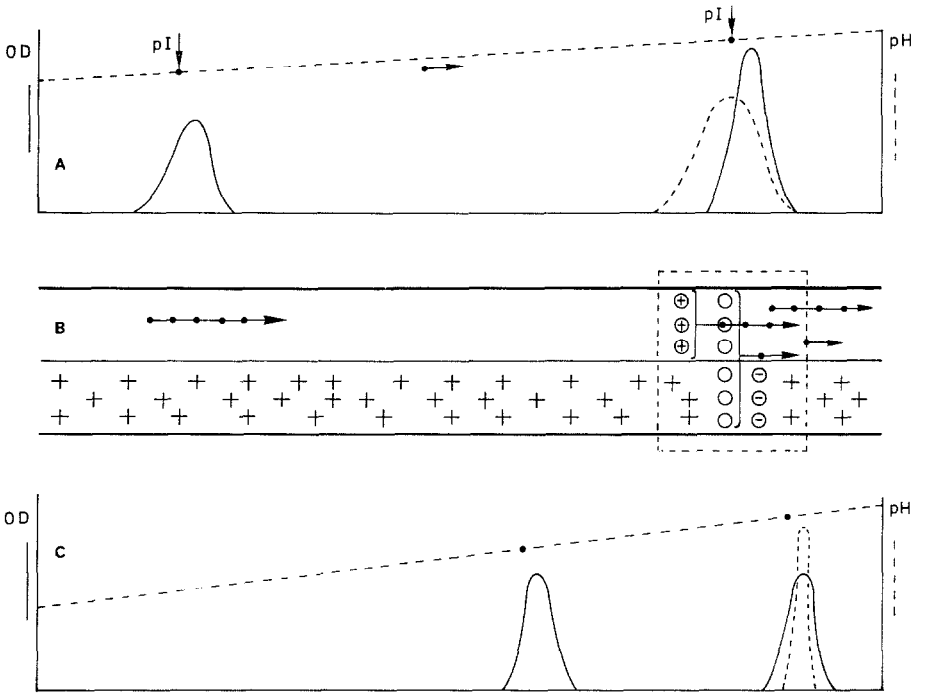

Fig. 1. Schematic representation of CF. (A) pH gradient and protein content along the length of the column shown in (B). (C) Location of protein bands in a pH gradient having twice the slope of the one in (A). Horizontal arrows indicate rates of displacement.

uncharged and therefore equally partitioned between the two phases. When the volumes of the two phases are equal, these molecules are moving at a rate half the rate of the mobile phase in the moving frame. Molecules in front of the centre are at higher pH and are negatively charged. They are therefore retained by the matrix and do not move in the frame. At the back of the centre the molecules are at a lower pH, are positively charged and are therefore expelled from the matrix. They move as rapidly as the liquid in the frame. It is evident that the band is compressed in this way. The compression is counteracted by the non-uniformity of flow, unavoidable in a column of beads. The final result is the bandwidth actually observed.

The width of a protein band depends on the steepness of its titration curve around its isoelectric point (Fig. 2). In order to carry a sufficient charge, Z, to be retained by the exchanger, protein b requires a larger pH deviation from its pI value than protein a. Therefore a larger displacement from the centre of the band is necessary for retention. Protein b will thus exhibit a wider band.

The higher the charge of the exchanger, the narrower will be the band, because less overall charge of the protein and therefore a smaller displacement from the centre of the band is needed for a molecule to be retained by the exchanger.

In Fig. 1C the pH gradient in the column is twice as steep as in Fig. 1A. The bands are, of course, twice as close as in Fig. 1A. The compression effect is also twice as large, because half the distance from the centre of the band is needed for a protein

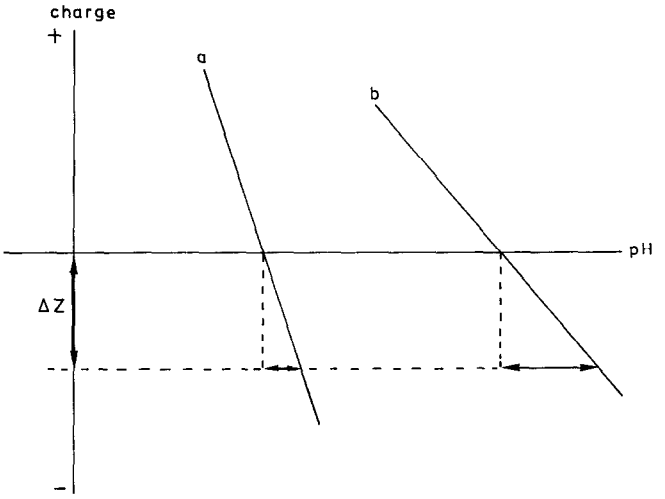

Fig. 2. Titration curves of two proteins around their p*I* values.

molecule to acquire the charge needed for retention. Unfortunately, a narrower band suffers more from non-uniformity of flow, and the result is a poorer separation than in Fig. 1A.

All of these factors are accounted for when the system is calculated from first principles (see Appendix I). The total width of a band, at 50% maximum, ΔpH, is described by:

$$\Delta\text{pH} = \frac{2.35}{1 - R_t}\sqrt{\frac{q}{\varphi} \cdot \frac{\text{dpH}/\text{d}V}{\text{d}Z/\text{dpH}}} \tag{1}$$

In eqn. 1 q denotes the coefficient of the non-uniformity of flow, φ the Donnan potential (multiplied by the factor F/RT) of the exchanger in the buffer concerned, dpH/dV the pH gradient, dZ/dpH the steepness of the titration curve of the protein around its p*I* and R_t the retardation factor, *i.e.*, the ratio of the rates of displacement of the pH gradient and the mobile phase, respectively.

When the pH gradient is not retarded, *i.e.*, when $R_t = 1$, ΔpH is infinite, signifying that no focusing occurs. The factor concerned approaches a constant when R_t decreases to realistic values like 0.1.

For optimum separation, smooth and constant pH gradients are needed. This requires proper tuning of the buffering capacity of the exchanger and of the buffer over a wide pH range. Research workers at Pharmacia managed to do so[3] and their materials are almost generally used[4].

An early application of CF is a separation of haemoglobin variants by Alexander and Neeley[5] (Fig. 3). They examined the separation on two columns of two different lengths, 26 and 54 cm. Of course, the separation is better on the longer column, as is usual in chromatography. It is interesting to compare the bandwidths; those for the protein, the exchanger and the buffer are identical; the pH gradient is the only

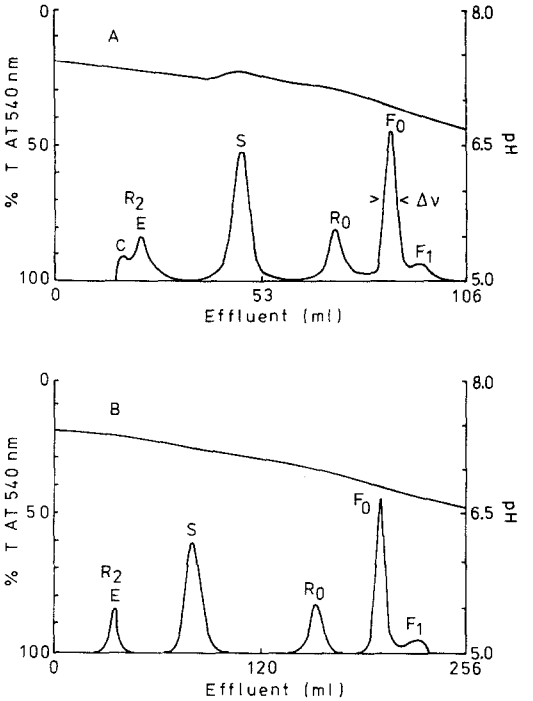

Fig. 3. Separation of haemoglobin variants. PBE 94 columns: (A) 26 cm × 0.9 cm; (B) 54 cm × 0.9 cm. Initial buffer: 0.025 *M* imidazole (pH 7.4). Limiting buffer: PB 96, 1:13 diluted, adjusted to pH 6.0. Flow-rate: 14.3 ml/h.

difference. This permits a check of eqn. 1 as far as the effect of the gradient is concerned, dpH/dV being calculated from the slope of the tangent to the pH gradient at the pH of elution, ΔpH by multiplying dpH/dV by ΔV, the bandwidth of the protein peak at half the maximum height in terms of the elution volume. The results, given in Table I, show a good correspondence between the observed and calculated ratios.

The smallest bandwidth was observed by Verbalis[6], who used a column of 100 cm and a narrow pH gradient to separate neurophysins (Fig. 4); the ΔpH was 0.015. Peaks differing in p*I* by no more than 0.02 units were reasonably separated.

Another relevant factor is the pH of an emerging protein solution, pI_{app}. It is evident from Fig. 1 that the compression causes the protein to be somewhat ahead of its isoelectric point; the protein is slightly negatively charged and partly retained by the exchanger. This permits the protein to keep pace with the slowly moving gradient.

TABLE I

EFFECT OF pH GRADIENT ON BAND WIDTH, ΔpH, OF THE HAEMOGLOBIN VARIANT F_0

	Short column	*Long column*	*Ratio*
ΔpH	0.040	0.025	obs. 1.60
Gradient	0.013	0.0043	calc. 1.75

However, there is another factor, one that causes the protein to lag behind. Owing to the positive charges, the pH is higher inside the beads than outside. Protein molecules that enter the beads will therefore undergo a shift in their overall charge in the negative direction and will thus be more retained by the beads than they would otherwise have been; they will emerge at a lower pH.

The sum of the two effects is represented by (see Appendix I):

$$\mathrm{p}I_{\mathrm{app}} - \mathrm{p}I = -\frac{\varphi}{4.6} + \frac{1}{\varphi|\,\mathrm{d}Z/\mathrm{dpH}|} \cdot \ln\frac{1 - R_t}{rR_t} \qquad (2)$$

r denoting the ratio of solvent volume inside the stationary phase and the void volumes. It is evident that this is a delicate balance that can easily turn in either direction, as borne out by the data in Table II. Low values of both φ and $\mathrm{d}Z/\mathrm{dpH}$ will cause a pronounced positive deviation (line 1), high values of both factors provide a negative deviation (line 3), whereas combinations of low φ with high $\mathrm{d}Z/\mathrm{dpH}$ (line 2) and high φ with low $\mathrm{d}Z/\mathrm{dpH}$ (line 4) tend to keep deviations low. In three of these cases $\Delta\mathrm{p}I$ can be calculated with satisfactory results.

FURTHER EXAMPLES OF SEPARATION

We now turn to a few other representative examples in the literature of what can be achieved with CF.

Aton *et al.*[8] separated rhodopsins, phosphorylated to different extents, by both IEF–PAGE (polyacrylamide gel electrophoresis) and CF. In Fig. 5 the results are plotted as $\Delta\mathrm{p}I$ *versus* electrophoretic pI values. They are remarkable; within a range of 0.8 pI units $\Delta\mathrm{p}I$ changes from $+0.5$ to -0.5, one complete unit upon the introduction

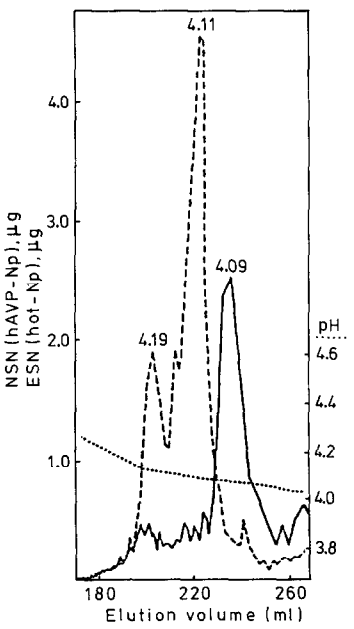

Fig. 4. Separation of neurophysins. For details see ref. 6.

TABLE II

*Δp*I VALUES, OBSERVED AND CALCULATED

	pI_{app}	pI	ΔpI	φ	dZ/dpH	R_t	ΔpI_{calc}
Myoglobin[a]	9.05	8.25	+0.8	0.65	1.8	0.16	+0.85
Papain[b]	9.75	9.60	+0.15	0.65	5.0	0.15	+0.2
Papain[c]	9.15	9.60	−0.45	3.2	5.0	0.03	−0.5
Myoglobin[d]	8.35	8.25	+0.1	high	1.8	–	–

[a] Fig. 7a of ref. 2.
[b] Fig. 12a of ref. 2.
[c] Fig. 5 of ref. 7.
[d] Fig. 6 of ref. 7.

of from two to eight phosphate groups. Since neither φ nor dZ/dpH is known, no definite explanation can be given as yet.

In many of the few hundred preparative papers published so far, CF was used as the final step in a purification sequence. When applied at an early stage, a ten-fold purification is quite usual. The highest purification achieved was 50-fold (the profile was not shown)[9]. As many as 29 components of eye lens crystallin were distinguished in one CF experiment[10]. The elution buffer contained 6 *M* urea to prevent precipitation of some of the components. A non-ionic detergent was also used for dissolving (membrane) proteins[11].

CF has also invaded the patent literature. Kato *et al.*[12] separated two human interleukins-2. One of them carries alanine, the other methionine as the N-terminal amino acid, the methionine being a one-unit extension of the chain. The Ala-terminal interleukin emerged first, at pH 8.0, the Met-terminal protein second, at pH 7.9 (Fig. 6), indicating that the Ala variant is the slightly more basic one. This corresponds nicely with the p*K* values of an alanyl dipeptide (8.2) and of a methionine dipeptide

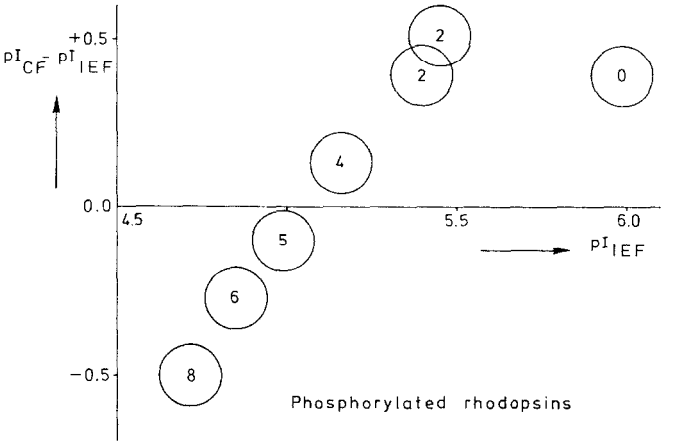

Fig. 5. Differences between p*I* values, determined by CF and by IEF, as a function of the values determined by IEF, for rhodopsins phosphorylated to different extents. The numbers indicate the number of phosphate groups per molecule. For details see ref. 8.

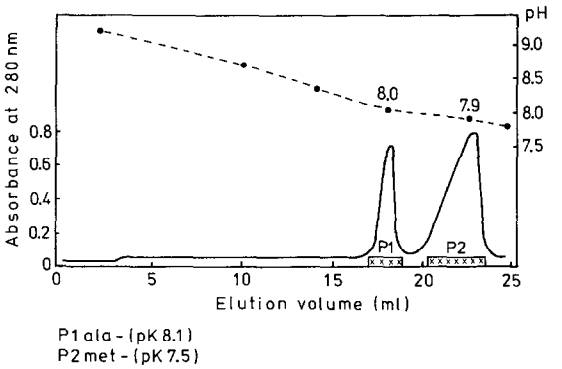

Fig. 6. The separation of two human interleukin-2 variants; pK values in parentheses indicate the values for related dipeptides.

(7.5), respectively. It is striking that such a small difference in the pK of one out of 34 charged groups in the two proteins permits their preparative separation.

DISCUSSION

It must be pointed out that eqns. 1 and 2 are equally applicable to all slowly-moving pH gradients, independent of whether they are produced with the aid of the buffering action of the column (CF) or entirely steered from the outside by appropriate gradient mixers (pH gradient ion exchange). Sometimes, a mixture of the two methods is used[13]. CF has the advantage of permitting the protein sample to be applied at the low pH of the range; no protein is subjected to a pH much higher than its pI value. Furthermore, CF allows an higher flow-rate[1].

The components of an ampholyte buffer behave much as proteins do, with the difference that a protein sample is added to the column only once and that the buffer is fed into it continuously. At the beginning of the separation, most of the components are strongly negative and are therefore gathered at the top of the column. When the pH in the column decreases, successive species lose most of their charge. All molecules of a particular species that had gathered that far move down and emerge as a single band. The approximate concentration of such just emerging ampholyte species in the fractions containing haemoglobin F_0 in Fig. 3 has been calculated (Appendix II). It was $3 \cdot 10^{-3}$ M, while the concentration of fixed charges at pH 7 in the last section of the column was 0.53 M[14]. The ratio is $6 \cdot 10^{-3}$, which is negligible; it is not possible that there is competition between protein and ampholyte species for such an abundance of fixed charges. It is therefore misleading to use the term displacement chromatography for this method. Displacement only starts after completion of CF. The elution volume between the end of CF and the start of the displacement is larger the lower is the concentration of negative buffer components in the elution buffer, *i.e.*, the nearer the terminal pH of the gradient is closer to the lower limit of the pH range covered by the ampholyte.

The concentration of an ampholyte species after its breakthrough point decreases to its level in the initial buffer. While more and more ampholyte species pass

TABLE III

CHANGE OF RESOLVING POWER DURING CF ON PBE 94 WITH POLYBUFFER 96, DILUTED 1:10, FROM pH 8.5 TO 6.0

Data from ref. 14.

pH	$[Cl^-]_m$ *(mM)*	P^+ *(mM)*	φ	$\sqrt{\varphi}$	*Ratio*
8.5	2.0	465	5.4	2.3	
					1.2
6.0	13.0	590	3.8	1.9	

through the column unretarded as positively charged molecules during CF, the concentration of counter ions in the emerging buffer increases gradually. Although the ion concentration in the mobile phase will affect the Donnan potential, the effect on the resolving power is small, as shown in Table III. The increase in chloride concentration is accompanied by an increase in charge inside the exchanger, P^+, as an increasing number of fixed amino groups is protonated with decreasing pH. Since $\varphi = \ln[Cl^-]_s/[Cl^-]_m$ and $[Cl^-]_s$ is virtually equal to P^+, φ can be calculated. It is found that φ does not change very much (Table III). Furthermore, ΔpH is inversely proportional to the square root of φ (see eqn. 1). Hence, the resolving power hardly changes in the range pH 8.5–6.

What improvements in resolving power might still be introduced? Increasing the maximum capacity of the exchanger three-fold would increase the buffering capacity three-fold and thus decrease dpH/dV three-fold. At the same time, φ would increase, but by no more than *ca.* 25%. Altogether, a nearly two-fold increase in resolving power would result. This is probably the most that can be obtained without reducing the pore size of the beads too much.

The non-uniformity of flow is quite limiting, but that is a general problem in chromatography. If diffusion were the only limiting factor (*cf.*, eqn. A3 of Appendix I), as in continuous gels, ΔpH for haemoglobin F_0 in Fig. 3B would theoretically have been *ca.* $1 \cdot 10^{-4}$[a]. This, of course, is unattainable, but even a few steps in that direction would be helpful.

APPENDIX I

Refinement of the equations describing bandwidth and ΔpI

In deriving eqn. 29 of ref. 1 it was tacitly assumed that the rate of movement of the pH gradient is negligible compared with the rate of the liquid flow. The equation can be made more general by introducing the ratio, R_t, of the two flows:

$$(dx/dt)_{pH} = R_t(dx/dt)_m \quad \text{(A1)}$$

Inside the moving frame the liquid flow is equal to

$$(dx/dt)_{m,mf} = (dx/dt)_m - (dx/dt)_{pH} = (dx/dt)_m(1 - R_t)$$

[a] The following values were used: $r = 2$, diffusion coefficient $D = 7 \cdot 10^{-7}$ cm^2 s^{-1}, $\varphi = 4.9$[14], $dZ/dpH = 8.7$[15], $dV/dt = 22.5$ ml h^{-1} cm^{-2} (*cf.*, Fig. 3) and $dpH/dV = 0.0043$ (Table I). R_t was neglected.

R_t being variable between 0 and 1. The effect of the moving frame can therefore be introduced by multiplying eqn. 12 of ref. 1 by $(1 - R_t)$:

$$\left(\frac{dx}{dt}\right)_{p,mf} = \frac{1 - R_t}{1 + rK}\left(\frac{dx}{dt}\right)_m \tag{A2}$$

K denoting the partition coefficient of the compound. Utilizing eqn. A2 instead of eqn. 12 of ref. 1 yields eqn. A3 instead of eqn. 29 of ref. 1:

$$(\Delta pH)^2 = \frac{1 + rK_0}{rK_0(1 + r)^2(1 - R_t)}\left[\frac{D}{dV/dt} + (1 + r)^2 q\right]\frac{dpH/dV}{\varphi \cdot dZ/dpH} \tag{A3}$$

K_0 being the partition coefficient at the center of a protein band. Furthermore, comparison of eqn. A1 with eqn. 5 of ref. 1 yields:

$$R_c = \frac{1 - R_t}{R_t} \tag{A4}$$

Inserting eqn. A4 into eqn. 31 of ref. 1 yields:

$$K_0 = \frac{1 - R_t}{rR_t} \tag{A5}$$

Insertion of eqn. A5 into eqn. A3 and neglecting the diffusion component yields eqn. 1 of this paper.

In eqn. 1 another alteration has been incorporated. In eqn. A3, ΔpH is the bandwidth from the centre to 60% of the maximum[1]. If the total bandwidth is taken to 50% of the maximum, as is more convenient, the factor 2.35 is added.

Experimentally, R_t is equal to the ratio of the volume of the column, v, and the elution volume, V_t, when the terminal pH, pH_t, emerges from the column. This is true only when the pH gradient is strictly linear (Fig. A1A). When this is not the case (which occurs most frequently), every point on the pH curve has its own V_t value, equal to the intercept of the tangent to the curve at the pH of elution with the volume axis (Fig. A1B).

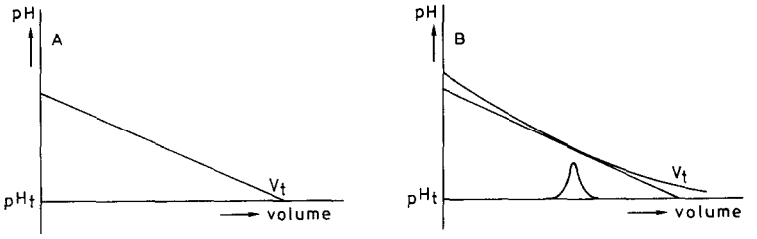

Fig. A1. Schematic representation of (A) an ideal pH gradient and (B) a non-linear pH gradient.

APPENDIX II

Calculation of the concentration of ampholyte species that are specifically eluted with a protein band

A protein is eluted at the elution volume, V_e, in a volume ΔV_e, that corresponds to $\Delta\text{pH} = (\text{dpH}/\text{d}V)\Delta V_e$. The amount of ampholyte specifically eluted with the protein can be derived as follows. The workable pH range of the ampholyte is i. It is assumed that the various buffer components are evenly distributed along this range. When they are arrayed in the order of their pI values, as in IEF, the interval ΔpH contains the fraction $\Delta\text{pH}/i$ of the total amount. The total amount of buffer introduced in the column is V_eM, where M denotes the molarity of the buffer solution. Hence, the interval ΔpH of the protein contains the amount of buffer $V_eM \cdot \Delta\text{pH}/i$. Since this amount is specifically eluted in the volume ΔV_e, its concentration, C_e, is:

$$C_e = \frac{V_eM}{i\Delta V_e} \cdot \Delta\text{pH} \approx \frac{V_eM}{i} \cdot \frac{\text{dpH}}{\text{d}V} \tag{A6}$$

Taking again haemoglobin as an example (F_0 in Fig. 3B), $V_e = 205$ ml, $\text{dpH}/\text{d}V = 0.0043$ (Table I). Polybuffer 96 appeared to contain 9.8% of dry weight material; the molecular weight was taken to be about 750 (ref. 16). The molarity of the undiluted buffer is therefore 0.13 M; after 1:13 dilution, 0.010 M. The workable pH range, $i = 3$ units. Inserted into eqn. A6, these data yield $C_e = 3$ mM.

Those components of the ampholyte that had already broken through the column are positively charged and do not enter the matrix.

ACKNOWLEDGEMENTS

The authors thank Dr. O. Elgersma, Philips, Eindhoven, The Netherlands, for discussing the alterations in the main equations.

REFERENCES

1 L. A. Æ. Sluyterman and O. Elgersma, *J. Chromatogr.*, 150 (1978) 17.
2 L. A. Æ. Sluyterman and J. Wijdenes, *J. Chromatogr.*, 150 (1978) 31.
3 L. Söderberg, T. Laao and D. Low, in H. Peeters (Editor), *Protides of Biological Fluids, Proceedings of the 29th Colloquium*, Pergamon, Oxford, 1982, p. 955.
4 *Chromatofocusing Handbook*, Pharmacia Fine Chemicals, Uppsala, 1981.
5 N. M. Alexander and W. E. Neeley, *J. Chromatogr.*, 230 (1982) 137.
6 J. G. Verbalis, *J. Chromatogr.*, 254 (1983) 309.
7 L. A. Æ. Sluyterman and J. Wijdenes, *J. Chromatogr.*, 206 (1981) 441.
8 B. R. Aton, B. J. Litman and M. L. Jackson, *Biochemistry*, 23 (1984) 1737.
9 M.-G. Yet and F. Wold, *J. Biol. Chem.*, 263 (1988) 118.
10 P. Body and H. Blocmendal, *FEBS Lett.*, 232 (1988) 39.
11 L. Wakefield, *J. Biochem. Biophys. Methods*, 9 (1984) 331.
12 K. Kato, T. Yamada and K. Kawahara, *Eur. Pat. Appl.*, 85,306,559.7 (1985).
13 T. H. J. Huisman and A. M. Dozy, *J. Chromatogr.*, 19 (1965) 160.
14 L. A. Æ. Sluyterman and C. Kooistra, unpublished results.
15 J. B. Matthew, G. I. H. Hanania and F. R. N. Gurd, *Biochemistry*, 18 (1979) 1919.
16 L. Söderberg, D. Buckley, G. Hagström and J. Bergström, *Protides Biol. Fluids*, 27 (1980) 687.