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CHROMATOFOCUSING: ISOELECTRIC FOCUSING ON ION-EXCHANGE COLUMNS

I. GENERAL PRINCIPLES

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SUMMARY

Theoretical considerations indicate the possibility of producing focusing effects in the ion-exchange chromatography of proteins in a pH gradient similar to those in the electrophoretic method of isoelectric focusing. A pH gradient can be produced "externally" by mixing two buffers of a different pH in a mixing chamber, or "internally" by taking advantage of the buffering action of the ion exchanger and running a buffer, initially adjusted to one pH, through a column initially adjusted to another pH. The latter method has the advantage of not subjecting the proteins to a pH more extreme than about its isoelectric pH value.

Equations are derived for the width of the protein bands, taking into account axial diffusion and axial dispersion by non-uniformity of flow and for the pH at which proteins are expected to emerge from the column. These equations serve as a guide for establishing the conditions for efficient separations.

INTRODUCTION

Isoelectric focusing by electrophoresis in ampholine buffers has proved to be a successful method of protein separation during the last 10 years[1]. The method is based on protein charges and a pH gradient developed in the column by the passage of a current. In ion-exchange chromatography of proteins also protein charges are involved and a pH gradient can be used. The latter method of protein separation is well known[2], although in most instances a salt gradient instead of a pH gradient is utilized.

The possibility of producing focusing effects in a pH gradient in an ion-exchange column has been examined, with a view to obtaining separations comparable to those of the electrophoretic system. In this paper, the principles by which such separations might be effected are explained. Firstly, a new method of producing a pH gradient, the "internal" method, in contradistinction to the "external" method, and the manner in which separations occur in this system are considered. Thereafter, in

a more refined model, apparent pI values are discussed and the theoretical bandwidth is calculated.

The symbols used are listed at the end of the paper.

THEORETICAL

Method of internally producing a pH gradient

The customary method of making a pH gradient inside a column is gradually to mix a buffer of one pH with a volume of buffer of another pH in a mixing chamber and to introduce the effluent from the mixing chamber into the column. This method is called here the "external" method. On the other hand, it is possible to produce a pH gradient "internally", by taking advantage of the buffering action of a suitable ion exchanger. This is done by passing a buffer of a certain initial pH through the column of the ion exchanger pre-adjusted to a different initial pH. The kind of gradient obtained in this way can be calculated in the following manner.

It is assumed as a first approximation that the composition of the buffer is such that the pH in the range concerned is proportional to added base, B_m:

$$pH_m = pH_0 + \frac{B_m}{a_m} \tag{1}$$

Although the ion exchanger normally contains only one kind of ionizing group with one intrinsic pK, in the absence of salt there will be a range of apparent pK values owing to the polyelectrolyte effect. Therefore, it is assumed that in a suspension of the exchanger also there is proportionality between pH and added base, B_s:

$$pH_s = pH_0 + \frac{B_s}{a_s} \tag{2}$$

When aliquots of both phases are mixed, the final pH will be

$$pH = pH_0 + \frac{B_m + B_s}{a_m + a_s} \tag{3}$$

B_m and B_s are solved from eqns. 1 and 2 and inserted into eqn. 3:

$$pH = \frac{a_m\, pH_m + a_s\, pH_s}{a_m + a_s} = \frac{pH_m + R_c\, pH_s}{1 + R_c} \tag{4}$$

This yields*

$$pH = \frac{a_m\, pH_m + a_s\, pH_s}{a_m + a_s} = \frac{pH_m + R_c\, pH_s}{1 + R_c} \tag{4}$$

* In an actual experiment, the increase in the pH of the buffer is due to retention of the more negatively charged components of the buffer from the mobile phase by the anion exchanger, the more basic components being left behind in the mobile pjase. The parameters B_m and B_s only serve the convenience of the calculation and are eliminated again in the final equation. The results are therefore independent of the actual mechanism.

Now, let us assume that the column of exchanger is divided into ten equal sections and the elution buffer into aliquots equal to the void volume of a column section. The pH of each aliquot passing the successive sections is re-adjusted in each section in accordance with eqn. 4. The results of the calculation for 19 aliquots are shown in Fig. 1. The initial pH of the buffer and column were 8.0 and 10.0, respectively. For the sake of convenient plotting, R_c was taken to be 1.0. The pH of an aliquot of buffer when it emerges from the column is equal to the pH in the last section. A graph of pH *versus* elution volume is shown in Fig. 2.

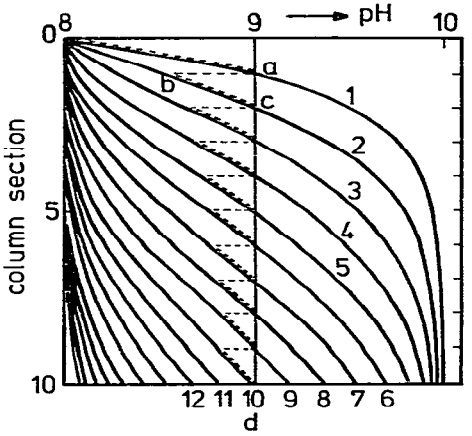

Fig. 1. Calculated pH of buffer aliquots 1–19, when running through the successive sections of a column with buffering action. The numbers inside and below the figure indicate the numbers of the aliquots. Broken line, transport of a protein of p*I* 9 through the column (see text). Initial pH of buffer, 8; initial pH of column, 10.

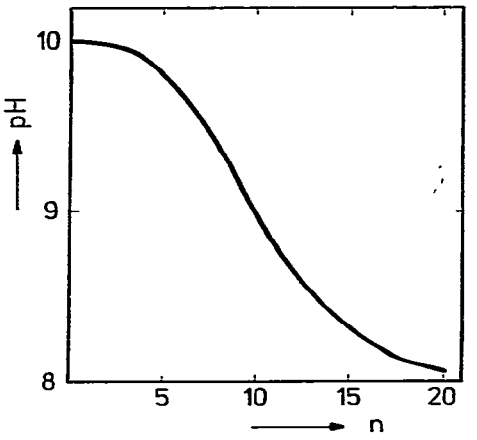

Fig. 2. pH of the eluent as a function of the elution volume expressed as the number of aliquots of buffer that have passed through the column (n).

From the same data, one can plot the progress of the pH gradient through the column (Fig. 3). The curves are drawn from the moment when the first aliquot of buffer reaches the last section of the column, *i.e.*, when aliquot 1 is in section 10, having the pH of section 10, aliquot 2 is in section 9, having the pH of section 9, aliquot 3 is in section 8, etc. It is evident from Fig. 3 that the gradient decreases as it advances along the column.

The rate of advance of the gradient can be calculated using eqn. 4. If the pH difference between two neighbouring column sections of length Δx is ΔpH, then the pH in the second section on shifting the mobile phase over the distance Δx (Fig. 4) is

$$\mathrm{pH_0} + \Delta' \mathrm{pH} = \frac{\mathrm{pH_0} + \Delta\mathrm{pH} + R_c\,\mathrm{pH_0}}{1 + R_c} = \mathrm{pH_0} + \frac{\Delta\mathrm{pH}}{1 + R_c}$$

$$\frac{\Delta\mathrm{pH}}{\Delta'\mathrm{pH}} = 1 + R_c \quad .$$

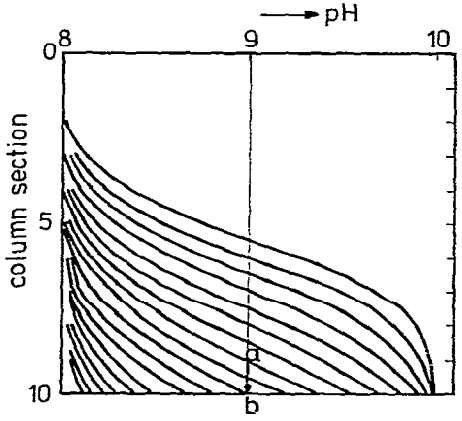

Fig. 3. pH distribution inside a column at the moment that the buffer aliquots 1–19 successively reach the end of a column with buffering action.

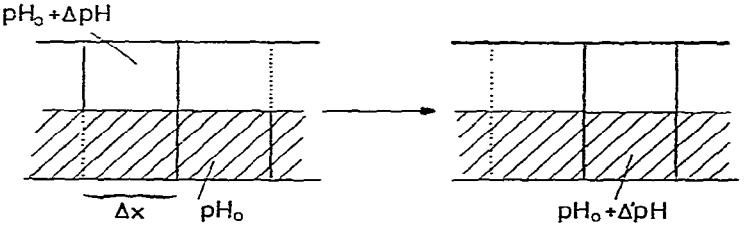

Fig. 4. Change in pH caused by a small shift of the mobile phase along a distance Δx. The shaded part represents the stationary phase.

As the gradient does not change appreciably on such a small shift, $\Delta\mathrm{pH}/\Delta x = \Delta'\mathrm{pH}/\Delta' x$, where $\Delta' x$ denotes the shift of the pH gradient. Therefore,

$$\frac{\Delta' x}{\Delta x} = \frac{\Delta' \mathrm{pH}}{\Delta \mathrm{pH}} = \frac{1}{1 + R_c}$$

or

$$\left(\frac{\mathrm{d}x}{\mathrm{d}t}\right)_{\mathrm{pH}} = \frac{1}{1 + R_c}\left(\frac{\mathrm{d}x}{\mathrm{d}t}\right)_{\mathrm{m}} \tag{5}$$

Even if R_c is not constant over the whole pH range of the separation, eqn. 5 is valid, R_c denoting the value at the pH of the emerging protein eluate.

The "all or none" model

The manner in which a protein passes through the column in the internally produced pH gradient can be understood from Fig. 1, making first the simplifying assumption that the protein either runs along with the buffer through the column when both protein and exchanger are positively charged, or is completely retained by the ion exchanger when they are oppositely charged ("all or none" movement). A protein with a p*I* of 9.0, contained in aliquot 1, will be carried along with this

aliquot up to the site of the column where the pH of the aliquot is slightly above 9.0 (point a); the protein is then negatively charged and is therefore retained by the positively charged anion exchanger. When aliquot 2 passes this site, the pH is lowered to the value of point b. The protein is then carried along by aliquot 2 until the pH of this aliquot in turn is slightly above pH 9.0 (point c). This process is repeated until the protein emerges from the column with the tenth aliquot at point d.

In an actual experiment, there will be an infinitely large number of infinitely small steps carrying the protein in its p*I* straight from point a to the end of the column. Proteins with different p*I*s (Fig. 5, components I, II and III) are eluted in the order of their p*I* values.

The family of curves of the type in Fig. 1 also enables one to predict the occurrence of focusing action (Fig. 6). Protein of p*I* 9.0 dissolved in aliquot 1 follows the

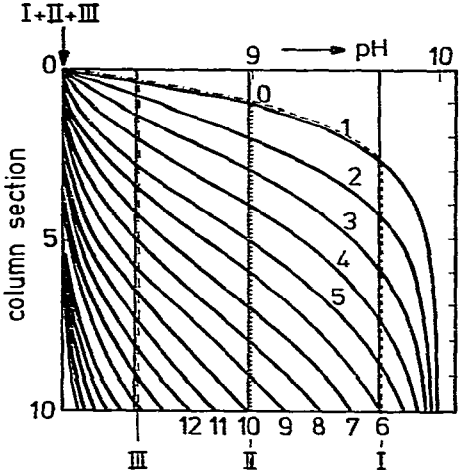

Fig. 5. Schematic representation of the separation of components I, II and III on a column with buffering action.

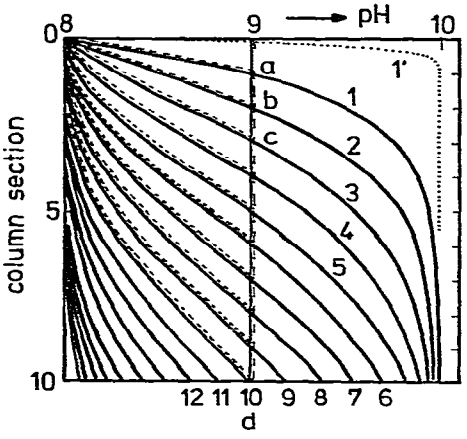

Fig. 6. Focusing action on a protein of p*I* 9.

course from the start to point a and to point d as described above. A sample of the same protein dissolved in the second aliquot travels along with this aliquot, joins the protein of the first aliquot at point b and subsequently follows the same course. Protein of p*I* 9.0 dissolved in the third aliquot joins the protein of the two preceding aliquots at point c, etc. Protein contained in aliquot 10 is the last portion that is able to join the protein from the preceding aliquots before emerging from the column; protein dissolved in aliquot 11, on the other hand, arrives "too late". Therefore, a protein of p*I* 9.0 contained in aliquots 1–10 converges into a single band before emerging from the column. This amounts to a focusing effect.

The maximum volume in which a protein may initially be dissolved in order to emerge as a single band, to be called the distribution volume, depends on the p*I* of the protein. It is 10 aliquots in the case examined above, whereas it is virtually zero if a protein emerges in the first aliquot, *i.e.*, when the p*I* of the protein is equal to the initial pH of the column. In such an instance the protein is nowhere retarded by the exchanger and the elution volume is equal to the void volume. The distribution volume is therefore equal to the elution volume of the protein minus the void volume of the column. However, with the gel bead model described in the next section, this conclusion has to be amended (see under *Distribution volume*).

If the pH gradient is produced externally, the proteins are applied to the column at the initial (high) pH and adsorbed there. The different components remain adsorbed until the pH of the incoming buffer is slightly below the p*I* of the protein. The protein is then carried down the column in the void volume. In this way, each component more or less "waits its turn" to be desorbed and eluted.

It can be concluded that in both methods for the production of pH gradients the proteins emerge from the column at a pH very near to their p*I* values when the "all or none" mechanism is operative.

Gel bead model

In the experiments to be described in Part II, the stationary phase consisted of swollen gel beads carrying positive or negative charges. A positively charged protein will be partitioned in favour of the mobile phase in the case of an anion exchanger, the more so the higher is the positive charge of the protein. An uncharged protein will be equally partitioned between the two phases. The partition is in favour of the stationary phase if the protein is negatively charged, the more so the higher is the negative charge. This has consequences for the apparent p*I* values, the bandwidth and the distribution volume. For the calculations considered in the next section, it is assumed that the classical equations for the Donnan potential are valid. As this is true only for low values of ψ, the calculations should be considered as only a first approach to a quantitative understanding.

Effect of the Donnan potential on the apparent pI

So far, it has been assumed that at a certain height of the column there will be a certain pH at a certain moment. Owing to the Donnan equilibrium, however, there is a difference in pH between the two phases caused by the fixed charges in the stationary phase. The pH of the mobile phase is the pH measured when the buffer emerges from the column. This causes a certain error, which can be calculated approximately as follows.

We assume that in the narrow pH range immediately around the pI value a protein particle, P, carries either no charge, or one positive or one negative charge. Therefore, the proton equilibria in this range can be described by

$$PH_2^+ \overset{K_1}{\rightleftarrows} PH + H^+ \overset{K_2}{\rightleftarrows} P^- + 2H^+$$

$$K_1 = \frac{[PH]\,[H^+]}{[PH_2^+]};\ K_2 = \frac{[P^-]\,[H^+]}{[PH]} \tag{6}$$

According to the Donnan equilibrium[3]

$$\frac{[PH_2^+]_m}{[PH_2^+]_s} = \frac{[P^-]_s}{[P^-]_m} = \exp(\varphi) \tag{7}$$

Combination of eqns. 6 and 7 yields

$$[P^-]_s = [P^-]_m \exp(\varphi) = \frac{K_2\,[PH]_m}{[H^+]_m} \exp(\varphi) \tag{8}$$

$$[PH_2^+]_s = \frac{[PH_2^+]_m}{\exp(\varphi)} = \frac{[H^+]_m\,[PH]_m}{K_1 \exp(\varphi)} \tag{9}$$

pI' is defined as the pH where

$$[P^-]_s + [PH_2^+]_s = [P^-]_m + [PH_2^+]_m \tag{10}$$

the uncharged species PH being equally distributed.

Insertion of eqns. 8 and 9 into eqn. 10 finally yields

$$[H^+]_m = \sqrt{K_1\,K_2 \exp(\varphi)}$$

$$pI' = -\frac{1}{2}\left[\log(K_1\,K_2) + \frac{\varphi}{2.3}\right]$$

From eqn. 6 follows the well known relationship

$$pI = -\frac{1}{2}\log(K_1\,K_2)$$

Hence

$$pI' - pI = -\frac{1}{4.6}\varphi = \frac{1}{2}(pH_m - pH_s) \tag{11}$$

It can be concluded that pI' will be lower than pI on an anion exchanger and higher than pI on a cation exchanger.

Distribution volume

As discussed under *The "all or none model"*, the distribution volume of a protein is equal to the elution volume of the protein minus the elution volume of a protein, the pI' of which is equal to the initial pH of the column. For demonstrative purposes the curves in Figs. 1–4 are rather widely spaced. More realistic for the first small aliquot of buffer that enters the column is the increase in pH indicated by curve 1′ in Fig. 6. A protein with pI' = 10, dissolved in aliquot 1′, soon finds itself in a medium of pH 10. It is then equally distributed between the two phases and has to traverse the whole volume of the column. The distribution volume of a protein is therefore equal to the elution volume of the protein minus the total accessible volume of the column. The same is true for the case of the externally produced pH gradient.

Theoretical bandwidth

If the simplifying assumption of an "all or none" mobility of a protein made in the discussions concerning Figs. 1 and 5 were correct, then infinitely sharp bands would be produced. A more realistic picture of the bandwidth can be derived from the gel bead model.

According to Consden *et al.*[4], a compound travels through a column at the rate

$$\left(\frac{dx}{dt}\right)_p = \frac{A_m}{A_m + K A_s}\left(\frac{dx}{dt}\right)_m = \frac{1}{1 + r K}\left(\frac{dx}{dt}\right)_m \tag{12}$$

if the volume of the dry column material (agarose) is neglected.

For the present purpose we are interested in the difference in the rate, w, of the protein molecules at the sides of the zone and the rate at the centre of the zone, where the partition coefficient is taken to be K_0:

$$w = \left(\frac{dx}{dt}\right)_p - \left(\frac{dx}{dt}\right)_p^0 = \frac{r K_0}{1 + r K_0} \cdot \frac{1 - K/K_0}{1 + r K/K_0}\left(\frac{dx}{dt}\right)_m \tag{13}$$

According to Albertson[5], the distribution coefficient, K, of a charged particle between two phases is governed by the equation

$$\ln K = -\dot{\varphi} Z \tag{14}$$

It is assumed that, within the limits of the zone, the charge of the protein is proportional to pI' — pH and that the pH gradient is constant. Eqn. 14 can therefore be converted into

$$\ln K = \ln \frac{C_s}{C_m} = -a x \tag{15}$$

in which

$$a = \varphi \cdot \frac{d Z}{d\,\mathrm{pH}} \cdot \frac{d\,\mathrm{pH}}{dx} \tag{16}$$

Insertion of eqn. 15 into eqn. 13 and applying the translation $x' = x - x_0$ gives

$$w = \left(\frac{dx}{dt}\right)_m \cdot \frac{r K_0}{1 + r K_0} \cdot \frac{1 - \exp(-a x')}{1 + r K_0 \exp(-a x')} \tag{17}$$

In this way, the protein runs along the column in a moving frame, whose point of reference is a distance of x_0 away from the moving point at which $K = 1$, *i.e.* where $pH = pI'$.

Effect of axial diffusion

It is assumed that diffusion occurs in the mobile phase only. The protein flux by diffusion per unit cross-sectional area is therefore

$$\left(\frac{dN}{dt}\right)_{diff} = -\frac{D A_m}{A_m + A_s} \cdot \frac{d C_m}{dx} = -\frac{D}{1 + r} \cdot \frac{d C_m}{dx} \tag{18}$$

In the steady state, the protein flux caused by diffusion is equal to the opposite flux caused by the liquid flow

$$C w = \frac{D}{1 + r} \cdot \frac{d C_m}{dx} \tag{19}$$

in which C denotes the mean concentration in the total cross-section:

$$C = \frac{A_m C_m + A_s C_s}{A_m + A_s} = \frac{C_m + r C_s}{1 + r} \tag{20}$$

According to eqn. 15

$$\frac{C_s}{C_m} = \exp(-a x) \tag{21}$$

Translation along $x' = x - x_0$ and application again of eqn. 15 yields

$$x' = x + \frac{\ln K_0}{a}$$

$$x = x' - \frac{\ln K_0}{a}$$

Insertion into eqn. 21 yields

$$\frac{C_s}{C_m} = \exp\left[-a\left(x' - \frac{\ln K_0}{a}\right)\right] = K_0 \exp(-a x') \tag{22}$$

By using eqn. 22, C_s in eqn. 20 is replaced by C_m:

$$C = \frac{C_m}{1 + r} [1 + r K_0 \exp(-a x')]$$

From this equation, C_m is solved and inserted into eqn. 19 and, furthermore, eqn. 17 is inserted into eqn. 19. Rearrangement finally yields the differential equation

$$\left[\left(\frac{dx}{dt}\right)_m \cdot \frac{1 - \exp(-a\,x')}{1 + r\,K_0} - D \cdot \frac{a}{r\,K_0 + \exp(a\,x')}\right] dx' = \frac{D}{r\,K_0} \cdot \frac{dC}{C}$$

Integration and insertion of the boundary condition $C = C_0$ when $x' = 0$ finally yields

$$\frac{D}{r\,K_0} \cdot \ln \frac{C}{C_0} = \left(\frac{dx}{dt}\right)_m \cdot \frac{a\,x' + \exp(-a\,x')}{a\,(1 + r\,K_0)} - \\ - \frac{D}{r\,K_0}\left[a\,x' - \ln \frac{r\,K_0 + \exp(a\,x')}{1 + r\,K_0}\right] \qquad (23)$$

Insertion of realistic values for the various entities demonstrates that the second term on the right-hand side can be neglected and that the left-hand exponential can be approximated by a three-term series. These approximations convert eqn. 23 into

$$\ln \frac{C}{C_0} = \frac{1}{2}\left(\frac{dx}{dt}\right)_m \cdot \frac{r\,K_0\,a}{D\,(1 + r\,K_0)} \cdot x'^2 \qquad (24)$$

This is the Gaussian distribution curve with its maximum at $x' = 0$. The bandwidth at the inflection points of the curve, where $\ln(C/C_0) = -\frac{1}{2}$ or $C = 0.6\,C_0$, is

$$(\Delta x)^2 = \frac{1 + r\,K_0}{r\,K_0\,a} \cdot \frac{D}{\left(\frac{dx}{dt}\right)_m}$$

Replacing a according to eqn. 16 yields

$$(\Delta x)^2 = \frac{1 + r\,K_0}{r\,K_0} \cdot \frac{D}{\left(\frac{dx}{dt}\right)_m} \cdot \frac{1}{\varphi \cdot \frac{dZ}{d\,pH} \cdot \frac{d\,pH}{dx}} \qquad (25)$$

Experimentally, it is convenient to express the bandwidth in terms of ΔV, according to

$$\Delta x = (1 + r)\,\Delta V$$

Therefore,

$$(\Delta V)^2 = \frac{1 + r\,K_0}{r\,K_0\,(1 + r)^2} \cdot \frac{D}{\frac{dV}{dt}} \cdot \frac{1}{\varphi \cdot \frac{dZ}{d\,pH} \cdot \frac{d\,pH}{d\,V}} \qquad (26)$$

The bandwidth in terms of ΔpH, which is equal to

$$\Delta pH = \frac{d\,pH}{d\,V} \cdot \Delta V$$

is decisive for a separation. Therefore,

$$(\Delta\mathrm{pH})^2 = \frac{1 + r\,K_0}{r\,K_0\,(1 + r)^2} \cdot \frac{D}{\frac{\mathrm{d}V}{\mathrm{dt}}} \cdot \frac{\frac{\mathrm{d\,pH}}{\mathrm{d}V}}{\varphi \cdot \frac{\mathrm{d}Z}{\mathrm{d\,pH}}} \tag{27}$$

Effect of non-uniformity of flow

The dispersion caused by non-uniformity of flow velocity in the mobile phase is similar to the effect of diffusion[6]. Whereas the diffusion is proportional to dt (eqn. 18), the dispersion is proportional to dx, the displacement of the protein in the mobile phase. Therefore, the protein flux by non-uniformity of flow, similar to eqn. 18, is described by

$$\left(\frac{\mathrm{d}N}{\mathrm{d}x}\right)_{\mathrm{disp}} = -q \cdot \frac{\mathrm{d}C_{\mathrm{m}}}{\mathrm{d}x}$$

where q is a constant of the column; the higher the quality of the column, the lower is q. This can be converted into

$$\left(\frac{\mathrm{d}N}{\mathrm{d}t}\right)_{\mathrm{disp}} = -q \left(\frac{\mathrm{d}x}{\mathrm{d}t}\right)_{\mathrm{m}} \frac{\mathrm{d}C_{\mathrm{m}}}{\mathrm{d}x}$$

because the protein is carried along with the mobile phase. Conversion of the flow-rate into units of V according to $\mathrm{d}x = (1 + r)\,\mathrm{d}V$ yields

$$\left(\frac{\mathrm{d}N}{\mathrm{d}t}\right)_{\mathrm{disp}} = -(1 + r)\,q \cdot \frac{\mathrm{d}V}{\mathrm{d}t} \cdot \frac{\mathrm{d}C_{\mathrm{m}}}{\mathrm{d}x}$$

The sum of the fluxes caused by diffusion and by non-uniformity of flow, $(\mathrm{d}N/\mathrm{dt})_{\mathrm{tot}}$, is given by

$$\left(\frac{\mathrm{d}N}{\mathrm{d}t}\right)_{\mathrm{tot}} = \left(\frac{\mathrm{d}N}{\mathrm{d}t}\right)_{\mathrm{diff}} + \left(\frac{\mathrm{d}N}{\mathrm{d}t}\right)_{\mathrm{disp}} = -\left[\frac{D}{1 + r} + (1 + r)\,q \cdot \frac{\mathrm{d}V}{\mathrm{d}t}\right] \frac{\mathrm{d}C_{\mathrm{m}}}{\mathrm{d}x} \tag{28}$$

Eqn. 28 is equal to eqn. 18 when $D/1 + r$ in eqn. 18 is replaced by the term between square brackets in eqn. 28. As the quantities in this term are constants, the same substitution can be carried out in the final eqn. 27. This yields

$$(\Delta\mathrm{pH})^2 = \frac{1 + r\,K_0}{r\,K_0\,(1 + r)^2} \left[\frac{D}{\frac{\mathrm{d}V}{\mathrm{dt}}} + (1 + r)^2\,q\right] \frac{\frac{\mathrm{dpH}}{\mathrm{d}V}}{\varphi \cdot \frac{\mathrm{d}Z}{\mathrm{dpH}}} \tag{29}$$

Difference between observed and real pI

As shown under *Effect of axial diffusion*, the maximum of a zone is located at $x' = x - x_0 = 0$ or $x = x_0$, *i.e.*, the maximum is a distance of x_0 away from the

moving site at which $K = 1$ and $\mathrm{pH} = \mathrm{p}I'$. This phenomenon produces an error in the observed value of $\mathrm{p}I'$, which can be calculated by using eqn. 14:

$$\ln K_0 = -\varphi Z = -\varphi \cdot \frac{\mathrm{d}Z}{\mathrm{dpH}} (\mathrm{pH} - \mathrm{p}I') \tag{30}$$

Under equilibrium conditions, the protein adapts its velocity (eqn. 12, $K = K_0$) to the velocity of the pH gradients (eqn. 5). Equating these equations yields

$$K = \frac{R_c}{r} \tag{31}$$

Insertion of eqn. 31 into eqn. 30 yields

$$\mathrm{p}I_{\mathrm{obs}} - \mathrm{p}I' = -\frac{1}{\varphi \cdot \frac{\mathrm{d}Z}{\mathrm{dpH}}} \cdot \ln \frac{R_c}{r} \tag{32}$$

R_c can be approximately related to a_e and a_b as derived from the titration curve of the ion exchanger and of the buffer, respectively. The buffer capacity of the stationary phase is the sum of the buffer capacity of the exchanger itself, measured per millilitre of packed gel, and of the buffer components inside the beads. The concentration of the latter is taken to be equal to the concentration in the mobile phase. This assumption is approximately correct if φ is not too high or if the buffer is a mixture of positive, negative and neutral buffering components, such as the ampholine buffers. In that case

$$R_c = \frac{a_s}{a_m} = \frac{(A_s + A_m)\, a_e + A_s\, a_b}{A_m\, a_b} = (1 + r)\, r_c + r \tag{33}$$

Insertion of eqns. 11 and 33 into eqn. 32 yields the total difference between the observed and real $\mathrm{p}I$:

$$\mathrm{p}I_{\mathrm{obs}} - \mathrm{p}I = -\frac{1}{4.6} \cdot \varphi - \frac{1}{\varphi \cdot \frac{\mathrm{d}Z}{\mathrm{dpH}}} \cdot \ln \left(1 + r_c + \frac{r_c}{r}\right) \tag{34}$$

DISCUSSION

It can be concluded that it is feasible to produce focusing effects in separations on ion-exchange columns under suitable conditions, which can be deduced from eqn. 29 (a high value of φ and a low pH gradient). A high value of φ can be obtained by utilizing exchangers of high capacity and keeping the ionic strength and therefore the buffer concentration as low as possible. Further, a low buffer concentration is coupled to a low pH gradient in the internal method of production of the gradient. On the other hand, the lowness of the buffer gradient will find a practical limitation in yielding very low protein concentrations in the eluent, as indicated by eqn. 26.

The theoretical resemblance of the chromatographic and electrophoretic

methods is also evident when the effect of axial diffusion is considered. The effect of the diffusion in the electrophoretic method is given by the equation

$$(\Delta\mathrm{pH})^2 \approx \frac{D \cdot \frac{\mathrm{dpH}}{\mathrm{d}x}}{-E \cdot \frac{\mathrm{d}u}{\mathrm{dpH}}}$$

This equation is similar to eqn. 27 if the electric field, E, replaces $(\mathrm{d}V/\mathrm{dt}) \cdot \varphi$ in eqn. 27 and the electrophoretic mobility, u, replaces Z.

It should be pointed out that eqns. 27 and 34 are valid regardless of the method of gradient production. The main advantage of the internal method of producing the pH gradient is the mildness of the separation conditions. No component is subjected to a pH higher than approximately its own pI value (Fig. 5), whereas in the external method the initial pH of the mixture on an anion exchanger has to be higher than the (often unknown) pI value of the component, of highest pI. A similar avoidance of extreme pH values can be achieved on a cation exchanger. This mildness is a common feature of the internal production of the pH gradient by both ion-exchange chromatography and electrophoresis.

Another advantage of employing the buffering action of the exchanger is the more efficient use of the separation time. In the internal method of gradient production all components travel from the start (see Fig. 5) at a rate equal to the rate of travel of the pH gradient. According to eqns. 5 and 33, this rate is

$$\left(\frac{\mathrm{d}x}{\mathrm{d}t}\right)_\mathrm{p} = \frac{1}{1 + r + r_c + r\,r_c} \cdot \left(\frac{\mathrm{d}x}{\mathrm{d}t}\right)_\mathrm{m}$$

If there is no appreciable buffering action of the exchanger, *i.e.*, if $r_c = 0$, and the external method of gradient production has to be applied, each protein more or less waits its turn to be desorbed and then runs down the column at a rate of $1/(1 + r)$ relative to the rate of the mobile phase. If, for example, $r = 2$ and $r_c = 2$, this leads to a difference between the two methods of a factor of 3. This means that, with identical elution profiles of pH *versus* volume, the internal method permits higher flow-rates and therefore shorter separation times than the external method, without increasing the risk of non-equilibrium of the proteins between the mobile and the stationary phases.

The essential difference between the two methods of pH gradient production is the advantage derived from the buffering action of the exchanger in the internal method. The buffering action of the column causes a continuous change in the pH of an aliquot of buffer when it runs down the column. This effect is maintained even when a combination is effected, *i.e.*, when the initial pH of the elution buffer, before being applied to the column, is further decreased as in the external method. Such a procedure could, for example, help to avoid the extremely low pH gradient at the end of the elution (Fig. 1, $n > 15$).

Eqn. 34 predicts that the pI values observed with chromatofocusing may not be the real values and should be considered as only approximately correct.

It is to be expected that focusing effects will also occur when the elution is carried out with a salt gradient. Calculation of the bandwidth for that case will be similar to the derivation of eqn. 34 but requires replacement of eqn. 14 with an

equation that describes protein distribution as a function of salt concentrations. It may not be easy to do so from first principles, owing to non-ideality of the fairly high salt concentration and to protein–salt interactions.

In Part II, the principles of the pH gradient elution presented here are verified in a number of experiments.

SYMBOLS

$A_{m,s}$	cross-sectional area of the mobile and the stationary phase, respectively
$a_{m,s}$	buffer capacity per unit of column length of the mobile and the stationary phase, respectively
$a_{b,e}$	buffer capacity per millilitre of buffer and of packed gel of exchanger, respectively
C	mean protein concentration
$C_{m,s}$	protein concentration in the mobile and the stationary phase, respectively
D	diffusion coefficient of a protein
ψ	Donnan potential
φ	$F\psi/RT$, where F, R and T have their conventional meanings
K	partition coefficient, C_s/C_m
K_0	partition coefficient at the centre of a protein band
m	suffix indicating the mobile phase
p	suffix indicating the protein
q	quality factor of the column
r	A_s/A_m
R_c	a_s/a_m
r_c	a_e/a_b
s	suffix indicating the stationary phase
V	elution volume per unit cross-sectional area
$\frac{dV}{dt}$	flow-rate per unit cross-sectional area
x	distance along the column
x'	$x - x_0$
Z	protein charge
dZ/dpH	slope of the titration curve of a protein near its isoelectric point.

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