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Note

Change of counter ion concentration and of resolving power in a chromatofocusing run

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In a pH gradient, the width of a protein band can most efficiently be expressed as ΔpH, the pH interval covered by the central part of the band, *i.e.*, the part within its boundaries at half the maximum height. It has been shown [1] that ΔpH, as far as the properties of the system are concerned, is proportional to

$$\Delta\text{pH} \propto \sqrt{\frac{\text{dpH/d}V}{\varphi}} \tag{1}$$

where dpH/dV is the pH gradient in terms of the eluent volume V and φ is the Donnan potential of the ion exchanger multiplied by F/RT. For monovalent ions such as Cl^- the Donnan potential ϕ is

$$\phi = \frac{RT}{F} \cdot \ln\left(\frac{[Cl^-]_s}{[Cl^-]_m}\right) = \frac{RT}{F} \cdot \varphi \tag{2}$$

where the subscripts s and m indicate the stationary and mobile phase, respectively. The composition of the emerging ampholite buffer is not constant, as an increasing number of buffer components emerge from the column during the course of a separation [2]. This is accompanied by an increase in counter ion concentration, as shown semiquantitatively in Fig. 26 in ref. 2. Such an increase will cause a decrease in Donnan potential. On the other hand, the charge of the matrix increases as more amino groups are protonated when the pH decreases. Further, the pH gradient is usually not absolutely constant during a run. The resolving power of the system may therefore not be the same during the course of a separation. The three factors involved and their combined effect, briefly discussed in a previous paper [1], are examined in more detail.

EXPERIMENTAL

A 20.2 × 1.0 cm I.D. column of PBE 94 (lot No. NL 05230) (Pharmacia,

0021-9673/90/$03.50

Uppsala, Sweden) was equilibrated with a 25 m*M* ethanolamine–HCl buffer solution of pH 9.0. The pH gradient was produced using a Polybuffer 96–HCl solution of pH 6.0 (dilution 1:10). The chloride concentration was measured by isotachophoresis [3].

The total binding capacity at pH 9.0 was measured by equilibrating the column again with the ethanolamine buffer. The chloride was eluted with 0.3 *M* potassium nitrate. The elution was terminated when the pH of the eluent rapidly increased. The amount of chloride bound by the exchanger was taken as equal to the total amount of chloride in the effluent minus the (slight) amount of chloride of the buffer in the void volume.

For measurement of the titration curve, a 14.2-ml portion of packed PBE 94 column was suspended in 50 ml of water and adjusted to pH 2.9 with 1 *M* hydrochloric acid. Aliquots of 0.75 ml of a 0.20 *M* sodium hydroxide solution were added under nitrogen; 5 min after each addition the stirrer was stopped, the material was allowed to settle and the pH of the supernatant was read.

The void volume was determined with blue dextran on a column of Sepharose 6B, the matrix on which PBE 94 is based [2].

RESULTS

A portion of PBE 94 ion exchanger was titrated in the absence of added salt (Fig. 1). The proton binding was found to be 200 μmol/ml packed gel between pH 3 and 11. As expected, this is lower than the value of 240 μmol/ml [2] observed when the titration

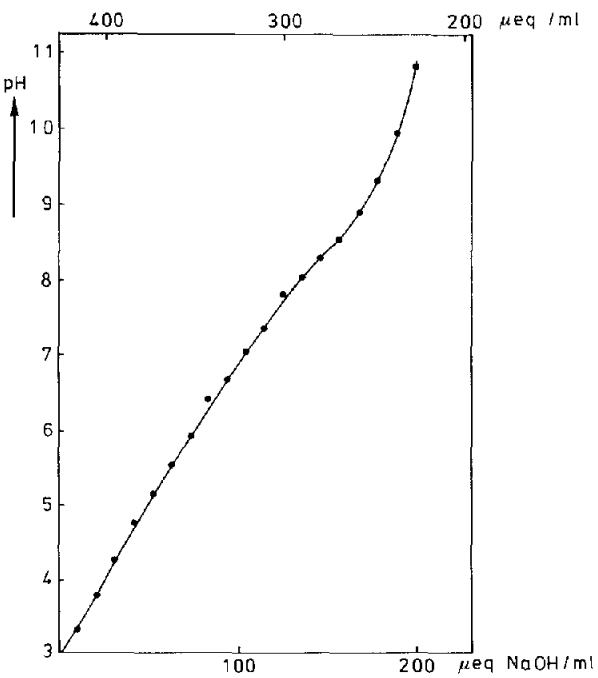

Fig. 1. Titration curve (bottom axis) and total charge as a function of pH (top axis) of PBE 94.

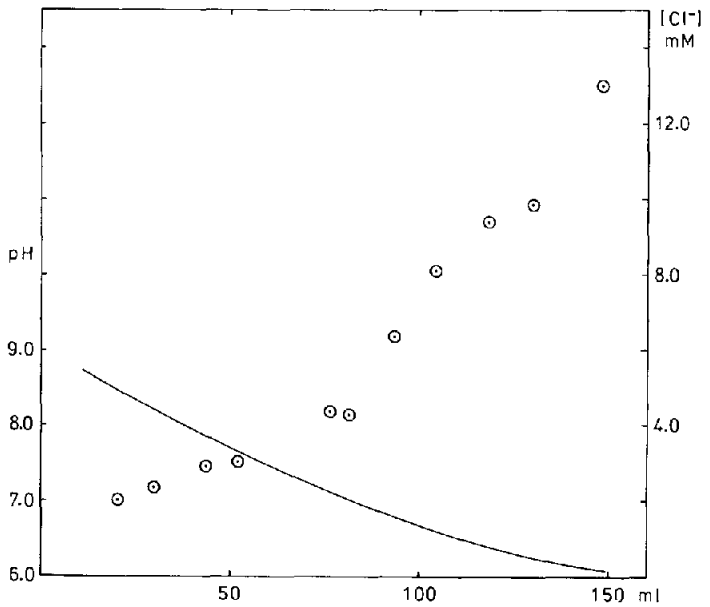

Fig. 2. pH (drawn curve) and accompanying chloride concentration (○) as a function of eluent volume.

was carried out in 1 *M* potassium chloride solution to suppress polyelectrolyte effects. The former value is more realistic, as the present titration conditions are close to the conditions of the separations.

The titration curve underestimates the total charge, as quaternary amino groups are not titrated. They were determined by measuring the total chloride binding capacity at pH 9.0, which proved to be 254 μmol/ml packed gel. Subtraction of the proton binding between pH 9 and 11, *i.e.*, 27 μmol/ml (Fig. 1), yields 227 μmol/ml packed gel as the content of fixed quaternary ions. The total charge as a function of pH is shown in Fig. 1 on the top axis. As the void volume of the column is 34% of the total volume, the internal charge of the matrix, P^+, is obtained by multiplying the values in Fig. 1 by 1.5.

A pH gradient was produced between pH 9 and 6 in the usual manner, utilizing PBE 94 ion exchanger and 10-fold diluted Polybuffer 96. Determination of chloride content was started when the pH began to decrease. The results are shown in Fig. 2. As expected, there was a continuous increase in chloride content as the pH decreased. The conductivity exhibited the same trend (not shown).

Values of φ were calculated from the data in Figs. 1 and 2 utilizing eqn. 2. As $[Cl^-]_m \ll P^+$, $[Cl^-]_s$ is virtually equal to P^+. The calculated values of φ are presented in Fig. 3 (curve a), together with the concomitant dpH/dV values (curve c) as derived from the curve drawn is Fig. 2.

DISCUSSION

There is a limited decrease in φ during chromatofocusing (Fig. 3, curve a),

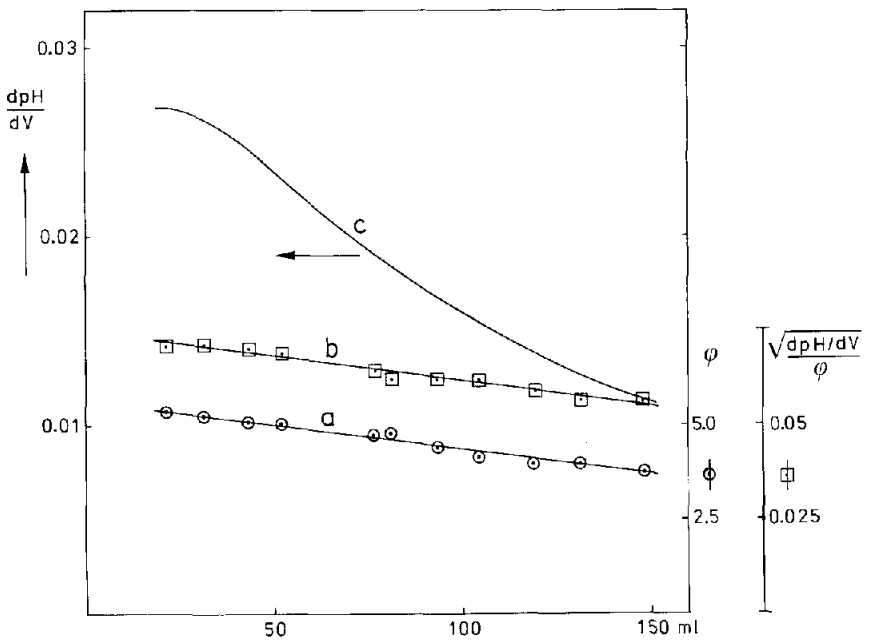

Fig. 3. pH gradient (c), φ (a) and square root of $(\mathrm{dpH/d}V)/\varphi$ (b) as a function of eluent volume.

suggesting some increase in ΔpH according to eqn. 1. This increase, however, is overruled by the effect of the decreasing values of $\mathrm{dpH/d}V$ (Fig. 3, curve c). The combined effect, calculated with eqn. 1, is shown in Fig. 3, curve b. It turns out that ΔpH decreases during a run, *i.e.*, the separation of components that emerge last from the column is better than that of components that are eluted at the beginning of the run. This, however, is only a small effect (a factor of no more than 1.25).

It can therefore be concluded that the combination of PBE 94 ion exchanger and Polybuffer 96 provides a system with a fairly constant separation capability during a run, notwithstanding an increase in counter-ion concentration in the course of a separation. This conclusion was confirmed by examination of numerous separations actually carried out [1,2]. There is, therefore, no need to choose the pH range of the separation in such a way that the desired component emerges from the column at the beginning, in the middle or at the end of the run.

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REFERENCES

1 L. A. Æ. Sluyterman and C. Kooistra, *J. Chromatogr.*, 470 (1989) 317.
2 *Chromatofocusing Handbook*, Pharmacia, Uppsala, 1981.
3 P. Boček, L. Miedziak, M. Deml and J. Janák, *J. Chromatogr.*, 137 (1977) 83.