Journal of Chromatography, 362 (1986) 101–112
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 650

CHROMATOPHORESIS: A NEW APPROACH TO THE THEORY AND PRACTICE OF CHROMATOFOCUSING

II. EXPERIMENTAL VERIFICATION

ANDREW MUREL*, SVETLANA VILDE, MARET PANK, IGOR SHEVCHUK and OSKAR KIRRET

Institute of Chemistry, 15 Akadeemia tee, 2000108 Tallinn, Eesti NSV (U.S.S.R.)

(Received March 12th, 1986)

SUMMARY

The buffering mechanism of chromatofocusing has been refuted by the lack of a pH gradient during elution with a non-amphoteric buffer and by the smooth pH gradient produced upon elution with ampholytes on non-buffering ion exchangers. The frontal development mechanism of pH gradient generation suggested previously was confirmed experimentally. Amino acids emerge from the column in accordance with their isoelectric points. A small inverse pH gradient accompanied by a decrease in ionic strength was observed when two amino acids were used to elute a third one from a column of a strong ion exchanger. The displacement of ampholytes by a base or an acid in chromatophoresis furnishes increasing and decreasing pH gradients on strong cation and anion exchangers, respectively. The influence of the displacer concentration on the pH gradient and on the ionic strength was found to be in accord with theory. Amino acids, carrier ampholytes and proteins were fractionated. The properties of ion exchangers suitable for chromatophoresis are discussed.

INTRODUCTION

In Part I[1], the buffering mechanism of chromatofocusing (CHF) suggested by the authors[2] of this method was questioned. The pH gradient formation was attributed to the frontal development mechanism well known from general chromatography texts.

Computer simulations of the ampholyte distribution, pH and ionic strength of the effluent in frontal and displacement developments suggest that the displacement technique has several advantages; it is disignated as "chromatophoresis" (CHPH) by analogy with isotachophoresis.

An even and low ionic strength throughout the entire pH gradient and simultaneous removal of salts from the sample and/or from synthetic carrier ampholytes are expected to be among the merits of CHPH. According to the computer data, chromatophoresis requires lower amounts of ampholytes and renders them in a pure (isoionic) state.

0021-9673/86/$03.50

Computer simulations in Part I were not related to any parameter of an actual chromatophoretic run, *e.g.* the theoretical plate number, partition coefficients, etc. However, the suggested frontal and displacement mechanisms of ampholyte focusing on ion-exchange resins can be verified experimentally.

EXPERIMENTAL

Materials

Polybuffers 74 and 96 were supplied by Pharmacia (Uppsala, Sweden). Amphoteric eluents (Ampholent) were supplied by Oktoober (Tallinn, U.S.S.R.). The ion exchangers employed were PBE 118, SP-Sephadex C-25, QAE-Sephadex A-25 (all from Pharmacia), Separon HEMA 1000 Q, 90–125 μm (Laboratorní Přistroje, Prague, Czechoslovakia) and DEAE-Toyopearl 650 M(Toyosoda, Tokyo, Japan). The agarose sulpho derivative was prepared from 25 ml of Agarose 4B (Experimental Works of Institute of Chemistry, Tallinn, U.S.S.R.) by overnight swirling of the beads (50–200 μm) with 12 ml of 1 *M* sodium hydroxide and 2 ml of epichlorhydrin (both analytical grade; Reachim, U.S.S.R.) at room temperature followed by addition of 2g potassium sulphite to the washed gel and heating for 30 min at 50°C with swirling. The resulting gel binds 7 μequiv. of carrier ampholytes and 10 μequiv. of Na^+ per millilitre.

Egg white was diluted in an appropriate buffer (1:5), filtered through a glass sinter and centrifuged at 3000 *g*. The amino acids used were "pure" grade (Reachim). All other materials were of "analytical" quality and were used without further purification.

Apparatus

Chromatographic columns with glass fabric discs on the bottom adaptors were designed and kindly supplied by Dr. Arvo Polokainen (Institute of Experimental Biology, Tallinn, U.S.S.R.). The flow-rates were regulated by NP-1M peristaltic pumps (SKB, Kiev, U.S.S.R.). The conductivity was monitored continuously by a REPPS-1M device (CKB, AMN, U.S.S.R.) in 0.1-ml flow cell. The fraction collector FCC-60 was purchased from Laboratorní Přistroje.

Continuous pH readings were made with a digital pH-meter 517 (Meratronic, Poland). A capillary flow-through glass electrode OP-0745 P was taken from an OP-212 pH-meter (Radelkis, Budapest, Hungary) and screened by a grounded aluminium foil (from an Anneke chocolate plate; Kalev, Tallin, U.S.S.R.). Fig. 1 shows the design of the flow-through unit attached to the EVP-08 reference electrode (Gomel, U.S.SR.). The pH detector was positioned behind the conductivity monitoring unit to avoid the effect of potassium chloride leakage from the reference electrode.

The pH and conductivity were recorded by means of a TZ-4221 two-channel recorder (Laboratorni Přistroje). Equipment from LKB (Bromma, Sweden) was used for protein separation: Max Coldlab 2021 adjusted to +4°C, Perpex pump, 2138 Uvicord S, 2070 Ultrorac II. The pH of fractions was measured by a PHM 84 Research pH-meter (Radiometer, Denmark).

Chromatographic procedure

Columns were packed with ion exchangers in 1 *M* sodium chloride solution

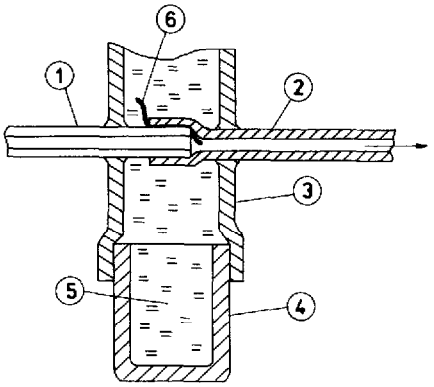

Fig. 1. Schematic representation of the flow-through unit attached to the EVP-08 reference electrode. 1 = Polyethylene tubing, 3 mm O.D.: 2 = silicone rubber tubing, 3 mm O.D.: 3 = silicone rubber tubing, 12 mm O.D.: 4 = polyethylene bung; 5 = saturated potassium chloride solution; 6 = cotton textile thread.

using the technique described previously[3] and were operated at room temperature (18–25°C) except for the protein tests. A 20% excess of 0.1 *M* acetic acid solution was passed through the SP-Sephadex and Agarose columns to convert the cation exchangers into the hydrogen form, followed by deionized water (about one third of the column volume). Similarly, Separon HEMA Q and QAE-Sephadex were treated with 0.1 *M* sodium hydroxide and 0.1 *M* ammonium hydroxide, respectively, to yield the hydroxide forms. Carrier ampholytes were applied in the form of 0.2–10% aqueous solutions. Sample ampholytes were allowed to pass into the gel and the elution buffer (or displacing constituent in CHPH) was carefully layered onto the damp surface of the gel. Protein samples contained carrier ampholytes when applied to a strong exchanger column.

Other procedures

Isoelectric focusing was performed with a Multiphor 2117 (LKB) in 1 × 25 × 105 mm acrylamide gel slabs as described earlier[4].

Thin-layer chromatography on Silufol plates (Kavalier, Czechoslovakia) was utilized to identify amino acids in the column effluent. The organic layer of a *n*-butanol–water–acetic acid mixture (4:5:1) served as a mobile phase.

The capacity of the ion exchanger was determined using a measured gel volume. An approximately two-fold excess of ampholytes or salts was passed through the washed gel (in salt-free form, see above) on a glass sinter and then rinsed with water. The concentration of bound base or acid was determined by potentiometric titration of the washes, yielding the capacity for anion or cation, respectively. The ampholyte contents were calculated from the optical density of washes at 280 nm and by measuring the light absorption of the solution when the bound ampholytes were displaced from the gel by an excess of the corresponding eluent. A coincidence of two measurements within 10% was regarded as satisfactory.

RESULTS

Elution with non-amphoteric buffer

A PBE 118 column was adjusted to pH 9.85 with triethylamine hydrochloride. Elution was carried out with a 0.5% (w/w) aqueous solution of either tetraethylenepentamine (Fig. 2a) or carrier ampholytes (Fig. 2b), both solutions being adjusted to pH 8.0 with hydrochloric acid. Both eluents exhibit similar buffer capacities (*ca.* 0.05 mequiv. per pH unit per ml of 1% solution) in the pH range investigated. However elution with non-amphoteric buffer resulted in a steep decrease in pH rather than the smooth pH gradient expected.

Thus, the buffer capacity of the eluent is of little use for chromatofocusing except for amphoteric compounds. Moreover, the buffer capacities of unfocused ampholyte mixtures are known to be generally much higher than those relating to the corresponding pH gradient[5]. In an attempt to rescue the "buffering mechanism" it could be suggested that the eluent buffer capacity in the equation for pH generation[2] corresponds to isoelectric ampholytes.

Elution on non-buffering ion exchangers

According to the "buffering mechanism", CHF can produce a smooth pH gradient only in those pH ranges where both the eluent and ion exchanger display

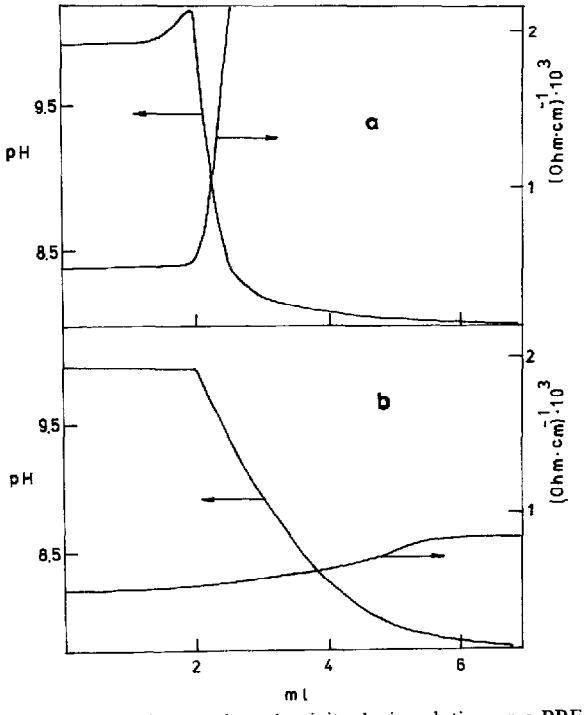

Fig. 2. pH gradient and conductivity during elution on a PBE 118 column with different buffers. Column bed: 152 mm × 5.5 mm. Elution conditions: starting buffer, 0.025 *M* triethylamine hydrochloride, pH 9.85; elution buffers (0.5%, w/w) adjusted to pH 8.0 with hydrochloric acid, (a) tetraethylenepentamine, (b) Ampholent 4-9; flow-rate 10 ml/h.

buffering action. Thus, DEAE-Bio-Gel A[6] was operated at pH 10.0–7.5. Specially designed exchangers possess an uniform buffer capacity from pH 11.0 to 4.0, and the pH range of CHF operation is correspondingly extended[3,7].

However, Serva has demonstrated superb CHF resolution of a complex protein mixture on a DEAE-SP 500 ion exchanger in the range pH 10.5–2.1[8]. This result conflicts with the buffering mechanism of chromatofocusing since the major part of the pH gradient was developed on a totally charged, *i.e.*, non-buffering, gel. Our results (Figs. 3 and 9) are in agreement with Serva's report, suggesting that a buffering gel is dispensable for pH gradient formation and for protein separation.

Thus, instead of a buffering mechanism, frontal development has been suggested[1] to explain the remarkable separation ability of chromatofocusing. If this suggestion is correct, some other phenomena (inverse pH gradient and decrease in ionic strength) may occur under certain experimental conditions.

CHF with amino acids on a strong ion exchanger

In order to investigate the possibility of an inverse pH gradient during CHF (Fig. 11 in Part I) a solution of two amino acids was employed to elute the sample (a third amino acid). Thin-layer chromatography revealed amino acids in the effluent, as shown by the solid lines at the top of Fig. 3. The inverse pH gradient is produced when the sample ampholyte emerges from the column and it is accompanied by a decrease in conductivity as predicted. However, the observed pH decrease lies at the limit of accuracy of our instrument, and therefore cannot be regarded as a verification of the inverse pH gradient.

Displacement development with ampholytes on strong ion exchangers—chromatophoresis (CHPH)

The CHPH principle involved, which was explained in detail in Part I, can be derived from the diagram in Fig. 4. Suppose that one column section (as represented by one wave on the diagram) is capable of binding one ampholyte molecule from the aliquot of mobile phase. Let the ampholyte concentration be one ampholyte per aliquot, the void volume of the column section and the aliquot volume being equal. Suppose, that affinities of the ampholytes for the exchanger decrease in the order

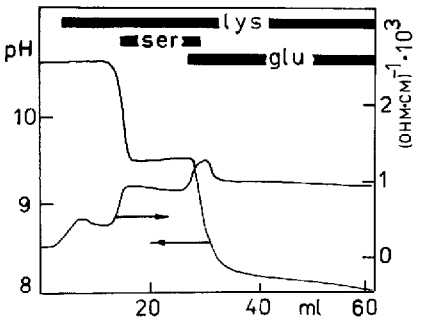

Fig. 3. CHF of serine. Column bed: 170 mm × 8.2 mm. Ion exchanger: Separon HEMA 1000 Q (90–125 μm), OH^-, capacity for Cl^-, 0.30 and 0.43 μequiv./ml (measured, and claimed by manufacturer, respectively). Sample: 0.5 mequiv. each of Ser, Lys and Glu; total molarity of solution 50 m*M*. Elution conditions: 50 m*M* solution of Lys and Glu (each 25 m*M*). Flow-rate: 25 ml/h.

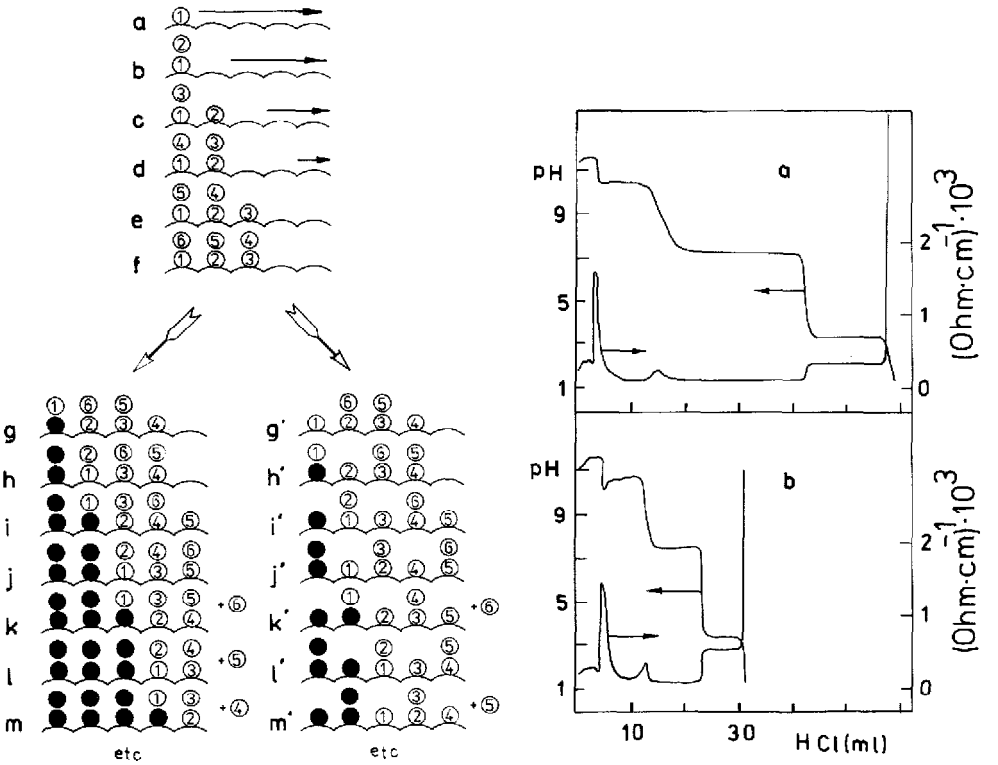

Fig. 4. Schematic representation of displacement development. For details see the text.

Fig. 5. CHPH of amino acids. Column and ion exchangers as in Fig. 3. Sample: 0.5 mequiv. each of Ser, Lys and Glu in 5 ml water. Flow-rate: 25 ml/h. Displacement: (a) 50 m*M*, (b) 100 m*M* hydrochloric acid.

$1>2>3>4>5>6$, *i.e.*, a lower-numbered mobile phase ampholyte should displace an higher-numbered one from the exchanger into the mobile phase.

Ampholytes are deliberately applied to the column one after another in the most unfavourable order for the purpose of demonstrating the resolution of the method. The solid arrow denotes the direction of eluent flow and at the same time the vanishing "relic" mobile phase (divisions a–d). The exchanger adsorbs the ampholytes and only pure water comprises the first five aliquots as they emerge from the column (divisions f–j).

Chromatophoresis begins upon addition of a constituent (filled circles) which has a greater affinity for the exchanger than any ampholyte. Divisions g–m and g′–m′ correspond to one displacer molecule per one aliquot and per two aliquots, respectively. Consequently, the ampholyte concentration in the effluent is 2 and 1 ampholytes per aliquot, respectively (see eqn. 11, Part I). The ampholytes are arranged in order of their affinity for the ion exchanger. Since the affinity is related to the isoelectric point, *i.e.*, to the ampholyte's charge at a given pH, a pH gradient is generated.

Amino acids were subjected to CHPH with different eluent concentrations (Fig. 5). Thin-layer chromatography shows that, unlike CHF (*cf.*, Figs. 3 and 5),

chromatophoresis furnishes amino acids in a pure, isoionic state. Serine (p*I* 5.68) is isoelectric over a broad pH range since it does not belong to the family of "good"[9] ampholytes. CHPH on anion and cation exchangers furnishes serine at pH 7.4 and 4.3, respectively, while CHF (Fig. 3) gives the apparent p*I* 9.6.

The conductivity increases in the zone of converged Lys and Ser in accordance with the theoretical model. However, no conductivity peak is detected between Ser and Glu, either due to complete resolution of zones or, more probably, because this peak is masked by the high conductivity of Glu solution. Salt contaminations in relatively crude amino acid reagents appear to be amenable for the first raise of conductivity just prior to Lys plato.

Removal of salts

Strong ion exchangers are known to cause dissociation of salts

$$G\text{–}SO_3H + NaCl \rightleftarrows G\text{–}SO_3Na + HCl \quad (1)$$

where G denotes the insoluble matrix of the exchanger. In the column this equilibrium is shifted to the right due to withdrawal of hydrochloric acid by the effluent flow. In the absence of carrier ampholytes, the salt dissociation results in extreme pH values, thus jeopardizing the native structure of proteins.

Desalting on an anion exchanger produces alkali hydroxides (Fig. 6). Two parallel experiments with salt-free and salt-containing ampholytes furnished very similar pH and conductivity profiles for the zones of carrier ampholytes. Sodium hydroxide does not bind to the anion exchanger and can be separated from the ampholyte "train" by a zone of pure water, providing the column capacity is high enough. In contrast, all components that bind to a strong ion exchanger display no electrolytic vacuum between them in CHPH.

Carrier ampholyte fractionation

A mixture of several Ampholent batches (6 ml of 20% solution, pH 4–10) was subjected to chromatophoresis on QAE-Sephadex column (14 ml). 0.1 *M* Hydrochloric acid was applied at a rate of 20 ml/h, and 50 fractions were collected. Iso-

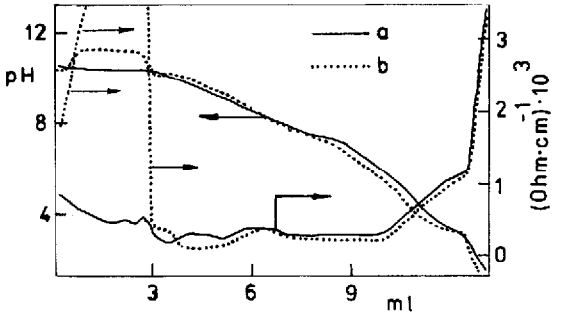

Fig. 6. CHPH of ampholytes on QAE-Sephadex A-25. Column bed: 110 mm × 11 mm. Flow-rate: 17 ml/h. Displacement: 40 m*M* hydrochloric acid. Samples are 4 ml of 3% Ampholent 4-10 containing (a) no salts, (b) 0.15 mequiv. sodium chloride.

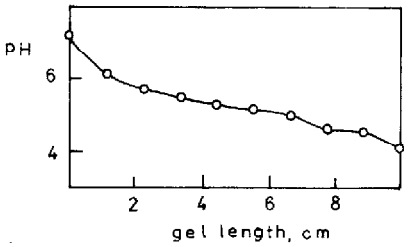

Fig. 7. Isoelectric focusing of combined ampholyte fractions (pH 4.7–5.7) from CHPH Ampholent fractionation.

electric focusing of the combined fractions (pH 5.7–4.7) resulted in a flat pH gradient (Fig. 7). The combined fractions of pH 8.5–8.7 furnished a narrow pH gradient (8.8–8.6) when being subjected to repeated CHPH on 3 ml QAE-Sephadex A-25 (results not shown).

The flat pH gradient produced by the carrier ampholyte fractions confirms the fact that in CHPH the ampholytes are arranged in order of their isoelectric points. In contrast to carrier ampholyte fractionation in an electric field, *e.g.*, refs. 10, 11, CHPH is much more simple and safe since it does not require high-voltage apparatus. This procedure is a conventional step in Ampholent preparation.

CHPH of proteins

All attempts to perform protein chromatophoresis on commercially available strong ion exchangers (Sephadexes SP C-25 and QAE A-25, Separon HEMA Q) totally failed. The pH gradient was devoid of proteins, and the latter were recovered as a single peak upon elution with 1 *M* sodium chloride.

In a search for an appropriate sorbent we have tried to perform CHPH on a weak ion exchanger. DEAE-Toyopearl furnished three and seven protein peaks when egg white was subjected to chromatophoresis and chromatofocusing, respectively (Figs. 8 and 9). However, only strong ion exchangers provide "true" chromatophoretic development (see Discussion). Therefore, a low capacity sulpho derivative of Agarose 4B was prepared (see *Materials*). Three protein peaks were revealed by chromatophoresis of egg white on this strong cation exchanger (Fig. 10). CHPH of commercial whale myoglobin (p*I* 7.1, 7.4, 7.6 for isoform and 7.68 for major component[12]) resulted in one major band near pH 8.2, and two meagre peaks on the ascending shoulder of the main band. A mixture of egg white and myoglobin was resolved into four peaks (results not shown).

DISCUSSION

Ampholyte desorption from ion exchanger

Usually, chromatographic techniques involve sorption–desorption mechanisms. The methods of ampholyte desorption from exchangers are conventionally divided into four types:

(1) alteration of ampholyte–eluent affinity (solubility), changing the partition equilibrium in favour of solvent. For instance, a decreasing pH gradient on DEAE exchangers produces positive charges on ampholytes thus increasing their solubility;

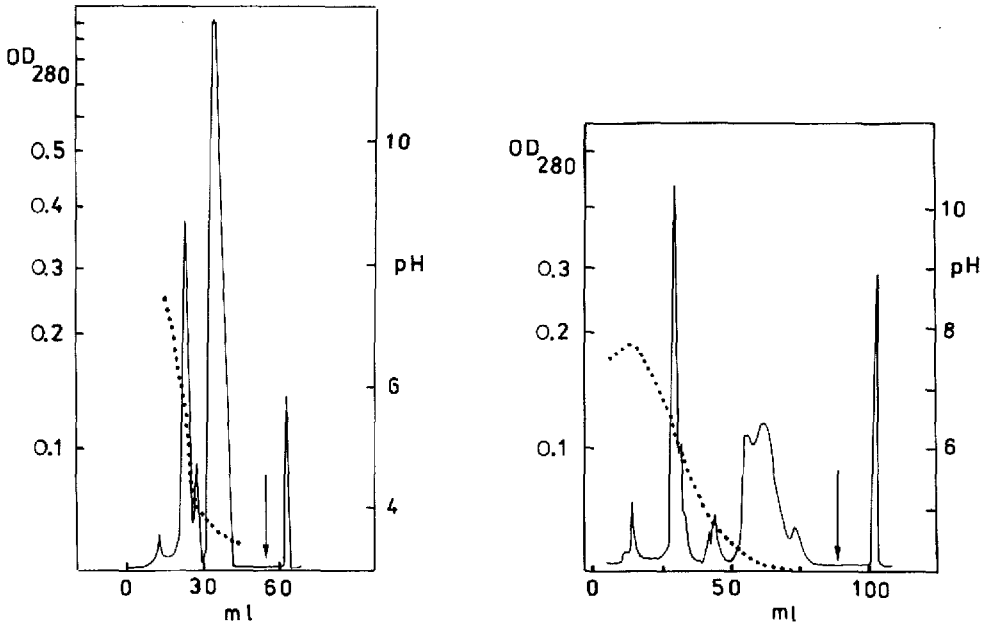

Fig. 8. CHPH of egg white on DEAE-Toyopearl. Column bed: 125 mm × 11 mm. Starting buffer: 25 m*M* imidazole hydrochloride, pH 7.4. Sample: 1.2 ml starting buffer containing 0.2 ml egg white. Ampholytes: 15 ml of Ampholent 4-7.5, 10 μmol per pH unit per ml. Displacement: 5 m*M* acetic acid. Flow-rate: 15 ml/h. Arrow indicates the end of CHPH and application of 1 *M* sodium chloride. Dotted curve denotes pH.

Fig. 9. CHF of egg white on DEAE-Toyopearl. Conditions as in Fig. 8, except for displacement step. Instead of displacement, frontal development (CHF) was performed. Elution buffer: 10 μmol per pH unit per ml Ampholent 4-7.5, adjusted to pH 4.0 with hydrochloric acid. Arrow indicates the end of CHF and application of 1 *M* sodium chloride. Dotted curve denotes pH.

(2) variations in mobile phase composition that do not affect ampholyte solubility but do change the affinity of the sorbent for ampholyte, *e.g.*, positive charges on DEAE gels are quenched above pH 10.0 by an increasing pH gradient;

(3) increase in the competitive ion (salt) concentration. Reversible formation of ion pairs with competitive ions reduces the possibility of direct ampholyte–ion exchanger interaction;

(4) displacement of one ampholyte (A_1) by another (A_2) on a strong ion exchanger according to a second order mechanism of ion substition (Si2):

$$G\text{–}SO_3^-A_1^+ + A_2^0 \rightleftharpoons G\text{–}SO_3^- \begin{cases} A_1^{\delta+} \\ A_2^{\delta+} \end{cases} \rightarrow G\text{–}SO_3^-A_2^+ + A_1^0 \downarrow \qquad (2)$$

Displacement on weak ion exchangers can proceed via a Si1 mechanism:

$$G\text{–}COO^-A_1^+ + H_2O \rightleftharpoons G\text{–}COOH + A_1^0 \qquad (3)$$
$$G\text{–}COOH + A_2^0 \rightleftharpoons G\text{–}COO^-A_2^+$$

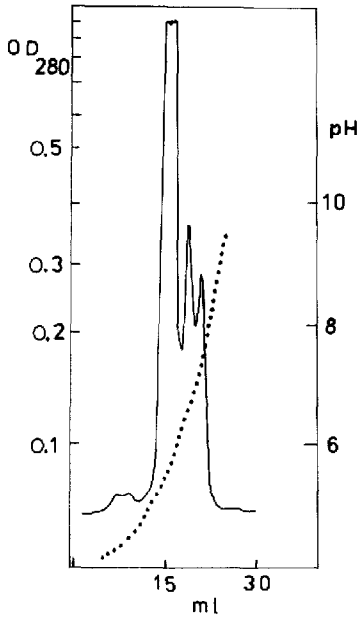

Fig. 10. CHPH of egg white on sulpho agarose. Column bed: 170 mm × 11 mm, sulpho derivative of Agarose 4B (50–200 μm), H^+. Sample: 0.3 ml of 20% egg white in diluted ampholytes. Ampholytes: PB 74 and PB 96 (1:1) diluted in 14 ml water (1:20). Displacement: 10 m*M* ammonium hydroxide. Flow-rate: 15 ml/h. Dotted curve denotes pH.

The latter mechanism is supported by the fact[13] that carrier ampholytes can be completely eluted from DEAE-Sepharose CL-6B by water. Consequently, the capacity, Q_a, of the weak ion exchanger for ampholytes is a dynamic criterion in eqn. 11, Part I.

In Part I the Si2 mechanism was postulated as the sole method of ion exchange in CHF and CHPH. However, all the above four mechanisms of ampholyte desorption are likely to be valid in actual protein chromatofocusing on buffering (weak) ion exchangers. The increasing ionic strength of the mobile phase and the decreasing negative charge of a protein molecule are countered by the growing positive charge of the buffering anion exchanger during the development of decreasing pH. The dwindling Donnan potential[2] and the formation of multiple ionic bonds between a protein and the gel complicates further the interpretation of the CHF process on the molecular level.

In contrast, CHPH on strong ion exchangers appears to be less ambiguous. The Si2 mechanism implies that electrolytic vacuum is not permissable in pH gradient formed by established ampholyte "train". An analogous law of pH monotony was postulated earlier[14] for isoelectric focusing.

The absence of destructive diffusion implies an high potential resolving power for CHPH. For comparison, the resolving power of isoelectric focusing depends on the balance between electrical and diffusional mass transport[15,16], *i.e.*, between focusing and dispersive forces, the latter being eliminated in CHPH due to the Si2 mechanism.

The large difference between the electric conductivities at neutral and extreme pH values (below 3.5 and above 10.5) is known to be a problem in isoelectric focusing of very basic or very acidic proteins. So far, CHPH practice has not revealed any obstacle to ampholyte separation in pH gradient extremes.

Ion exchanger

Synthetic carrier ampholytes, proteins and some amino acids have more than one group capable of ionic interaction with an ion exchanger. These ampholytes may form multiple ionic bonds with the resin provided the latter has two or more adsorption sites within reach of the ampholyte molecule. Since the affinity of an adsorbed molecule for the stationary phase has an exponential dependence on the bond number, the charge density of the ion exchanger should be kept low. Otherwise the ampholyte order in the CHPH "train" will be governed not only by p*I* values but also by the number of ionizable groups and by the geometric dimensions of the ampholytes. However, a low charge density is dispensable when chromatophoresis is performed with molecules of similar sizes, *e.g.*, only amino acids (Fig. 5), or only carrier ampholytes (Fig. 6).

How small should the capacity of the ion exchanger be to prevent an ampholyte molecule from binding to more than one site? Assuming that the charged groups of the exchanger are uniformly distributed in the gel volume provides the following data:

Ion exchanger capacity (μM/ml)	1	5	10	50	200	1000
Distance (nm) between two adjacent ionizable groups	12	7	5.5	3.2	2	1.2

These should be compared with the ampholyte dimensions (Table I-4 in ref. 17): alanine, 0.5 nm (MW 89); myoglobin, 3.6 nm (MW 17000); haemoglobin, 6.8 nm (MW 63000); glutamate dehydrogenase, 13 nm (MW 1000000).

Egg proteins (albumin, MW 43000) were not eluted during CHPH on SP-Sephadex C-25 (300 μM/ml) with either a pH gradient or with ammonium hydroxide solution. Multiple protein–exchanger bonds were cleaved by 1 *M* sodium chloride. The Agarose sulpho derivative was the only exchanger to resolve proteins according to the CHPH principle. The poor resolution may be attributed to contamination of relatively crude Agarose gel and possibly to the too high capacity of the sulpho derivative.

Though commercially available strong ion exchangers have been used for carrier ampholyte fractionation they are far from ideal CHPH supports. For instance, Sephadex gels swell up to 20% of the bed volume and are not stable at extreme pH. Spheron and Separon glycol methacrylate gels retain excellent chemical and physical stability even at extreme pH and ionic strength, and in the presence of organic solvents[18]. Unfortunately, the carrier ampholytes turned out to be too large (MW *ca.* 1000) to penetrate the micropores of the Spheron-type gels. As a result, the capacities for carrier ampholytes and for chloride ion differ dramatically (25 and 300 μM/ml, respectively, on Separon HEMA Q), which makes these resins inefficient for carrier ampholyte fractionation. Moreover, the faint hydrophobicity of the available Spheron batches makes protein CHPH at low ionic strength impossible. New modifications

of glycol methacrylate sorbents with reduced non-specific activity (GCL-type Sepharon gels) have recently been reported by Laboratorni Přistoje (personal communication).

CONCLUSIONS

Fractionation of amino acids, carrier ampholytes and proteins on strong ion exchangers proceeds according to the proposed CHPH principle. The low capacity exchangers needed for protein separation require the application of larger column volumes as compared with other types of protein chromatography. However, the relative consumption of expensive carrier ampholytes per protein load in CHPH is the same or even lower than in chromatofocusing. The slope of the pH gradient is dependent only on the ampholyte composition and its length is dictated by the concentration of the inexpensive displacer and by the ampholyte load.

So far, chromatophoresis has been efficiently applied only for carrier ampholyte fractionation. We hope that the development of congruent ion exchangers will enable further application of CHPH to various protein analyses and separations. These strong ion exchangers should meet the following demands:

(1) absence of non-specific interaction with ampholytes, (2) high chemical and mechanical stability at extreme pH, (3) similar capacities for all applied ampholytes and for the eluent.

REFERENCES

1 A. Murel, S. Vilde, M. Pank, I. Shevchuk and O. Kirret, *J. Chromatogr.*, 347 (1985) 325–334.
2 L. A. AE. Sluyterman and O. Elgersma, *J. Chromatogr.*, 150 (1978) 17–30.
3 *Chromatofocusing with Polybuffer and PBE*, Pharmacia, Uppsala, 1982.
4 A. Murel, S. Vilde, A. Kongas and O. Kirret, *Electrophoresis*, 5 (1984) 139–142.
5 S. Fredriksson, *J. Chromatogr.*, 135 (1975) 441–446.
6 L. A. AE. Sluyterman and J. Wijdenes, *J. Chromatogr.*, 150 (1978) 31–44.
7 L. A. AE. Sluyterman and J. Wijdenes, *J. Chromatogr.*, 206 (1981) 441–447.
8 *Catalog 1984/85*, Serva Feinbiochemica, Heidelberg.
9 H. Svensson, *Acta Chem. Scand.*, 16 (1962) 456–466.
10 O. Vesterberg, *Acta Chem. Scand.*, 23 (1969) 2653–2666.
11 E. Gianazza, M. Pagani, M. Luzzana and P. G. Righetti, *J. Chromatogr.*, 109 (1975) 357–364.
12 P. G. Righetti and T. Caravaggio, *J. Chromatogr.*, 127 (1976) 1–28.
13 J. P. Emond and M. Pagé, *J. Chromatogr.*, 200 (1980) 57–63.
14 H. Svensson, *Protides Biol. Fluids*, 15 (1967) 515–522.
15 H. Svensson, *Acta Chem. Scand.*, 15 (1961) 325–341.
16 O. Vesterberg, *Acta Chem. Scand.*, 21 (1967) 206–216.
17 A. L. Lehninger, *Biochemistry*, Worth Publishers, New York, 1972.
18 O. Mikeš, P. Štrop, J. Zbrošek and J. Čoupek, *J. Chromatogr.*, 119 (1976) 339–354.