ELSEVIER

Journal of Chromatography A, 676 (1994) 409–420

JOURNAL OF CHROMATOGRAPHY A

New approaches to concentration on a microliter scale of dilute samples, particularly biopolymers with special reference to analysis of peptides and proteins by capillary electrophoresis I☆. Theory

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First received 20 December 1993; revised manuscript received 21 March 1994

Abstract

New methods are described for the concentration of ionic analytes, particularly ampholytes, such as peptides and proteins. In most of these methods the sample is depleted (partially) of strong electrolytes concomitantly with the concentration. The methods are based on the fact that electrophoretic migration velocities decrease upon decreasing the absolute value of the zeta potential of a solute and the pore size of the electrophoresis medium and upon increasing the cross section of the electrophoresis chamber, the viscosity and the electrical conductivity of the electrophoresis medium. We have also utilized the zone-sharpening properties of displacement electrophoresis in combination with a hydrodynamic counter flow to create a stationary zone where the sample solutes can be collected continuously.

In practice, the whole electrophoresis tube is filled with the sample solution to be concentrated. When a voltage is applied the solutes begin to migrate, but finally cease to move as they approach the end of the tube, provided that the above-mentioned parameters in that section of the tube have been given appropriate values. By means of this technique the sample can be concentrated into a zone of a width of 0.2–0.5 mm. Accordingly, a 400–1000 fold concentration is obtained when a 200 mm long tube is filled completely with the sample and still more if also an electrode vessel (or a vessel connected to this electrode vessel) is loaded with sample. The narrow sample zone can be withdrawn from the tube and subjected to further studies or used as a starting zone for an in-tube zone electrophoresis. The tendency for broadening of the very narrow starting zone during the initial phase of this electrophoresis step can be counteracted by a short mobilization step involving displacement electrophoresis, electrophoresis in a steep pH gradient, or on-tube dialysis against a (diluted) buffer. This step can be omitted when the concentration is accomplished by a combination of displacement electrophoresis and a counter flow.

In Part II we show how the theory developed in this paper can be utilized practically.

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☆ For Part II see ref 1.

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SSDI 0021-9673(94)00316-2

1. Introduction

Every analysis method has a lower detection limit under which one cannot obtain accurate analysis data. Therefore, one must sometimes perform a preconcentration step which usually entails large losses when the sample volume is 1–10 μl or less. The most common methods for the concentration of solutes of biochemical interest (lyophilization [2], ultrafiltration [3,4], partition between two polymer aqueous phases [5], osmotic removal of water [6] and chromatographic adsorption–desorption of the solute) are not applicable on such a small scale.

There is, accordingly, a need for efficient preconcentration techniques applicable for minute-volume samples. Five such methods, based on electrophoretic zone sharpening, have recently been presented [7,8]. They are described in detail in this paper (references to related methods are found in Discussion). A requirement is that the solutes to be concentrated are ionic polymers or weak electrolytes, preferably ampholytes, such as proteins, or can be converted to such by complex formation. When the concentrated zone is to be used as the starting zone for a subsequent electrophoresis the concentration can take place in the electrophoresis chamber, for instance, a capillary.

2. The theory of the concentration (step I), the mobilization of the concentrated zones (step II) and the electrophoretic analysis (step III)

The discussion below refers to an analysis by high-performance capillary electrophoresis (HPCE). However, following the concentration step the sample can be withdrawn from the fused silica tubing and processed by other techniques than HPCE.

2.1. Sample treatment

When the sample is in the form of a solution, for instance a biological sample, it can often with advantage be applied directly into the electrophoresis tube, provided that pH is such that the solutes migrate electrophoretically in the desired direction and not excessively slowly. A pH adjustment may be necessary. If displacement electrophoresis is utilized for the concentration the sample has to contain an appropriate leading (terminating) ion. One must then add to the sample a small volume of a highly concentrated solution of the leading (terminating) buffer. In cases when it is uncertain whether the pH and the background electrolyte of the sample might disturb the concentration and electrophoresis steps the sample should be dialyzed (desalted) by micro methods, as will be described elsewhere [9]. These methods can be used for larger peptides and macromolecules and are based on dialysis in small-pore polyacrylamide tubes [8,9]. For determination of the pH and electrical conductivity in minute (sample) volumes, see Discussion.

2.2. Step I: concentration of the dilute sample

The electrophoretic velocity v of a solute is determined by the expression

$$v = u \frac{I}{q\,\kappa} \quad (1)$$

where the mobility u can be calculated from the equation

$$u = \frac{\epsilon\,\zeta}{4\,\pi\,\eta} \quad (2)$$

(I = current, q = cross-sectional area of the electrophoresis chamber, κ = electrical conductivity, ϵ = dielectric constant, ζ = zeta potential of the solute and η = viscosity.)

An electrophoretically migrating zone can be concentrated if its front can be forced to move slower than its rear. According to Eqs. 1 and 2, this requirement will be fulfilled if the zone is permitted to migrate toward a section of the electrophoresis chamber where u decreases or/and q, κ, or η increase. Different approaches to manipulating these parameters to attain a narrow starting zone, *i.e.*, a concentration, are described below, along with an approach based on a combination of displacement electrophoresis and a hydrodynamic counter flow. Unless otherwise

stated, the discussion refers to anions in the sample. Cations are treated in an analogous way.

Alternative a

Concentration by electrophoresis toward a steep, non-buffering pH gradient (isoelectric focusing). The whole capillary is filled with the sample solution and the cathode vessel with buffer BC (for instance, 0.01 *M* Tris–HCl, pH 8.5) and the anode vessel with buffer Ba (for instance, 0.5 *M* tris–HCl, pH 2.5). A steep, non-buffering pH gradient at the anodic end of the capillary is created immediately when the voltage is switched on (see Fig. 1a and Fig. 2a, step I). At the same time the anions in the sample begin migrating toward the anode. The strong anions pass through the pH gradient, *i.e.*, the sample is freed of strong electrolytes, whereas ampholytes will concentrate into very narrow zones. Weak electrolytes, such as carboxylic acids, will also concentrate in the pH gradient. However, they are non-charged at low pH ($pH < pK \pm 1.5$), where ampholytes become positively charged. The diffusional broadening of weak electrolytes is therefore not counteracted by the zone sharpening that is characteristic of ampholytes in a pH gradient (isoelectric focusing). Examples of appropriate experimental conditions (pH, buffers, etc.) given in this and other sections are taken from our subsequent paper [1].

Alternative b

Concentration by electrophoresis toward a small-pore polyacrylamide gel. The whole capillary is filled with the sample and a short plug of the gel is introduced into one end of the capillary as described in ref. 1. The solutes migrate toward the gel plug when a voltage is applied but stop migrating when they come into contact with the gel because it is impermeable to large molecules, *i.e.*, a concentration takes place at the gel surface (see Fig. 1b and Fig. 2b, step I).

Alternative c

Concentration by electrophoresis toward a piece of dialysis tubing that permits the passage of current but not of the analytes. A short dialysis tubing is prepared and attached to one end of the fused silica tubing as described in ref. 1 in section 3.4. This concentration technique is depicted in Fig. 1c and Fig. 2c, step I. When the solutes leave the fused-silica tubing the field strength decreases abruptly (q in Eq. 1 increases) and their migration velocities become

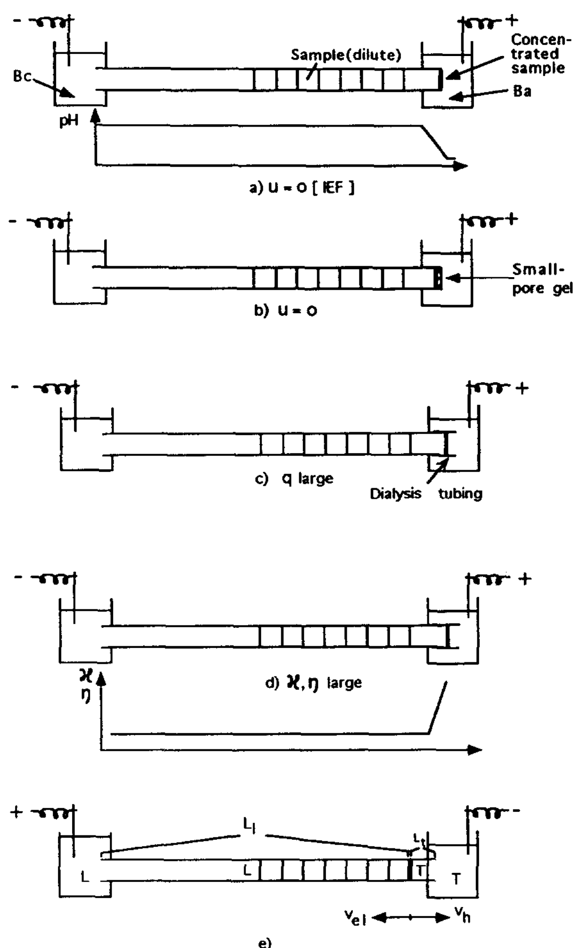

Fig. 1. Different approaches to concentrate a dilute sample solution (step I). (a) By isoelectric focusing in a pH gradient; (b) by zone electrophoresis toward a small-pore gel; (c) toward a gradient in effective cross-section (q); (d) toward a gradient in electrical conductivity (κ) or viscosity (η); (e) by a combination of displacement electrophoresis and a counter-flow. In the mobilization (step II) and the electrophoretic analysis (step III) the polarity of the electrodes is reversed [except in (e)], *i.e.*, the anode is to the left (see Fig. 2, a–d).

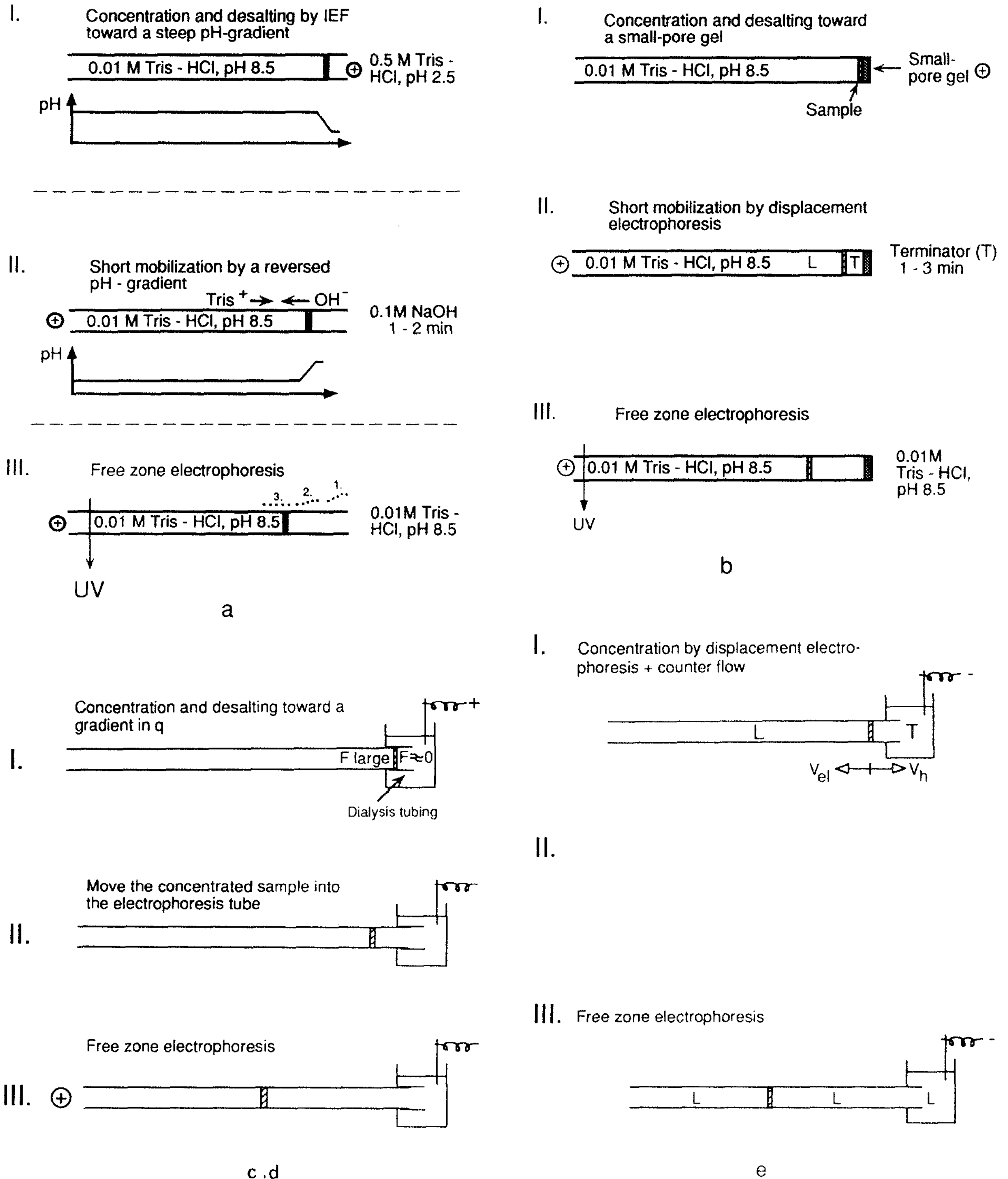

Fig. 2. Different approaches to mobilize (step II) samples concentrated as outlined in Fig. 1 (step I). Step III: analysis of the sample by free zone electrophoresis. All notations are the same as in Fig. 1.

virtually zero. The solutes will, accordingly, become concentrated into a narrow zone. In this step low-molecular weight ions in the sample are removed by diffusion through the pores in the dialysis tubing.

Alternative d

Concentration by electrophoresis toward a gradient in conductivity. If the right-hand electrode vessel in Fig. 1d or Fig. 2d, step I, is filled with a buffer of high ionic strength the sample in the capillary will become concentrated in the conductivity (concentration) gradient formed at the tube end. A similar concentration can be obtained by means of a viscosity gradient.

Alternative e

Concentration by a combination of displacement electrophoresis and a counter flow. The electrophoresis tube is filled with the sample dissolved in the leading buffer or transferred to that buffer as described in ref. 2 (see Fig. 1e and Fig. 2e, step I). A hydrodynamic flow is generated in the electrophoresis tube in a direction opposite to the migration direction of the boundary between the leading and the terminating ions. The magnitude of the counter flow is adjusted such that the boundary becomes stationary. If the sample ions have mobilities between those of the leading and the terminating ions, they will become concentrated at the boundary, because a sample ion which by diffusion or convection enters the terminating (leading) buffer will move back into the concentrated sample zone, since the field strength —and thereby the velocity of the sample ion— is higher (lower) in the terminating (leading) buffer than in the sample zone.

In the initial phase of the displacement electrophoresis step a fast-moving solute can have an electrophoretic velocity which is higher than the velocity of the counter flow. A population of this solute located close to the end of the capillary may, therefore, leave it. As the width of the terminating zone increases the field strength in the leading buffer zone decreases (see below). The velocity of the solute therefore decreases successively until no further solute molecules migrate out of the capillary. To eliminate or suppress this loss of fast-moving solutes one should decrease the concentration of the sample-containing leading buffer in the capillary (but not that of the leading buffer in the anode vessel). The field strength then decreases at this end of the capillary and thereby also the electrophoretic velocities of the proteins as they approach the anolyte. Another alternative is to mix the sample with the terminator instead of with the leading buffer. Part of the very slowly migrating sample ions may then, however, leave the capillary at its cathodic end. The loss can be minimized by decreasing the concentration of the terminator in the cathode vessel (but not that in the capillary).

The hydrodynamic flow should be adjusted so that the boundary layer (the concentrated sample) between the leading and terminating buffers is located about 1–2 cm from one end of the capillary (the right end in Fig. 1e). At a cursory glance, one might expect a small decrease in the hydrodynamic flow to move the boundary layer with the sample in Fig. 1e significantly toward the left, and an increase in the flow to displace the sample out of the capillary. However, moderate changes in the flow will affect the position of the sample only slightly, as shown below.

Assume that the distance from the left end of the capillary in Fig. 1e to the stationary, concentrated, narrow sample zone is L_l cm (= the length of the leading buffer zone) and the distance from the right end to the sample zone is L_t (= the length of the terminating zone). Since the leading ion and the terminating ions migrate with the same velocity

$$u_l \frac{V_l}{L_l} = u_t \frac{V_t}{L_t} \tag{3}$$

where u_l and u_t are the mobilities of the leading and terminating ions, respectively, and V_l and V_t are the voltages over the leading and terminating zones, respectively.

Rearrangement of the factors in Eq. 3 and substitution of V_t for $V - V_l$, where V is the voltage applied over the capillary, gives

$$\frac{L_l}{L_t} = \frac{u_l}{u_t} \frac{V_l}{V - V_l} \tag{4}$$

From the relation $v_l = u_l V_l/L_l$, where v_l = the electrophoretic velocity of the leading ion (= the electrophoretic velocity of the concentrated zone = velocity of the hydrodynamic counter flow when this zone is stationary with respect to the capillary wall) one obtains an expression for V_l which can be introduced into Eq. 4. Solving for L_t following substitution of L_l with $L - L_t$, gives

$$L_t = \frac{u_t}{u_l - u_t}\left(V\frac{u_l}{v_l} - L\right) = \frac{u_t L}{u_l - u_t}\left(\frac{V}{L}\frac{u_l}{v_l} - 1\right) \quad (5)$$

Putting $V/L = F$ (F is the field strength obtained when the capillary is filled completely with terminating or leading buffer) Eq. 5 can be transformed to

$$L_t = \frac{u_t L}{u_l - u_t}\left(F\frac{u_l}{v_l} - 1\right) = \frac{u_t L}{u_l - u_t}\left(\frac{F}{F_l} - 1\right) \quad (6)$$

Insertion of appropriate values in Eqs. 5 or 6 shows that L_t is roughly inversely proportional to v_l. For instance, when the differences between the buffer levels in the electrode vessels in two experiments are 25 and 20 mm, the decrease in the hydrodynamic flow rate is 20%. If the width of the terminating zone is 12 mm in the first experiment it is, accordingly, about 14 mm in the second experiment, *i.e.*, the change in the position of the solute zone is only 2 mm, or in other words, the adjustment of the buffer levels is not critical. It is easy to explain this finding in qualitative terms. If the flow decreases the sample zone moves to the left in Fig. 1e. The terminator zone becomes longer and the leading buffer zone shorter. The current will then decrease, since the former zone has a higher ohmic resistance per cm than has the latter zone. A decrease in current means that the field strength F_l in the leading buffer zone decreases and therefore also the migration velocity of the boundary layer v_l ($= u_l F_l$), which counteracts the above displacement of the sample zone toward the left. Analogously, an increase in hydrodynamic flow (which displaces the sample zone toward the right) gives rise to an increase in the current and thereby an increase in the velocity of the sample zone. It will, accordingly, be displaced electrophoretically to the left. The net movement of the sample zone is, therefore, nearly negligible.

2.3. Step II: short mobilization of the concentrated sample zone

For an electrophoretic analysis of the concentrated sample zone the polarity of the electrodes must be reversed (except in alternat.ve e). However, this very narrow zone will broaden considerably if the polarity is changed immediately after the concentration is finished. To counteract this broadening, a short mobilization step should be introduced between the concentration step and the final electrophoretic step. Some different approaches to accomplish the mobilization are described below.

Alternative a

Mobilization by displacement electrophoresis. Following concentration of the sample, buffer Ba in the right electrode vessel in Fig. 1 is replaced by a terminating buffer, for instance, 0.03 *M* glycine–NaOH, pH 10 (Fig. 2b, step II).

Upon reversing the polarity of the electrodes the anion in the terminating buffer (glycine in our example) forms a migrating boundary with the anion in the buffer in the electrophoresis tube (for instance, chloride). The requirements for displacement electrophoresis become fulfilled if the anions in the sample have a mobility lower than those of the anions in the buffer in the electrophoresis tube and higher than those of the anions in the terminating buffer. In this displacement step the very narrow concentrated zone will move without zone spreading toward the left (the anode is at the left in the mobilization and analysis steps; see Fig. 2b, step II). When the subsequent analysis is based on zone electrophoresis (rather than displacement electrophoresis) the displacement step should be interrupted as soon as the concentrated zone begins to move toward the left (or preferably somewhat earlier). Otherwise, the onset of the zone electrophoresis step (Fig. 2b, step III; see also step

III, alternative b, below) will be delayed, which gives rise to an unnecessarily long analysis time.

Alternative b

Mobilization by a reversed, non-buffering pH gradient. The buffer in the right-hand electrode vessel used for the concentration is exchanged for a solution of high pH (for instance 0.1 *M* NaOH) to make the acidic solutes migrate toward the positive pole in the mobilization step (see Fig. 2a, step II); for basic solutes which migrate toward the negative pole a solution of low pH is chosen (for instance, 1 *M* HCl).

Alternative c

Mobilization by zone electrophoresis following concentration by electrophoresis toward a dialysis tubing. Prior to the subsequent electrophoresis step the concentrated sample in the dialysis tubing is moved hydrodynamically one or two mm into the electrophoresis tube (see Fig. 2c, step II), for instance, by raising the right-hand electrode vessel. Due to the low field strength in the dialysis tubing the sample zone will otherwise broaden when it migrates into the electrophoresis tube upon reversal of the direction of the current. Alternatively, one can move the electrophoresis tube toward the left so that the section of the dialysis tubing that contains the concentrated sample zone will be in contact with air rather than buffer.

Alternative d

Mobilization following concentration toward a gradient in conductivity. Following this concentration (Fig. 1d) the sample should be moved into the dialysis tubing for removal of salt by dialysis against a dilute buffer in the electrode vessel and then back into the capillary (upon reversing the polarity of the electrodes a zone sharpening occurs when the voltage is switched on if the buffer in the sample has a conductivity lower than that of the buffer in the electrophoresis tube). A similar approach can be employed for concentration toward a viscosity gradient when the added viscosity-increasing agent is dialyzable.

One should note that most electrode vessels used in HPCE have an effective cross-sectional area much larger than that of the capillary (see alternative c above). Accordingly, in almost all apparatus, the field strength in the dialysis tubing is sufficiently low without an increase of the ionic strength (or the viscosity) in the electrode vessel. In fact, one can even have a lower ionic strength in the electrode vessel than in the capillary, and thereby omit the above dialysis step aimed at giving a narrow starting zone in the subsequent zone electrophoresis.

2.4. Step III: electrophoretic analysis

Alternative a

Analysis by displacement electrophoresis. In this case the mobilization according to alternative a is not interrupted, but is allowed to proceed until all of the solutes have passed the detector.

Alternative b

Analysis by zone electrophoresis following mobilization by displacement electrophoresis or electrophoresis in a reversed pH gradient (step II, alternatives a or b). When the concentrated starting zone, mobilized as in step II, alternatives a or b, is to be subjected to zone electrophoresis the solution in the right-hand electrode solution is exchanged for a buffer of the same composition as that in the capillary and in the left electrode vessel (see Fig. 2b, step III and Fig. 2a, step III).

Alternative c

Analysis by zone electrophoresis following concentration by a combination of displacement electrophoresis and a counter flow. In this approach the separate mobilization step can be omitted: one can proceed directly from the concentration step to the zone electrophoresis step without reversing the direction of the current following substitution of the terminating buffer in the cathode vessel with the leading buffer (see Fig. 2e, step III).

3. Discussion

A comparison among some concentration methods. On-line electrophoretic concentration of ionic substances by transferring the sample to a buffer with a conductivity lower than that of the electrophoresis buffer was first described by Haglund and Tiselius [10]. The effect can be reinforced by proper choice of the pH of the sample [11]. A concentration based on such manipulations can be performed as a one-step procedure by using two or more buffers, the first step being a concentrating displacement electrophoresis which in a second stage is transformed automatically into an analytical zone electrophoresis. This elegant approach was introduced by Ornstein for concentration of a protein sample in polyacrylamide gel electrophoresis [12]. The same degree of concentration can, however, often be achieved in a simpler way by lowering the conductivity of the sample [13].

The design of Ornstein's discontinuous buffer system is partly based on the fact that a protein migrates more slowly in a sieving gel of polyacrylamide than in free solution. Accordingly, this buffer system cannot be used in carrier-free electrophoresis of high-mobility proteins or peptides. For free electrophoresis of this class of substances we have, therefore, introduced new buffer systems based on the use of terminators, the mobilities of which span over a wide range when the pH is varied [11]. An example of an appropriate terminator is diaminopimelic acid, used in the experiment shown in Fig. 2b in ref. 1 (for similar methods based on displacement electrophoresis, see refs. 14–20). An advantage of the concentration methods described herein over those based on discontinuous buffer systems is that the whole electrophoresis tube, rather than only a fraction of it, can be filled with the sample solution and, if so desired, also an electrode vessel. The degree of concentration is, accordingly, higher.

A counter flow has been used in electrophoresis for several purposes; for instance, in order to compensate for the hydrodynamic flow which attends the electroendosmotic flow in a closed electrophoresis tube or/and to increase the effective length of the electrophoresis tube [21–26].

A method based on displacement electrophoresis, electroendosmotic flow and a counter flow is described in ref. 27. The degree of concentration accomplished by this technique was limited, since only (part of) the capillary, but not the electrode vessel was filled with sample. The authors reversed the polarity of the electrodes when the displacement electrophoresis was replaced by a zone electrophoresis. It was emphasized that "correct timing of the voltage switching is important" to avoid that "the sample zones will migrate out of the capillary" or broaden. In the method presented herein the polarity is the same throughout the experiment and any change in the hydrodynamic flow rate is counteracted by an automatic adjustment of the electrophoretic migration velocity of the concentrated solute zone, *i.e.*, there is no risk that it will leave the capillary (see the discussion of Eqn. 5). For an interesting concentration method based on zone electrophoresis and a hydrodynamic counter flow, see ref. 28.

A sharpening of the starting zone upon decreasing the field strength by increasing the effective cross-section of the electrophoresis medium has been utilized in paper electrophoresis of proteins [29]. The method is very efficient [30]. The same principle was used in the approach presented in Fig. 1c.

The use of a gradient in viscosity and conductivity for concentration purposes has been described earlier [31]. This method differs from that in Fig. 1d, in that the gradient was created inside the electrophoresis tube rather than at the boundary between the electrophoresis tube and an electrode vessel. Stabilization against convection in the tube was accomplished by a sucrose gradient. In a method for recovery of proteins following polyacrylamide gel electrophoresis the protein zone of interest was cut out, the protein eluted eletrophoretically and then concentrated in a conductivity gradient [32]; compare Fig. 1d.

The following considerations are important for the design of the buffer systems used for the concentration of solutes.

(1) *Concentration toward a non-buffering, steep pH gradient (step I, alternative a; Fig. 1a).* When the concentration is performed by means of an anolyte of low pH the number of protons,

N_{H^+}, entering the capillary per time unit from the anode vessel is of interest, since it determines the position and the progress of the pH gradient in the capillary. This number is governed by the expression

$$N_{H^+} = v_{H^+}\, q\, n_{H^+} \quad (7)$$

where v_{H^+} = the migration velocity of the protons in the anolyte, q = the cross-sectional area of the capillary and n_{H^+} = the number of protons per volume unit in the anolyte.

Since $v_{H^+} = F\, u_{H^+} = I\, u_{H^+}/q\, \kappa$ (F = the field strength in the capillary, u_{H^+} = the mobility of the proton in the anolyte, I = the current and κ = the electrical conductivity)

$$N_{H^+} = \frac{I\, u_{H^+}\, n_{H^+}}{\kappa} \quad (8)$$

The conductivity κ is determined by the expression

$$\kappa = \sum c_i \cdot u_i \quad (9)$$

where c_i is the concentration of ion i (in coulomb/ml). For the 0.5 *M* Tris–HCl solution of pH 2.5 (the stop solution) used in the experiment outlined in Fig. 2a we get

$$\kappa = c_{H^+} \cdot u_{H^+} + c_{Tris^+} \cdot u_{Tris^+} + c_{OH^-} \cdot u_{OH^-} + c_{Cl^-} \cdot u_{Cl^-} \quad (10)$$

To obtain an efficient concentration (isoelectric focusing) the pH of the stop solution should be at least one pH unit below the p*I* value of the solute in the sample that has the lowest pI value [c_{H^+}, the first term in Eq. 10, cannot, accordingly, be chosen arbitrarily]. The third term in this equation can be neglected, since c_{OH^-} is very small at low pH values. The remaining second and fourth terms in Eq. 10 can, accordingly, be used to manipulate the conductivity, *i.e.*, the Tris–HCl solution (pH 2.5) in Fig. 2a should be adjusted to a molarity that gives the solution a proper conductivity. When the molarity decreases the number of protons entering the capillary increases, according to Eqs. 8 and 10. At excessively low molarity the pH gradient in the capillary will, therefore, migrate toward the cathode during the concentration, and at very high molarity little or no change in pH will occur in the capillary (*i.e.*, the solutes migrate out of the capillary). Experiments have shown that the molarity of the stop solution should be such that a virtually stationary pH gradient is created at a distance of 0.5–3 mm from the end of the capillary. The suitable molarity can be established experimentally by the use of coloured solutes and transparent glass capillaries.

(2) *Mobilization by a reversed, non-buffering pH gradient (step II, alternative b; Fig. 2a, step II).* Following the concentration of solutes by isoelectric focusing in a steep pH gradient or by zone electrophoresis toward a small-pore gel one can mobilize the solute zone by a solution of high pH, for instance, 0.1 *M* NaOH (Fig. 2a, step II). This solution should contain no other negatively charged ions than hydroxyl ions. Sodium, potassium or ammonium hydroxides are, therefore, recommended. The only ions entering the capillary are then hydroxyl ions, which have a very high mobility and, accordingly, change the pH very rapidly at the cathodic end of the capillary. This is important when the concentration is achieved by isoelectric focusing in a pH gradient, since the focused solute zone will broaden upon reversal of the polarity of the electrodes until the initial pH gradient is abolished (a pH gradient gives a zone sharpening only when the pH increases in the direction of the current). When a sufficiently large number of hydroxyl ions have entered the capillary a reversed pH gradient has been created which gives rise to a zone-sharpening. This pH gradient migrates toward the anode with the proteins gathered in a very narrow zone (about 0.2 mm wide); see Fig. 2a, step II. At this stage, which takes only 1–2 min to attain, the mobilizing high-pH solution should be replaced by the electrophoresis buffer (the same buffer as in the anode vessel and in the capillary). The hydroxyl ions continue to migrate as a plug toward the anode but their concentration decreases as they meet and react with the buffering cations in the capillary as indicated by the broken pH profiles in Fig. 2a, step III (Tris ions, for example, which are fed continuously into the capillary from the anode vessel by electrophoresis). If it is essential to minimize the analysis time one should fill the cathode vessel with the electrophoresis buffer

somewhat before the complete zone-sharpening (stacking) outlined in Fig. 2a, step II, has been achieved, because the large number of hydroxyl ions in the capillary at its cathodic end continue to make the pH gradient still narrower at the same time as the buffer anions (chloride) migrate into the capillary.

Eq. 11, which corresponds to Eq. 8, gives the number of hydroxyl ions, N_{OH^-}, that enter the capillary per time unit during the mobilization (step II in Fig. 2a).

$$N_{OH^-} = \frac{I\, u_{OH^-}\, n_{OH^-}}{\kappa} \quad (11)$$

Using the same reasoning as for Eq. 8 one can show the importance of choosing an appropriate value of κ, the conductivity, to cause a rapid increase in the pH at the cathodic end of the capillary during the short mobilization step, thereby rapidly replacing the initial zone-broadening pH gradient by a new, reversed, zone-sharpening one. Experiments have shown that 0.1 *M* sodium hydroxide is a suitable mobilizing solution. The pH in the capillary at its cathodic section will not become 13 (that of 0.1 *M* NaOH), since the concentration of hydroxyl ions in the capillary is reduced quickly by the buffering ions in the electrophoresis buffer. Therefore, and also because the solutes start to migrate as soon as the pH rises above their p*I* values, the solutes will not become located in an area of extremely high pH and the risk of denaturation is negligible, particularly since the mobilization and thereby the possible exposure to elevated pH has a duration of only 1–2 min. Mobilization by 0.1 *M* sodium hydroxide is very simple and requires no preexperiments to establish optimum conditions for every new type of electrophoresis buffer. For mobilization at low pH we use 1 *M* HCl.

The concentration of proteins and peptides can be performed also with a pH gradient created with carrier ampholytes in the same way as in isoelectric focusing [25]. However, the high buffering capacity of these ampholytes makes it difficult to abolish the pH gradient quickly. Mobilization, therefore, leads to broadening of the narrow, concentrated zone.

(3) *Mobilization by displacement electrophoresis (step II, alternative a; Fig. 2b, step II).* The mobilizing solution discussed above should contain no anions other than hydroxyl ions. However, buffering anions must be present if mobilization is to be accomplished by a brief displacement electrophoresis step. In the experiment shown in Fig. 1b in the succeeding paper [1] the anion glycine was chosen as terminator. To be useful for displacement of the concentrated zone of sample anions (acidic peptides and proteins) the terminator (in that experiment glycine, p*K* 9.8) should —besides having a mobility lower than that of the leading ion (chloride) and those of the solutes— have a p*K* value 1.0–1.7 units higher than the p*K* value of the leading buffer cation (in that experiment Tris, p*K* = 8.1) and the pH value of the leading buffer (in that experiment Tris–HCl, pH 8.5) should be close to the p*K* value of its cation (Tris) to give optimum buffer capacity. If these requirements are fulfilled a short plug of terminating ions (glycine) will quickly enter the capillary during the brief mobilization step (Fig. 2b, step II). These ions will be rapidly titrated to the pH of the electrophoresis buffer, since upon their migration toward the anode they continuously meet new protons and new buffer cations (Tris) migrating in the opposite direction. The terminating ion (glycine) will thus quickly acquire a low net charge and will, therefore, migrate more slowly than all or most of the sample zones, *i.e.*, the requirements for displacement electrophoresis are fulfilled. A very narrow sample zone is, accordingly, created. The terminating buffer (glycine–NaOH) can then be exchanged for the electrophoresis buffer (Tris–HCl), the anion of which (Cl^-) should have a high mobility to overrun very quickly the sharp protein zones and destack them. The transition from displacement to zone electrophoresis is, accordingly, very rapid. In the experiment shown in Fig. 2b in ref. 1 diaminopimelic acid was used as terminator instead of glycine.

For the concentration of basic ampholytes an

appropriate buffer system has been described in ref. 1 in section 3.1. The theoretical considerations are analogous to those presented above for acidic peptides and proteins. For instance, the different buffers should be designed such that the terminator (for instance Bis–Tris, $pK = 6.7$), introduced as a short plug in the displacement step, rapidly loses most of its positive charge as it meets the leading buffer (= the zone-electrophoresis buffer = 0.05 *M* EPPS-NaOH, pH 7.5, in the above buffer system) in the final analytical zone electrophoresis step.

(4) *Estimation of conductivity (ionic strength) and pH of minute-volume samples.* Some of the described concentration methods work only within limited conductivity and pH ranges. Direct measurements of these parameters by means of conductivity and pH probes is not possible owing to the small sample volumes. An indirect estimation can, however, be done as follows. The conductivity (κ) can be determined by filling a capillary with the sample solution and measuring the current I and voltage V as in a conventional HPCE experiment [11]:

$$\kappa = \frac{I L'}{V \pi R^2} \quad (12)$$

where L' is the length of the capillary and R its radius.

For the determination of pH one can utilize (1) the equilibrium between two easily detectable ions (for instance two species of vanadate ions) the concentrations of which are pH dependent [33], or (2) the fact that the spectra of pH indicators change with pH [34]. With such ionic compounds as analytes one can rapidly determine the pH of the sample solution. The conductivity and pH may be estimated conveniently in the same run.

Acknowledgements

The work was financially supported by the Swedish Natural Science Research Council and the Knut and Alice Wallenberg and Carl Trygger Foundations.

References

[1] J.-L. Liao, R. Zhang and S. Hjertén, *J. Chromatogr. A*, 676 (1994) 421.
[2] T.W.G. Rowe, *Ann. N.Y. Acad Sci.*, 85 (1960) 641–681.
[3] H. Laurell, *Studies on Preparative Vertical Zone Electrophoresis (Dissertation)*, Almqvist and Wiksell, Uppsala, 1958.
[4] B. v. Hofsten and S.-O. Falkbring, *Anal. Biochem.*, 1 (1960) 436 .
[5] E.C.J. Norrby and P.Å. Albertsson, *Nature*, 188 (1960) 1047 .
[6] P.Flodin, B. Gelotte and J. Porath, *Nature*, 188 (1960) 493 .
[7] J.-L. Liao and S. Hjertén, *Concentration of Biological Samples on a Microliter Scale and Analysis by Capillary Electrophoresis*, US Patent Application, filed January 26, 1993.
[8] S. Hjertén, J.-L. Liao, A. Palm, L. Valtcheva and R. Zhang, *5th Int. Symp. on High-Performance Capillary Electrophoresis, Orlando, FL, January 25–28, 1993*, Abstracts, p. 56.
[9] S. Hjertén, L. Valtcheva and Y.-M. Li, *J. Cap. Electroph.*, submitted for publication.
[10] H. Haglund and A. Tiselius, *Acta Chem. Scand.*, 4 (1950) 957–962.
[11] S. Hjertén, *Electrophoresis*, 11 (1990) 665–690.
[12] L. Ornstein, *Ann. N.Y. Acad. Sci.*, 121 (1964) 321–349.
[13] S. Hjertén, S. Jerstedt and A. Tiselius, *Anal. Biochem.*, 11 (1965) 219–233.
[14] S. Hjertén, K. Elenbring, K. Kilár, J.-L. Liao, A. Chen, C. Siebert and M.-D. Zhu, *J. Chromatogr.*, 403 (1987) 471.
[15] C. Schwer and F. Lottspeich, *J. Chromatogr.*, 623 (1992) 345.
[16] F. Foret, E. Szoko and B.L. Karger, *J. Chromatogr.*, 608 (1992) 3.
[17] D. Kaniansky, J. Marák, V. Madajková and E. Simunicová, *J. Chromatogr.*, 638 (1993) 137.
[18] T. Hirokawa, A. Ohmori and Y. Kiso, *J. Chromatogr.*, 634 (1993) 101.
[19] J.L. Beckers and M.T. Ackermans, *J. Chromatogr.*, 629 (1993) 371.
[20] F. Foret, E. Szökö and B.L. Karger, *Electrophoresis*, 14 (1993) 417.
[21] S. Hjertén, *Arkiv för Kemi*, Band 13, Nr 16 (1958) 151–152.
[22] S. Hjertén, *Prot. Biol. Fluids (Proceedings of the 7th Colloquium, Bruges, 1959)*, Elsevier, Amsterdam, 1960, pp. 28–30.
[23] B.P. Konstantinov and O.V. Oshurkova, *Zh. Tekhn. Fiz.*, 36 (1966) 942 .
[24] W. Preetz, *Talanta*, 13 (1966) 1649 .
[25] S. Hjertén, *Chromatogr. Rev.*, 9 (1967) 122–219.
[26] F.M. Everaerts, *J. Chromatogr.*, 49 (1970) 262 .

[27] N.J. Reinhoud, U.R. Tjaden and J. van der Greef, *J. Chromatogr.*, 641 (1993) 155–162.
[28] A. Hori, T. Matsumoto, Y. Nimura, M. Ikedo, H. Okada and T. Tsuda, *Anal. Chem.*, 65 (1993) 2882–2886.
[29] S. Hjertén, *Biochim. Biophys. Acta*, 32 (1959) 531–534.
[30] C.J.O.R. Morris and P. Morris (Editors), *Separation Methods in Biochemistry*, Pitman and Sons Ltd., London, 1963, p. 748.
[31] S. Hjertén, *Biochim. Biophys. Acta*, 237 (1971) 395–403.
[32] S. Hjertén, Z.-Q. Liu and S.-L. Zhao, *J. Biochem. Biophys. Meth.*, 7 (1983) 101–113.
[33] T. Groh and K. Bächmann, *J. Chromatogr.*, 646 (1993) 405–410.
[34] S. Hjertén and K. Elenbring, in preparation.