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# THEORETICAL AND EXPERIMENTAL STUDY OF HIGH-PERFORMANCE ELECTROPHORETIC MOBILIZATION OF ISOELECTRICALLY FOCUSED PROTEIN ZONES

STELLAN HJERTÉN*, JIA-LI LIAO* and KUNQUAN YAO**
*Institute of Biochemistry, University of Uppsala, Biomedical Center, P.O. Box 576, S-751 23 Uppsala (Sweden)*
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## SUMMARY

In an earlier paper we showed that it is possible to mobilize a train of isoelectrically focused proteins and thus detect them on-tube or off-tube. The mobilization was performed in different ways, for instance electrophoretically by exchanging the anolyte for the catholyte or *vice versa*. In this paper we treat the electrophoretic mobilization theoretically, originating from the conditions of electroneutrality. The information thus gained was used to design anolytes and catholytes of appropriate compositions for mobilization of focused proteins. The usefulness of these electrode solutions is illustrated by focusing-mobilization experiments performed in free solution in a glass tube of length 110 mm. Since the inside diameter of the tube and its wall thickness were only 0.05 mm, the Joule heat was efficiently removed, which allowed the use of high field strengths (270 V/cm). The focusing time was therefore as short as 6 min. The time required for mobilization was about 15 min (360 V/cm). The mobilized protein zones were detected on-tube by absorbance measurements at 280 nm. The glass tube was treated with non-cross-linked polyacrylamide to eliminate electroendosmosis and adsorption of proteins onto the tube wall. The following conclusions drawn from the theoretical studies were experimentally verified: mobilization toward the anode (cathode) can be accomplished by selecting an anolyte (catholyte) containing a cation (anion) other than the proton (hydroxyl ion); the cation (anion) will then electrophoretically migrate into the separation tube and continuously increase (decrease) the pH from the anodic (cathodic) end of the tube. The pH of the electrode solution toward which the mobilization takes place is critical for off-tube, but not for on-tube detection. With the aid of the electroneutrality condition that applies in isoelectric focusing, one can easily explain the generation of the so-called plateau phenomenon.

* Permanent address: Biology Department, Jinan University, Guangzhou, China.
** Permanent address: Department of Pharmacology, Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing, China.

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## INTRODUCTION

In high-performance electrophoresis the solute zones are detected either "on-tube" as they pass a stationary UV detector or "off-tube" when they have left the separation chamber and have been transferred to the flow cuvette of a conventional high-performance liquid chromatography (HPLC) monitor. Accordingly, the method does not permit recording of the stationary protein zones in isoelectric focusing experiments. To adapt it to such experiments, we have recently developed techniques to mobilize the focused protein zones[1]. In this paper we describe some alternative mobilization methods and their theoretical basis.

## EXPERIMENTAL AND RESULTS

### *Materials and equipment*

Myoglobin from horse heart and alcohol dehydrogenase from baker's yeast were obtained from Sigma (St. Louis, MO, U.S.A.) and chymotrypsinogen A and the carrier ampholytes Pharmalyte™ from Pharmacia Fine Chemicals (Uppsala, Sweden). Enolase from baker's yeast was a gift from Dr. Göran Pettersson of this institute, and human transferrin from Dr. Lars-Olov Andersson, KABI/Vitrum, Stockholm, Sweden. Human haemoglobin was prepared from outdated blood. Reagents of electrophoresis purity, namely acrylamide, ammonium persulphate and N, N, N′, N′-tetramethylethylenediamine (TEMED), and the carrier ampholytes Bio-Lyte™ were obtained from Bio-Rad (Richmond, CA, U.S.A.). Silane-174 (γ-methacryloylpropyltrimethoxysilane) was obtained from LKB-Products (Bromma, Sweden).

The separation tube had a length of 110 mm, inside diameter of 0.05 mm and a wall thickness of 0.05 mm. The on-tube detector was a modified spectrophotometer (PM QII; Carl Zeiss, Oberkochen, F.R.G.). The electrode (403-30-M8) for measurement of the pH in a gel slab was from Ingold AG (Urdorf, Switzerland).

### *Mobilization of proteins by addition of salt to the anolyte*

The separation tube was coated with non-cross-linked polyacrylamide as described recently[2], in order to eliminate electroendosmosis and adsorption of proteins onto the tube wall. The tube was filled with a 2.5% solution of Pharmalyte, pH 3–10, containing human transferrin (5 μg/μl) and human haemoglobin (5 μg/μl). 0.02 *M* phosphoric acid served as the anolyte and 0.02 *M* sodium hydroxide as the catholyte. The focusing was performed at 3000 V for 6 min; during this time the current decreased from 15 to 1 μA. The mobilization of the focused protein zones was done at 4000 V for about 15 min following replacement of the 0.02 *M* phosphoric acid in the anode vessel by 0.02 *M* phosphoric acid containing 0.08 *M* sodium chloride. During the mobilization, the current rose from 1 to 200 μA. The migrating zones were monitored at 280 nm as they passed a stationary UV detector (a modified Zeiss spectrophotometer). The separation pattern is shown in Fig. 1, diagram B.

### *Mobilization of proteins by other compositions of anolyte or/and catholyte*

The experiment shown in Fig. 1, diagram B, was then repeated with the difference that the mobilization of the focused transferrin and haemoglobin was per-

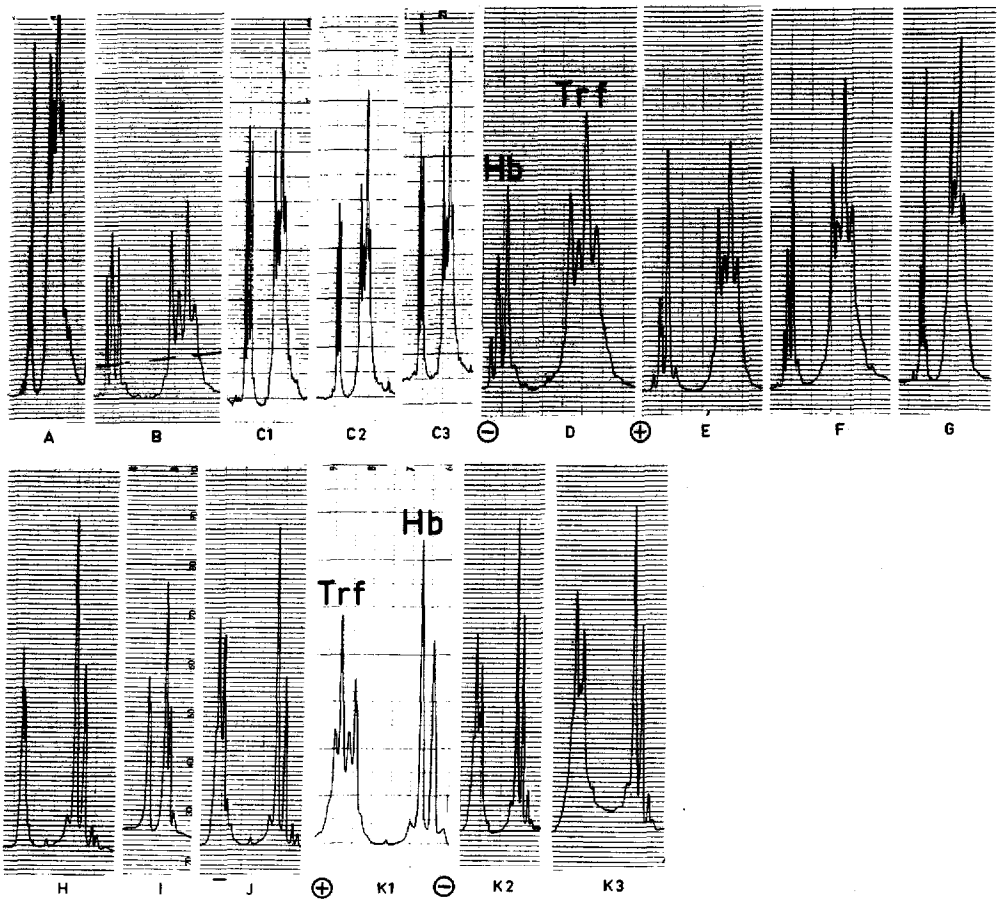


Fig. 1. Mobilization of focused proteins with the aid of some different anolyte and catholyte solutions. The sample consisted of human haemoglobin (Hb) and transferrin (Trf). The glass electrophoresis tube had dimensions 0.05 mm (I.D.) × 0.15 mm (O.D.) × 110 mm. In the focusing step the catholyte was 0.02 *M* sodium hydroxide and the anolyte of 0.02 *M* phosphoric acid. The protein zones were detected on-tube by a stationary UV detector. The letters A–K refer to the compositions of the anolyte and catholyte during the mobilization step (see Table I). The same sample was employed in all experiments except in those shown in B, C1, C2 and C3, where another batch of haemoglobin was used. These diagrams should therefore not be compared with the others as far as the haemoglobin pattern is concerned.

formed with other compositions and pH values of the anolyte or/and catholyte (Fig. 1, diagrams A, C1–K3).

*The change in the pH gradient during the mobilization*

The experiment corresponding to Fig. 1, diagram B was repeated in the free zone electrophoresis apparatus[3]; see Fig. 2. When the focused protein zones had migrated about 10 cm, 1-cm fractions were withdrawn from the rotating quartz tube and the pH was determined in each fraction. Fig. 2c gives the time course of the pH gradient (and the scanning pattern) at one particular stage in the mobilization, namely when transferrin was about to leave the range for scanning of the quartz tube

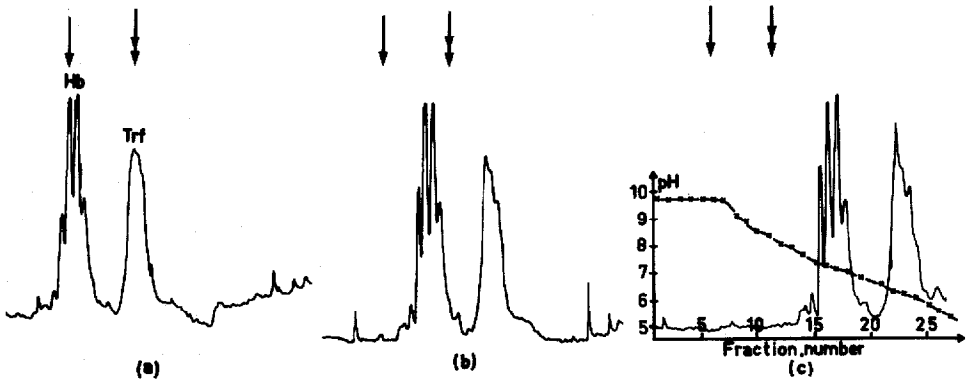


Fig. 2. Isoelectric focusing (a) of haemoglobin (Hb) and transferrin (Trf) in the free zone electrophoresis equipment[3] and subsequent mobilization (b,c) of the focused proteins by supplementing the anolyte with sodium chloride. Dimensions of the revolving quartz tube: 3 mm (I.D.) × 8 mm (O.D.) × 400 mm. In the focusing step the anolyte consisted of 0.02 *M* phosphoric acid and catholyte of 0.02 *M* sodium hydroxide. Carrier ampholyte: 1% Pharmalyte, pH 3–10. Voltage during the focusing: 500 V. Mobilization of the focused proteins was achieved by replacing the 0.02 *M* phosphoric acid with 0.02 *M* phosphoric acid containing 0.1 *M* sodium chloride. The mobilization was performed at a low voltage (500 V) to utilize the night for mobilization of the proteins. The scanning was carried out after the steady state had been attained (a) and during the mobilization (b and c). The arrows indicate the steady-state positions of the proteins.

(compare Fig. 3 in ref. 1). However, to monitor the changes in the pH gradient during the entire mobilization, the following experiment was performed.

A polyacrylamide gel with a thickness of 2 mm and total concentration, $T = 6\%$ and cross-linking concentration, $C = 3\%$ was cast in a 2.5% solution of Bio-Lyte (pH 3–10) on a water-cooled microscope slide with the dimensions 1 mm × 26 mm × 76 mm (the parameters $T$ and $C$ are defined in ref. 4). Focusing of the

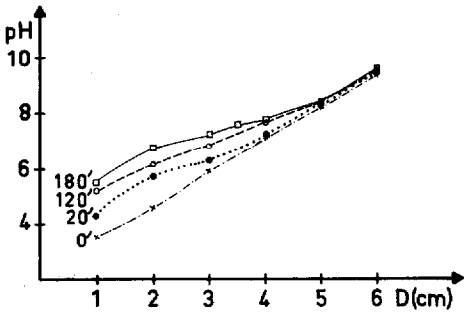


Fig. 3. The pH gradient at different mobilization times. The focusing of the carrier ampholytes was performed in a polyacrylamide gel with 0.02 *M* phosphoric acid as the anolyte and 0.02 *M* sodium hydroxide as the catholyte. The mobilization of the ampholytes was achieved by replacing the 0.02 *M* phosphoric acid by 0.02 *M* phosphoric acid containing 0.08 *M* sodium chloride (composition B in Table I; composition F gave a similar pattern). The pH at different distances, *D*, from the anodic end of the gel was measured with a surface electrode at the mobilization times indicated. The pH increase is caused by the sodium ions, since these are the only ions (except hydrogen ions) that can electrophoretically enter the gel from the anode vessel. As seen, the hydroxyl ions (which are the only ions that can migrate electrophoretically from the catholyte into the gel) are of less (or no) importance for the increase in pH. The same conclusion can be drawn from Fig. 2.

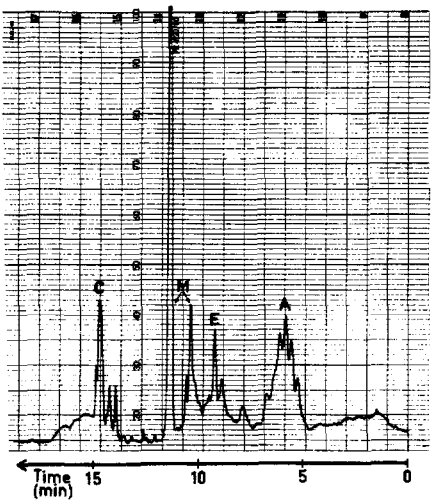


Fig. 4. Focusing-mobilization experiment with a complex protein mixture. The sample consisted of alcohol dehydrogenase (A), enolase (E), myoglobin (M) and chymotrypsinogen A (C). For experimental conditions, see Fig. 1. For mobilization of the focused proteins the anolyte composition A in Table I was used.

TABLE I

EXAMPLES OF THE ANOLYTE AND CATHOLYTE COMPOSITIONS IN THE MOBILIZATION STEP

In the focusing step the catholyte was 0.02 *M* sodium hydroxide and the anolyte 0.02 *M* phosphoric acid.

| | *Anolyte* | *Catholyte* | *Migration direction* |
|---|---|---|---|
| A | 0.02 *M* NaOH | 0.02 *M* NaOH | Anodic |
| B | 0.02 *M* $H_3PO_4$ + 0.08 *M* NaCl | 0.02 *M* NaOH | Anodic |
| C1 | 0.02 *M* Sodium phosphate, pH 3.6 | 0.02 *M* NaOH | Anodic |
| C2 | 0.02 *M* Sodium phosphate, pH 6.8 | 0.02 *M* NaOH | Anodic |
| C3 | 0.02 *M* Sodium phosphate, pH 11.5 | 0.02 *M* NaOH | Anodic |
| D | 0.02 *M* Ethanolamine titrated to pH 7 with $H_3PO_4$ | 0.02 *M* NaOH | Anodic |
| E | 0.02 *M* Ethanolamine, pH 11 | 0.02 *M* NaOH | Anodic |
| F | 0.02 *M* Sodium phosphate, pH 6.8 | 0.02 *M* Glycine titrated to pH 9.0 with NaOH | Anodic |
| G | 0.02 *M* NaCl | 0.02 *M* Glycine titrated to pH 9.0 with NaOH | Anodic |
| H | 0.02 *M* $H_3PO_4$ | 0.02 *M* $H_3PO_4$ | Cathodic |
| I | 0.02 *M* $H_3PO_4$ | 0.1 *M* NaCl | Cathodic |
| J | 0.02 *M* $H_3PO_4$ | 0.02 *M* Sodium phosphate. pH 6.8 | Cathodic |
| K1 | 0.02 *M* $H_3PO_4$ | 0.02 *M* NaOH + 0.02 *M* NaCl | Cathodic |
| K2 | 0.02 *M* $H_3PO_4$ | 0.02 *M* NaOH + 0.04 *M* NaCl | Cathodic |
| K3 | 0.02 *M* $H_3PO_4$ | 0.02 *M* NaOH + 0.08 *M* NaCl | Cathodic |

carrier ampholytes was performed at 500 V for 45 min with 0.02 *M* phosphoric acid as the anolyte and 0.02 *M* sodium hydroxide as the catholyte. The pH was then measured for each cm of the gel using a surface electrode. Following replacement of the 0.02 *M* phosphoric acid in the anode vessel by 0.02 *M* phosphoric acid containing 0.08 *M* sodium chloride a voltage of 500 V was again applied. The measurements of pH in the gel were repeated at certain time intervals. Fig. 3 shows the time course of the pH gradient during the mobilization step.

*Focusing-mobilization of an artificial mixture of proteins*

Fig. 4 shows a focusing-mobilization experiment where the mobilization was achieved by replacing the acid at the anode by sodium hydroxide (mobilization condition A in Table I). The sample was a mixture of alcohol dehydrogenase (A), enolase (E), myoglobin (M) and chymotrypsinogen A (C). Other experimental conditions were similar to those described above for haemoglobin and transferrin as model proteins (see Mobilization of proteins by addition of salt to the anolyte).

## THEORETICAL

The electroneutrality condition at steady state in the separation tube during the focusing is

$$C_{H^+} + \Sigma C_{NH_3^+} = C_{OH^-} + \Sigma C_{COO^-} \quad (1)$$

where $C_{H^+}$, $C_{OH^-}$, $C_{NH_3^+}$ and $C_{COO^-}$ are the concentrations in equivalents per litre (or Coulomb/cm$^3$) of protons, hydroxyl ions and positive and negative groups in the carrier ampholytes, respectively. We will now investigate how the electroneutrality condition for the focusing step should be modified in order to be applicable to the mobilization step. The discussion below refers to mobilization toward the anode.

The requirement for anodic mobilization of the ampholytes is that $\Sigma C_{NH_3^+} < \Sigma C_{COO^-}$ for any pH, *i.e.*, that they acquire a negative net charge. This can be accomplished only if the pH increases, which is equivalent to a decrease in $C_{H^+}$; at the same time $C_{OH^-}$ will increase since $C_{H^+} C_{OH^-}$ = constant. Under these conditions, the left side of eqn. 1 —which obtains only for the focusing step— will have a smaller value than the right side. Equality, and thereby mobilization, can, however, be achieved by adding a positive term to the left side of eqn. 1, which then takes the form

$$C_{X^{n+}} + C_{H^+} + \Sigma C_{NH_3^+} = C_{OH^-} + \Sigma C_{COO^-} \quad (2)$$

where $X^{n+}$ ($n$ is the valency) represents a cation. This equation illustrates one way to accomplish anodic mobilization, namely by supplementing the anolyte used for focusing with a cation which by electrophoresis can enter the tube. The experiments described above indicate that this is an efficient approach (see Fig. 1, diagrams A–G).

The analogous expression for cathodic mobilization is

$$C_{H^+} + \Sigma C_{NH_3^+} = C_{OH^-} + \Sigma C_{COO^-} + C_{Y^{m-}} \quad (3)$$

where $Y^{m-}$ is an anion. Examples of the utilization of this equation for mobilization are presented in experiments H–K3 in Fig. 1.

The condition for electroneutrality in the cathodic section of the separation tube, before the arrival of cation $X^{n+}$, is

$$C'_{H^+} + \Sigma C'_{NH_3^+} = C'_{OH^-} + \Sigma C'_{COO^-} \quad (4)$$

*i.e.* formally the same condition as for the focusing step (eqn. 1). Eqn. 4 differs principally from eqn. 2 inasmuch as it lacks a term corresponding to $C_{X^{n+}}$, which affects the value of the term $C_{H^+}$. One can therefore expect a slower change in pH at the cathodic end of the separation tube than at the anodic end when mobilization takes place toward the anode, *i.e.* the cations in the anode vessel are much more important for the mobilization of focused proteins than are the hydroxyl ions in the cathode vessel. This inference has been verified experimentally (see Figs. 2 and 3 herein, and Fig. 3 in ref. 1).

For cathodic mobilization an equation identical to eqn. 4 is valid for the anodic section of the separation tube, and analogous conclusions can be drawn.

The above considerations regarding the electroneutrality conditions indicate that the cations (anions) entering the separation tube will cause a pH change, but they do not reveal the course of events. Such information can, however, be obtained from the following theoretical treatment of how the composition of the anolyte affects the flux of protons into the separation tube.

Consider the boundary between the anolyte and the medium in the separation tube at the steady state in the focusing step. The number of protons, $N_{H^+}$, from the anolyte passing electrophoretically the boundary per time unit can be expressed by

$$N_{H^+} = v_{H^+} q n_{H^+} \quad (5)$$

where $v_{H^+}$ = the migration velocity of the protons in the anolyte, $q$ = cross-sectional area of the tube and $n_{H^+}$ = the number of protons per volume unit in the anolyte. Since $v_{H^+} = E u_{H^+}$ and $E = I/q\kappa$ (where $E$ = the field strength, $u_{H^+}$ = the mobility of the proton in the anolyte, $I$ = the current and $\kappa$ = the conductivity in the anolyte), eqn. 5 takes the form:

$$N_{H^+} = I u_{H^+} n_{H^+} / \kappa \quad (6)$$

For the mobilization step we get a similar expression

$$N'_{H^+} = I' u'_{H^+} n'_{H^+} / \kappa' \quad (7)$$

where $\kappa'$ is the conductivity in the anolyte used for the mobilization, $n'_{H^+}$ the number of protons in the same anolyte and $I'$ the current in the tube (primed parameters refer to the mobilization step and non-primed ones to the focusing step). The current I' has —in the initial phase of the mobilization— about the same value as $I$ in the focusing step, since at this stage of the mobilization the focused ampholytes, which have a high electrical resistance, take up almost the whole tube. Accordingly, the mobilized ampholytes (which have a much lower resistance than the focused am-

pholytes) and the sodium chloride added to the anolyte give rise to very little change in current during the first minutes after the anolyte composition has been altered in order to induce mobilization. Therefore, and also because $u_{H^+} \approx u'_{H^+}$, a good approximation is:

$$\frac{N_{H^+}}{N'_{H^+}} = \frac{\kappa'}{\kappa} \cdot \frac{n_{H^+}}{n'_{H^+}} \tag{8}$$

Let us first consider the case where 0.02 *M* phosphoric acid is used as the anolyte in the focusing step and 0.02 *M* phosphoric acid containing 0.08 *M* sodium chloride in the mobilization step (experiment B in Fig. 1). Eqn. 8 can then be simplified to:

$$\frac{N_{H^+}}{N'_{H^+}} = \frac{\kappa'}{\kappa} \tag{9}$$

Since in this case $\kappa \ll \kappa'$, $N'_{H^+} \ll N_{H^+}$. One can therefore state that due to the increase in conductivity achieved by supplementing the phosphoric acid with salt, the number of protons entering the tube from the anolyte decreases, which gives rise to a pH increase in the tube. Eqn. 8 is also applicable to the case where the 0.02 *M* phosphoric acid is replaced by 0.02 *M* sodium hydroxide (experiment A in Fig. 1). Even if the conductivity in the anolyte does not change as much as in the previous case, the ratio between the number of protons in 0.02 *M* phosphoric acid and 0.02 *M* sodium hydroxide, $(n_{H^+}/n'_{H^+}) \gg 1$. Accordingly $N_{H^+}/N'_{H^+} \gg 1$, *i.e.*, an increase in pH in the tube is obtained also in this case.

An analogous treatment can be performed for the other anolyte compositions shown in Table I. As stated in Theoretical, a decrease in proton concentration in the separation tube without utilization of a cation ($X^{n+}$ in eqn. 2) is not sufficient for mobilization. The flux of these cations, $N'_{X^{n+}}$, from the anolyte into the separation tube is governed by the relationship:

$$N'_{X^{n+}} = \Gamma u'_{X^{n+}} n'_{X^{n+}} / \kappa' \tag{10}$$

Combination of eqns. 7 and 10 gives:

$$\frac{N'_{X^{n+}}}{N'_{H^+}} = \frac{u'_{X^{n+}}}{u'_{H^+}} \cdot \frac{n'_{X^{n+}}}{n'_{H^+}} \tag{11}$$

Application of this equation to experiment B in Fig. 1 (Table I) and insertion of the mobilities of the protons and sodium ions gives:

$$\frac{N'_{X^{n+}}}{N'_{H^+}} = 0.14 \cdot \frac{n'_{X^{n+}}}{n'_{H^+}} \tag{12}$$

Since 0.02 *M* phosphoric acid containing 0.08 *M* sodium chloride has a pH of about 2, the ratio between the number of sodium ions and the number of protons entering the tube is about 1 (when the anolyte has a higher pH, see Fig. 1, diagrams Cl–G,

this ratio is considerably larger). However, in all the mobilization experiments these sodium ions are of decisive importance for the mobilization as pointed out in Theoretical. They are of much greater importance than the anolyte pH, in agreement with the experimental finding that mobilization can be achieved equally well with 0.02 *M* sodium hydroxide (composition A in Table I, and Fig. 1, A) and 0.02 *M* phosphoric acid containing 0.08 *M* sodium chloride (composition B).

Since $X^{n+}$ in eqn. 2 can be any cation (except the proton) this equation is applicable to all of the anolytes (A–G) in Table I. It is therefore unimportant whether the sodium ion comes from sodium hydroxide (anolyte A), sodium chloride (anolytes B and G), or sodium phosphate (anolytes C1–C3, F) or whether the sodium ion (anolyte G) is replaced by ethanolamine (anolytes D and E).

An increase in the concentration of sodium chloride in the anolyte should be accompanied by an increase in the concentration of sodium ions in the separation tube and cause an increase in pH according to eqn. 2, *i.e.*, a faster mobilization of the pH gradient, provided that the voltage gradient is kept constant. This was experimentally verified (not shown herein). In an analogous way one can make the cathodic mobilization faster by increasing the concentration of sodium chloride in the catholyte (Fig. 1, diagrams K1–K3). However, to avoid thermal zone deformation caused by the Joule heat, we seldom use a concentration of sodium chloride higher than 0.1 *M* —and not lower than 0.02 *M* to avoid long mobilization times.

## DISCUSSION

The above theoretical considerations and also Figs. 2 and 3 indicate that the pH changes in the cathodic section of the tube are relatively small during the mobilization. It may accordingly be difficult to mobilize proteins with p*I* values in the range 9–10 toward the anode. For focusing experiments with such basic proteins we therefore supplement the commercial carrier ampholytes with TEMED, which extends the pH gradient to pH 12 as reported by Guo and Bishop[5]; another possibility is to mobilize the proteins towards the cathode with the aid of the cathode solutions H–K3 in Table I.

In most focusing experiments the mobilization toward the anode has been achieved by changing the composition of the anolyte only. However, the composition of both anolyte and catholyte can be changed, provided that the pH of the catholyte is above the p*I* of the proteins to be mobilized (examples are found in Fig. 1, diagrams F and G).

From the above discussions, supported by the diagrams A–G in Fig. 1, one can summarize (1) that mobilization of focused proteins toward the anode can be achieved easily by means of cations other than protons (for instance sodium or ethanolamine) in the anolyte and (2) that any pH of the anolyte can be chosen. By analogous reasoning, mobilization toward the cathode can be accomplished by means of a catholyte of any pH containing an anion other than the hydroxyl ion (for instance chloride or phosphate). This is illustrated in Fig. 1, diagrams H–K3.

When the mobilization is based on the addition of salt (for instance sodium chloride) to the anolyte (catholyte), the proteins will stop migrating when they come within a few mm of the anodic (cathodic) end of the separation tube, since the pH there will be close to that in the anolyte (catholyte), *i.e.*, below (above) the p*I* of the proteins. For recovery of all the focused proteins by the migration elution technique[6],

the acidic anolyte (basic catholyte) must therefore be replaced by a sodium chloride containing anolyte (catholyte) of a pH higher (lower) than the p*I* of the most basic (acidic) protein in the sample.

It is tempting to try to use displacement electrophoresis (isotachophoresis) to mobilize the train of focused proteins. The experiment shown in Fig. 1, diagram F, should be viewed with this in mind. Phosphate may serve as a leading ion and form a moving boundary with the most rapidly migrating ampholyte or protein, whereas glycinate may be a terminating ion, displacing the most slowly migrating ampholyte or protein. However, mobilization can be achieved only by changing the pH in the separation tube such that the proteins acquire a negative net charge, which the leading and terminating ions cannot accomplish (since phosphate ions are negatively charged they cannot electrophoretically enter the tube and glycinate ions must migrate slower than the proteins if they are to function as a terminator). The primary increase in pH in the tube must therefore be achieved by the sodium ions in the anolyte (compare the experiment shown in Fig. 1, diagram B). Besides changing the pH in the tube, the sodium ions may serve as counter ions for the carrier ampholytes and the proteins in a displacement electrophoresis. When the pH in the tube increases the ampholytes and the proteins will begin to move and it is therefore difficult to decide whether, or to what extent, the subsequent migration is caused by displacement electrophoresis or ordinary zone electrophoresis. However, it is obvious that if displacement effects are involved in the mobilization procedure they are not of steady-state character, since all the ampholyte species do not move with the same velocity, which is evident from Fig. 3 (a similar pattern was obtained when the mobilization was achieved by the sodium phosphate–sodium glycinate system, *i.e.*, electrolyte composition F in Table I).

It should be stressed that electrophoretic mobilization is not limited to focusing experiments in free solution. The technique is also applicable to (preparative) experiments in supporting media, for instance polyacrylamide gels (see Fig. 3 herein and Fig. 4 in ref. 1), including those with immobilized pH gradients (not shown).

The experiments presented in Figs. 4 and 1 show that isoelectric focusing in the high-performance electrophoresis apparatus in combination with the mobilization technique described herein is a rapid, highly resolving method for the analysis of proteins. An obvious disadvantage with all methods based on isoelectric focusing is, however, that many proteins precipitate at their isoelectric points. Peaks corresponding to these precipitates can easily be distinguished from peaks corresponding to proteins in solution, since they are recorded as extremely narrow peaks (or as lines) when the focusing is performed in a carrier-free medium and the on-tube detection method is used. A characteristic of peaks originating from protein precipitates is also that they change both in size and position when the focusing experiment is repeated (in gels, precipitates clog the pores of the gel and thereby cause the well known streaking phenomena). To suppress the tendency for precipitation, we supplement the ampholytes with ethylene glycol (10–50%, v/v)[7,8] or G 3707 (0.5–2%, w/v), an efficient neutral and non-UV-absorbing detergent[9], or a mixture of both. As an alternative to G 3707 we have also used Brij 35[10].

The purpose of our mobilization studies has been to develop methods to mobilize in a single step not only one or a few of the focused proteins but all of them, without impairing the resolution obtained during the isoelectric focusing. The results

presented in this paper and in ref. 1 indicate that these attempts have been successful.

McCormick *et al.*[11,12] have shown that it is possible to elute selectively one of the protein bands from a polyacrylamide gel following isoelectric focusing by "selection of anolyte and catholyte with pH values as closely adjacent as required by the p*I*s of the proteins to be separated". Hjelmeland and Chrambach[13] have shown that isotachophoresis of weak acids and bases can create pH gradients of defined stabilities for focusing of proteins, provided that protons or hydroxyl ions are the sole counter ions. With obvious modifications, the electroneutrality conditions in eqns. 1–4 are applicable to such pH gradients as well, although in their present form they refer to the conventional synthetic carrier ampholytes. One can therefore conclude that the former, isotachophoretically generated pH gradients can be mobilized in the same way as the latter, *i.e.*, toward the anolyte (catholyte), by supplementing with cations (anions). It is interesting that a similar conclusion has been drawn by Buzás *et al.*[14] from other theoretical considerations of isotachophoretically generated pH gradients, although they express it differently: "pH gradients can be expected to be mobilized by replacing the common solvent counter ion of all constituents by any other counter ion".

These authors continue: " This rationale accounts for the mobilization of pH gradients previously observed upon replacing strongly acidic and basic anolyte and catholyte by a buffer constituent". In view of the above discussion of eqns. 1–4 and the experiments presented herein, we can now be more specific regarding the term "buffer constituent". For instance, when 0.01 *M* sodium cacodylate (pH 6.2) or 0.01 *M* sodium glycinate (pH 10.5) is used as anolyte to achieve mobilization of the pH gradient (as in ref. 11 for selective elution of one protein), in both cases it is the sodium ions that cause the mobilization, not the cacodylate or glycinate ions. The pH of the anolytes mentioned is not critical for the mobilization of the pH gradients, but must, of course, be above the p*I* of the protein if it is to be eluted from the separation tube.

At pH 7 the concentration of protons is equal to the concentration of hydroxyl ions, and $\Sigma C_{NH_3^+} = \Sigma C_{COO^-}$ according to eqn. 1. The same equation shows that at pH > 7, *i.e.*, $C_{H^+} < C_{OH^-}$, $\Sigma C_{NH_3^+} > \Sigma C_{COO^-}$. Ampholytes located in the region of the tube where the pH is above 7 are consequently positively charged and will therefore move toward the cathode. Analogously, one can conclude that ampholytes positioned in the region of the tube where the pH is below 7 will move toward the anode. We can thus, in a simple way, explain the so-called plateau phenomenon[15]. An important corollary is that not even theoretically can one expect the pH gradient to be stable (experimentally this has been shown by many investigators). When a sufficiently large cathodic flow is superimposed upon the plateau phenomenon (for instance by electroendosmosis) only a "cathodic drift" is observed[16].

For further applications of the focusing-mobilization method, see ref. 17.

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