Electrically controlled electrofocusing of ampholytes between two zones of modified electrolyte with two different values of pH

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ABSTRACT

A method is described for the separation and concentration of ampholytes from their mixtures with other ionic species. In a quadrupole electromigration column, two zones of different pHs are created by using controlled flows of the solvolytic ions (H^+ and OH^-) from appropriate electrode chambers at opposite sides of the column. Thus, a time-variable interface is created between two zones with a sharp change of pH. The position of the interface in the column, the direction and velocity of its movement and the difference in pH across the interface–pH gap can be adjusted by electric currents. This arrested interface is reasonably stable with time and has the following separation properties: ampholytes with pI values between the pHs of both zones are focused into a zone at interface; and other types of ampholytes and other weak or strong ions are not trapped at the interface. The basic properties of the above system are described and experiments showing the effects of the type of sample (ampholyte, weak ion), time, the concentration of primary electrolyte and the additives changing the viscosity, solubility or pI of ampholyte are given. The method proposed offers the following advantages: the ampholytes in the sample may be concentrated several hundred-fold; the focused zones have sharp boundaries (zones 0.1 mm in length were prepared) and high concentrations of the trapped species; the zone of a trapped ampholyte contains the ampholyte proper and simple ions of primary electrolyte (KCl) only; and the zones can be shifted to any selected position in the column (potentially to the location of a detector cell or a collection device).

INTRODUCTION

Separation and concentration of amphoteric substances play an important role in biochemistry. Both steps can be carried out gently in one run using an electromigration separation method such as isotachophoresis (ITP) or isoelectric focusing (IEF). A free solution variant of these techniques is especially valuable for the isolation of enzymes and peptides without losing their biological activity.

In ITP, when the steady state is reached, the substances separated according to their effective mobilities migrate through the column in the form of consecutive adjacent zones. To prevent remixing of the zones with the separated im-

purities during collection, spacers are used, spatially separating the zones from each other [1]. The selection of suitable spacers, with known mobilities and pK values, and the effective mobility of the substance of interest, complicates this method.

IEF, in contrast to ITP, has been accepted as a standard procedure for characterizing and separating ampholytes from their mixtures [2]. Substances, separated according to their pIs in the pH gradient created by a mixture of synthetic carrier ampholytes (SCAMs) are always contaminated by SCAMs, which must be removed by other separation techniques, if necessary.

The cost of SCAMs and their ability to create complexes with analytes led to attempts to create stable pH gradients without SCAMs. Such gradients were created by self-diffusion or mixing of

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buffers having different pHs [3–8]; electrolysis or electromigration separation of a mixture of buffers [9–17]; or by changing the pK value of the buffering component by controlling the temperature, dielectric constant and/or complexation [18–21].

The preparation of a pH gradient by diffusion or mixing of two buffers of different pH belongs to the oldest techniques. MacDonald and Williamson [3] created these gradients by using HCl or citric acid on one side and NaOH or sodium citrate on the other side of the separation chamber. A sample, dissolved in distilled water, was applied between them. Kolin [4] used acetate or barbital buffer with the pH adjusted with HCl, Hoch and Barr [5] used phosphate buffer. The advantages of this arrangement are the very short time of analysis (Kolin reported a few minutes) and simple preparation; the disadvantage is movement of the gradient under the influence of electric current. An improvement in the system stability was achieved by Martin and Hampson [6] and Rilbe [7], by maintaining constant flows of the buffer components from electrode chambers. This was achieved in Rilbe's steady-state rheoelectrolysis by mixing the contents of electrode vessels. Stable systems were suggested and verified by computer simulation and experimentally by Mosher et al. [8], who pointed out that the stability of the system decreases with increasing pH differences on both sides of the column.

pH gradients created by electrolysis are used on the evolution of solvolytic ions by the decomposition of electrolyte on the electrodes. Solvolytic ions penetrate into the column and thus create a pH gradient. This is the principle of classical IEF, as was suggested by Svensson [9]. Williams and Waterman [10] used a fourteen-chamber apparatus, Tiselius [11] used his classical apparatus and Sova [12] developed a large-scale apparatus (tens of litres in volume) for industry. The theory describing such a system was published by Hagedorn and Kuhr [13].

pH gradients created by moving boundary electrophoresis of mixtures of strong and weak acids, bases or simple ampholytes were used by Petterson [14], Stenmann and Grasbeck [15] and Chrambach and Nguyen [16]. This method, called buffer electrofucosing, gives sufficiently stable and linear pH gradients. Buffer premixtures are available commercially. The theory, developed by Hjelmeland and Chrambach [17], enables one to find the optimum composition of the buffer mixture; a further advantage is the possibility of gradient engineering.

Luner and Kolin [18], Lundahl and Hjertén [19] and Lochmüller et al. [20] created pH gradients based on the dependence of the pK value of the buffer on temperature. A temperature gradient superposed along the column with electrolyte of a constant composition gives a rise to a pH gradient. The preparation of the gradient is very rapid and, as mentioned by Kolin, the pI of the separated substance can be determined from the temperature of the solution.

Troitski et al. [21] used the dependence of the pK value of the buffering component on the dielectric constant or on complexation with polyols. Complexes of polyols and boric acid were also used for continuous IEF fractionation.

All the methods of IEF described so far can be characterized by a situation where, in the steady state, a balance exists between the diffusion flow of an ampholyte out of a zone and electromigration flow into it. The result is a Gaussian distribution profile of concentration of the focused substance. For the separation and subsequent isolation it is convenient to create a sharp zone of the substance with constant concentration, similarly to ITP. This requirement is fulfilled in the method of Righetti et al. [22], where the sample components are separated by isoelectric membranes in a multi-compartment electrolyser. Only amphoteric species with pI values that lie between the pH values of the neighbouring membranes can be found in the respective compartment. The precision of the determined pI values was 0.001 pH unit.

In this paper, an isoelectric separation method in free solution is suggested, where the zone of a purified ampholyte is sharp, *i.e.*, its concentration profile is pulse-like, it has a constant composition and contains in addition to focused substances only easily separable low-molecular-mass ions.

THEORETICAL

It is of key importance for the study of the separation and isolation of substances in pH gradients to investigate first the fundamental aspect, *i.e.*, the neutralization reaction boundary, where sharp pH changes occur. Such a boundary, for the accomplishment of successful separations of ampholytes, must have the following separation properties: weak and strong acids and bases pass through the boundary; ampholytes with pI values within the range of pH values on both sides (pH gap) are trapped at the boundary; and ampholytes with pI values outside the pH gap pass through the boundary. Further, the boundary should also fulfil the following demands: it must have fixed position in the column; the span of the pH gap must be regulated; and the boundary must be sufficiently stable with time.

Let us discuss the properties of the boundary first. Without the presence of ampholytes in the electrolyte system, only one neutralization boundary can be created in the column. Its velocity is given by the magnitudes of the flows of solvolytic ions. With respect to the stoichiometry of the neutralization reaction, the boundary is stationary if the flows are equal, *i.e.*,

$$J_H = J_{OH} \tag{1}$$

where J_H and J_{OH} are flows of H$^+$ and OH$^-$ ions, respectively. To ensure some background conductivity along the column and to give the system the possibility of controlling the pH gap at the boundary, the presence of a primary (background) electrolyte (*e.g.*, KCl) in the column is necessary.

As shown in earlier work [23], the magnitude of the ion flows can be regulated in a column equipped with pairs of electrode chambers on each side. This enables one to control the magnitude of flow of H$^+$, J_H, by setting the ratio of the flows of H$^+$ and its co-ion on one side and the magnitude of the flow of OH$^-$, J_{OH}, by setting ratio of the flows of OH$^-$ and its co-ion on the other side. In such a way, the span of the pH gap is easily controlled and it changes

symmetrically around *ca*. pH 7 for a strong electrolyte (KCl).

For the determination of the time stability of the boundary (system), it is convenient to start from a description of the system using flows of ions as shown in Fig. 1. In our considerations, we chose a case that is as simple as possible: the strong primary electrolyte, KCl, is modified from the left, anodic, side by a flow of cations H$^+$ and from the right, cathodic, side by a flow of anions OH$^-$. Hence from the anodic side, the flows of H$^+$ and K$^+$, J_H and J_K, enter the boundary and the flow of Cl$^-$, J_{Cl}, leaves the boundary. From the right side, by analogy, there is input J_{OH} and J_{Cl} and output J_K. Owing to the presence of a chemical reaction at the boundary,

$$H^+ + OH^- \rightleftharpoons H_2O \tag{2}$$

the flows of K$^+$ and Cl$^-$ on the two sides of the stationary boundary are not equal:

$$J_K^A \neq J_K^C \tag{3}$$

The flows of potassium and chloride in modified zones can be derived from the knowledge of solvolytic flows. The anodic flow of potassium, J_K^A, is equal to the flow of potassium in the non-modified electrolyte, J_K^P, decreased by the flow of potassium replaced by H$^+$. The cathodic flow of potassium, J_K^C, is equal to the flow of
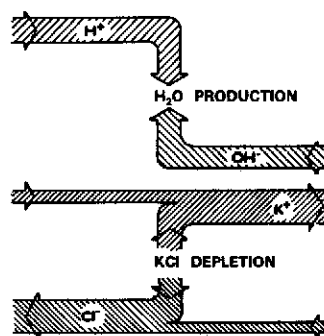

Fig. 1. Scheme of the flows of ions in the separation column. Anode is on the left, cathode is on the right. For further explanation, see text.

potassium in the non-modified primary electrolyte, J_K^P, which is decreased by the flow of potassium relevant to the flow of chlorides, replaced by OH^-, and increased by the flow of potassium, relevant to replacement by OH^-. Because the flows are substituted into the ratios of transference numbers, we can write

$$J_K^A = J_K^P - J_H^A \cdot \frac{T_K^{KCl}}{T_H^{HCl}} \qquad (4)$$

$$J_K^C = J_K^P - J_{OH}^C \cdot \frac{T_K^{KCl} - T_K^{KOH}}{T_{OH}^{KOH}} \qquad (5)$$

where T_x^y is the transference number of ion x in the electrolyte y. Similarly, we can derive for the flows of chloride in the cathodic and anodic part of the column:

$$J_C^C = J_C^P - J_{OH}^C \cdot \frac{T_C^{KCl}}{T_{OH}^{KOH}} \qquad (6)$$

$$J_C^A = J_C^P - J_H^A \cdot \frac{T_C^{KCl} - T_C^{HCl}}{T_H^{HCl}} \qquad (7)$$

For the depletion flows of the potassium and chloride from the boundary site, we can write

$$\Delta J_C = J_C^C - J_C^A; \quad \Delta J_K = J_K^C - J_K^A \qquad (8)$$

By combining previous equations, we obtain the expressions

$$\Delta J_C = J_S \cdot \frac{T_{OH}^{KOH}(T_C^{KCl} - T_C^{HCl}) - T_H^{HCl}T_C^{KCl}}{T_H^{HCl}T_{OH}^{KOH}} \qquad (9)$$

$$\Delta J_K = J_S \cdot \frac{T_H^{HCl}(T_K^{KCl} - T_K^{KOH}) - T_{OH}^{KOH}T_K^{KCl}}{T_{OH}^{KOH}T_H^{HCL}} \qquad (10)$$

and thus

$$\Delta J_C = \Delta J_K = -J_S \cdot \frac{U_K U_C(U_H + U_{OH})}{U_H U_{OH}(U_K + U_C)}$$

$$= J_S \cdot \text{constant} \qquad (11)$$

where U_K, U_C, U_H and U_{OH} are ionic mobilities of potassium, chloride, hydrogen and hydroxyl ions, respectively, and J_S is flow of solvolytic ions H^+ or OH^-. From eqn. 11, it follows that depletion flows of potassium and chloride from the non-moving neutralization boundary are equal, and only neutral KCl is depleted from the

boundary site. The depletion rate of KCl is dependent on the magnitudes of flows of solvolytic ions. The numerical value of the constant in eqn. 11 is 0.29, which means that 0.29 mol of KCl is depleted from the boundary per mole of neutralization water formed. Comparing the concentration of water ($55.5\ M$) with the concentration of primary electrolyte (*e.g.*, $0.01\ M$), it is seen that the effect of neutralization water created on the boundary is negligible. The neutralization boundary behaves as an ideal sink of solvolytic ions and a source of ions of primary electrolyte, *e.g.*, K^+ and Cl^-, which maintains the background conductivity of the system. Because electric current passes through the boundary, depleted KCl must be replenished to a certain extent. This is possible only by diffusion from the surroundings of the boundary. With constant current, a diffusional flow of KCl, J_{dif}, must be equal to electromigration depletion:

$$J_{dif} = \Delta J_K = \Delta J_C \qquad (12)$$

A break of the electric circuit at the boundary occurs with time, when the concentration of the background electrolyte (KCl) decreases to zero. This time and the variation of the concentration profile of KCl with time can be easily calculated if a model of linear diffusion is adopted, *i.e.*, with a constant gradient of concentration. For the evaluation of the time dependence of the concentration profile of the electrolyte along the x-axis, eqn. 13 can be used [24]:

$$C_{KCl} = C_0 - \frac{2\Delta J T^{1/2}}{(D\pi)^{1/2}}$$

$$\cdot \exp\left\{-\frac{x^2}{4Dt} + \frac{\Delta J}{D} \cdot \text{erfc}\left[\frac{x}{2(DT)^{1/2}}\right]\right\} \qquad (13)$$

where C_0 is the initial concentration of KCl, D is the diffusion coefficient, T is time and ΔJ is the mass flow from the boundary. After rearrangement for $x = 0$ and $C_{KCl} = 0$, we obtain

$$t = \pi D \left(\frac{C_0}{2\Delta J}\right)^2 \qquad (14)$$

It can be seen from eqn. 14 that the time stability of the boundary t is proportional to the square of the concentration of primary elec-

trolyte and inversely proportional to the square of the depletion flow. There are possibilities for controlling the time stability, namely by decreasing the driving current, which decreases the absolute value of the depletion flow and yielding diffusion, or by increasing the concentration of the primary electrolyte.

EXPERIMENTAL

Apparatus

A three-pole column, described previously [23], was rebuilt and now permits the regulation of any solvolytic flow from both sides of the capillary and ensures its constancy. The apparatus consists of a high-voltage (HV) power supply, equipped with two regulators of the ratio of driving currents and of the separation column. The power supply electric circuit provide the column by four adjustable and measured electric currents. These currents pass through two pairs of electrode chambers, each on the proper side of the separation capillary according to the polarity. In each pair, one chamber is filled with primary electrolyte and the other is filled with a modification electrolyte. The anodic or cathodic modification electrolyte is a solution of strong acid or base. The flows of ions from the electrolyte chambers pass through the washed membranes into the separation channel, which is a glass capillary of $100 \text{ mm} \times 0.3 \text{ mm}$ I.D. A scale glued on the side of the capillary serves for measuring the position and length of the zones. Sample can be introduced via a septum on each side of the column.

Chemicals

All chemicals were purchased from Lachema (Brno, Czech Republic) with the exception of the model substances, synthetic low-molecular-mass pI markers, which were a kind gift from Tessek (Prague, Czech Republic). The electrolyte systems used were 0.01 and 0.05 M solutions of KCl modified with H^+ and OH^-.

Focusing procedure

After filling the electrode chambers with the modification and primary electrolytes and switch-

ing on the membrane washing (to prevent any electrolysis products from entering capillary), a separation channel was filled with the sample dissolved in the primary electrolyte. The separation was started by setting up the ratio of the driving currents on both regulators and switching on the main driving current. Solvolytic ions penetrated into the channel and brought about the dissociation of the sample ampholyte, which migrated to the centre of the column and created the zone. The zone was photographed and its length and position were evaluated from the photograph.

RESULTS AND DISCUSSION

The first part of the experimental work was aimed at verification of the possibility of creating the stationary neutralization boundary in the column with the outlined separation properties.

The working pH range is depicted in Fig. 2a, where the dependence of the pH for both modified electrolytes on the ratio of the driving current in cathodic part of the column, as calculated for equal flows of solvolytic ions, is shown. This case corresponds to a stationary boundary. A concentration of the primary electrolyte (KCl) is parametrically selected. It can be seen that a pH gap ranging from *ca.* 1 to 13 pH units can be prepared in the given electrolyte system. It is symmetrical around the value pH 7.12.

The practical range of pH is limited by the stability with time. As follows from Table I, the time stability of the boundary was calculated for 0.01 and 0.05 M KCl as the primary electrolyte. In each instance the times needed to reach total depletion of the ions of KCl from the boundary (100%) and the times needed to reach 10% depletion of the ions (the pH is decreased by *ca.* 0.1) are given. It is clear from Table I that the practically useful ranges of pH are 10–4 in 0.01 M electrolyte and 11–3 in 0.05 M electrolyte. In Fig. 2b the dynamics of the KCl depletion from the boundary are illustrated. Under reasonable conditions, *i.e.*, pH range 4.26–10, the boundary is fairly stable.

Experimental verification of the stability of the boundary was carried out by adding a small amount of pH indicator (bromothymol blue or
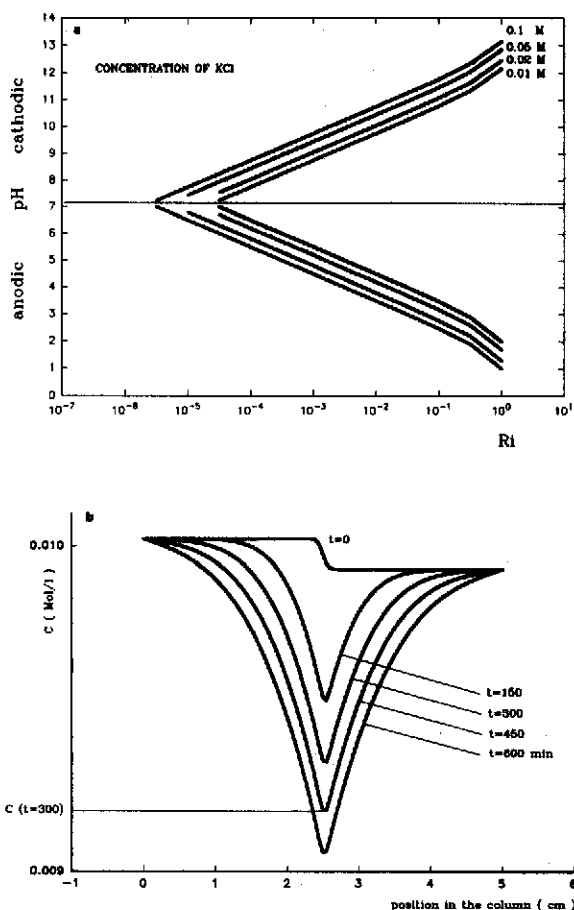
Fig. 2. (a) Dependence of the pH_a and pH_c on the ratio of the driving current on the cathodic side of the column for a different concentration of the primary electrolyte (KCl). (b) Calculated dependence of the concentration of KCl on time and position in the column. $I = 10$ A/m². Program kindly provided by Dr. W. Thormann (see ref. 8).

phenol red) at a concentration of 0.0001 *M* to the primary electrolyte so that the position of the boundary was made visible (Fig. 3). A boundary with a pH gap of 4.26–10 was stable for *ca.* 90 min in the column. By changing the ratio of the driving currents the boundary is mobilized or shifted.

It is not easy to find a suitable model coloured ampholyte with known p*I*; finally, we selected methyl red, which is a commonly used p*I* indicator in classical isoelectric focusing. Fig. 4 shows a photograph of the focused zone of methyl red. The indicator was loaded as a solution in the primary electrolyte. During the

TABLE I

TIME STABILITY OF THE BOUNDARY FOR 0.01 AND 0.05 *M* KCl AS A PRIMARY ELECTROLYTE AS A FUNCTION OF pH ON CATHODIC SIDE, pH_c

pH_c	Time (s)			
	0.01 *M* KCl		0.05 *M* KCl	
	100%	10%	100%	10%
8	$4.72 \cdot 10^7$	$4.72 \cdot 10^5$	$2.96 \cdot 10^1$	$2.96 \cdot 10^8$
9	$4.72 \cdot 10^5$	$4.72 \cdot 10^3$	$2.94 \cdot 10^8$	$2.94 \cdot 10^6$
10	$4.75 \cdot 10^3$	$4.76 \cdot 10^1$	$2.96 \cdot 10^6$	$2.95 \cdot 10^4$
11	$5.07 \cdot 10^1$	$5.0 \cdot 10^{-1}$	$2.99 \cdot 10^4$	$3.00 \cdot 10^2$
12	$2.0 \cdot 10^{-1}$	$2.0 \cdot 10^{-3}$	$3.3 \cdot 10^2$	$3.30 \cdot 10^0$

focusing in the column the concentration of the methyl red exceeded its solubility and a precipitate was formed. To prevent this precipitation, the addition of 75% ethylene glycol to the primary electrolyte was used. It is obvious that the methyl red created a sharp focus in the column. By changing the ratio of the driving current, the focused zone may be shifted along

Fig. 3. Neutralization boundary created in the column revealed by addition of 0.0001 *M* phenol red to the primary electrolyte. Conditions: primary electrolyte, 0.01 *M* KCl; modifying electrolytes, 0.01 *M* KCl + 0.001 *M* HCl, 0.01 *M* KCl + 0.02 *M* ammonia solution; total current through column, 400 μA; $I_H = 50$ μA; $I_{OH} = 100$ μA. Photograph taken in 5 min.

Fig. 4. Focused zone of methyl red (p*I* standard). Conditions: primary electrolyte, 0.05 *M* KCl; modifying electrolytes, 0.05 *M* KCl + 0.0025 *M* HCl, 0.05 *M* KCl + 0.1 *M* ammonia solution, all in 75% ethylene glycol; total current through column, 400 μA; $I_H = 160$ μA; $I_{OH} = 400$ μA. Photograph taken in 60 min.

the column, with a velocity that can be controlled and is proportional to the bias of the magnitude of the solvolytic flows from their balance. Up to a certain extent methyl red moves within the neutralization boundary; however, if the velocity of the boundary is higher than the maximum electromigration velocity attainable by methyl red, the dye lags behind the boundary and migrates in a zone electrophoretic mode.

In the next experiments, model coloured ampholytic substances readily soluble in water were chosen. Substances now produced commercially as pI markers are available with a broad range of pI values and have high absorption in the visible part of the spectrum. This enables us to avoid an expensive detection device. Fig. 5 shows the focused zone of a model substance (Tessek pI Marker 6) of pI 6. The substance focuses very well in the column.

Fig. 6 shows the separation of an ampholyte and strong ion. First, a zone of ampholyte was focused in the column and then the sample was injected (ferroin cationic indicator) via septum on the anodic part of the column. It is clear that ferroin passed through the focused zone of ampholyte without disturbing it. A slight movement of the focused zone was caused by side-effects, due to mechanical movement during the injection and also to the effect of the pH of the sample, which was not identical with that in the column.

The possibility of influencing the span of the pH gap and to utilize it for the separation a reaction slurry of Tessek pI Markers with a span of pI 6–9 is demonstrated in Fig. 7. The sample was first focused into one zone at the boundary having a sufficiently large span of the pH gap
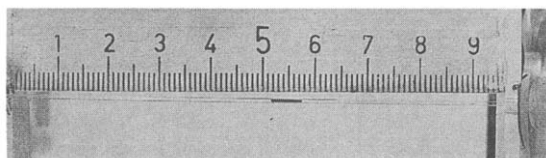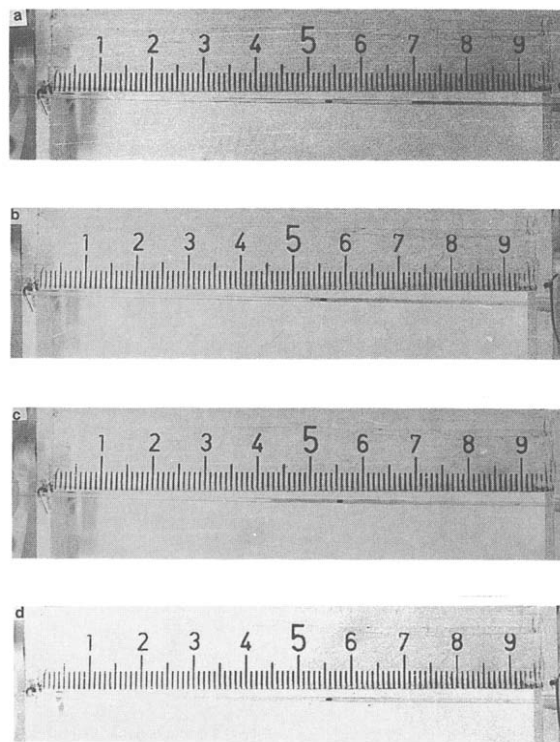

Fig. 6. Separation of the ampholyte and strong ion. Strong ion ferroin injected from the cathodic side of the column passes through focused zone of ampholyte (Tessek pI Marker 6). Conditions: primary electrolyte, 0.05 M KCl; modifying electrolytes, 0.05 M KCl + 0.0025 M HCl, 0.05 M KCl + 0.1 M ammonia solution; total current through column, 400 μA; $I_H = 35$ μA; $I_{OH} = 100$ μA. Photographs (a), (b), (c) and (d) taken in 30, 32, 33 and 35 min, respectively.

(4.25–10). Then, by changing the solvolytic flows, the span of the pH gap was reduced (ca. 5.75–8.5), which matches the focusing of the ampholyte of pI 6 only. As can be seen, more basic ampholytes migrate out of column, and the ampholyte of pI 6 remains focused. This depicts the way in which ampholytes can be separated step by step.

The described method, in contrast to classical isoelectric focusing, exhibits some characteristic features of isotachophoresis. With a steady state of the solvolytic flows, the length of the zone of an ampholyte is proportional to the amount of a sample injected and to the concentration of the primary electrolyte at a constant ratio of the driving currents. This feature is depicted in Fig. 8. The dependence of the length of zone on the

Fig. 5. Focused zone of Tessek pI Marker 6. Conditions, primary electrolyte, 0.01 M KCl; modifying electrolytes, 0.01 M KCl + 0.001 M HCl, 0.01 M KCl + 0.02 M ammonia solution; total current through column, 400 μA; $I_H = 20$ μA; $I_{OH} = 50$ μA. Photograph taken in 20 min.
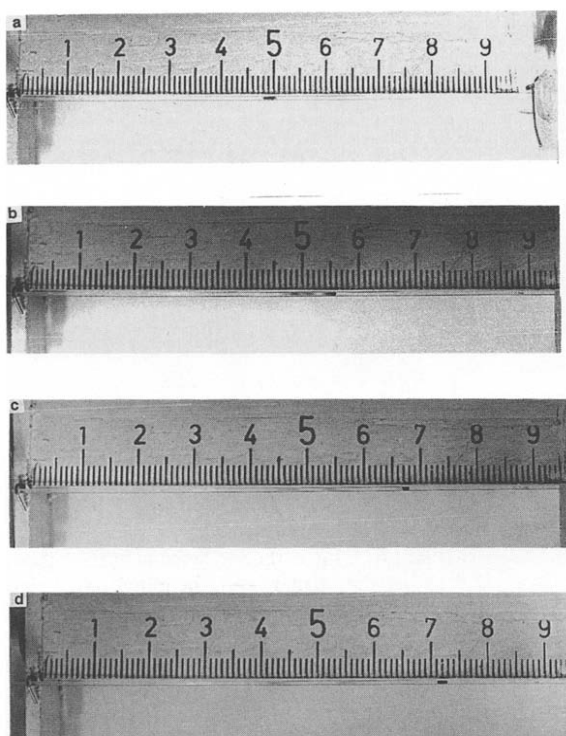
Fig. 7. Separation of a reaction slurry of ampholytes by a change in the span of the pH gap. Focused zone at larger span (a) is separated after its decrease (b–d). Conditions: primary electrolyte, 0.05 M KCl; modifying electrolytes, 0.05 M KCl + 0.005 M HCl, 0.05 M KCl + 0.1 M ammonia solution; total current through column, 400 μA; $I_H = 60$ μA and $I_{OH} = 200$ μA up to 27 min, then decreased to $I_H = 6$ μA and $I_{OH} = 10$ μA. Photographs (a), (b), (c) and (d) taken in 27, 30, 32 and 34 min, respectively.
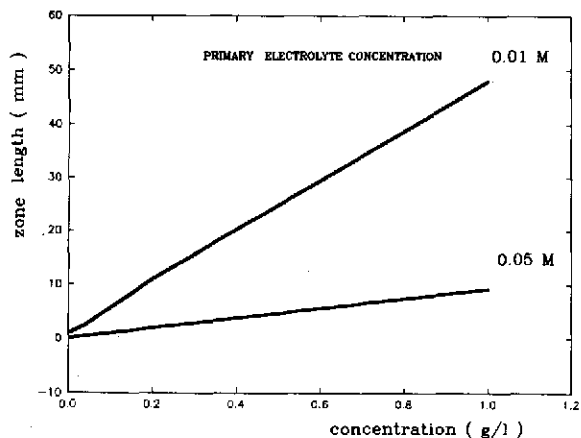

Fig. 8. Calibration lines of Tessek pI Marker 6 for 0.01 M KCl and 0.05 M KCl as a primary electrolyte. For conditions see Figs. 5 and 6, respectively.

injected amount for 0.01 M KCl is fairly linear; the small intercept is of experimental origin, as the focused zones of low load were only 50 μm in length, and this length was difficult to read from photographs. For the similar calibration line for 0.05 M KCl serving as primary electrolyte the dependence is fairly linear with a five times smaller slope.

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