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ZONE ORDER IN ISOTACHOPHORESIS

THE CONCEPT OF THE ZONE EXISTENCE DIAGRAM AND ITS USE IN CATIONIC SYSTEMS

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SUMMARY

The migration behaviour of zones of strong and weak bases in cationic iso-tachophoresis is considered. Based on the protolytic effects on the effective mobilities, the criteria decisive for the migration mode and zone order are formulated and the concept of the zone existence diagram is introduced, where zone pH *vs.* effective mobility is plotted for u_{BH} and K_{BH} as parameters. From such a diagram (for a given leading electrolyte terminator system) one may predict whether a given base will or will not migrate in stack and what the migration mode and zone order of a given pair of bases are. Based on this diagram, a general discussion is given of possible migration modes of a pair of bases, distinguishing (i) classical isotachophoretic migration, (ii) migration with inversion of effective mobilities or pH, (iii) steady-state mixed zone migration and (iv) enforced migration. Further, a new view of the formation and migration of the steady-state mixed zones is introduced and discussed. It is shown that if a steady-state mixed zone may be formed in the system then the character of the isotachopherogram is strongly affected by the ratio of the substances sampled. Based on the above-mentioned knowledge, a detailed classification is given of zone patterns potentially formed by a two-component sample of cathodically migrating bases in an isotachophoretic electrolyte system. It is shown that the elaborated principles may also be used in multi-component cationic analysis and the necessary criteria are given in the form of explicit mathematical equations.

INTRODUCTION

The basic qualitative information obtained from an electrophoretic analysis is data on relative effective mobilities. In classical zone electrophoresis (*e.g.*, ref. 1), these data can be obtained in a simple way by comparing the migration paths of a given species with that of a suitable internal standard.

In isotachophoresis, the step heights on the record (expressing the magnitudes of the useful detection signal) are obtained as the primary qualitative information. Qualitative isotachophoretic analysis is therefore based on the comparison of the step

heights of sample substances with those of standard substances; thermocouple[2], conductivity[3] and potential-gradient[4] detection records are currently evaluated in this way. As the magnitude of the detection signal is closely related to the effective mobility of the sample substance in all the above cases, mobilities may be determined as qualitative parameters from the record of the analysis. Using, *e.g.*, potential-gradient detection[5], the relative effective mobility is calculated as the reciprocal ratio of the detection signals in the zones of the sample substance and the standard.

In addition to the effective mobilities, the migration order of zones represents a general qualitative parameter in electrophoresis. Frequently in practice, a knowledge of the migration order of the zones under given conditions is of key importance for qualitative analysis in zone electrophoresis, although the migration order of zones always correlates with the respective sequence of effective mobilities.

In isotachophoresis, the classical view (see, *e.g.*, ref. 6) that zones arrange themselves in order of effective mobilities is not valid in every instance, as demonstrated in a number of experimentally observed cases[7–14]. Therefore, in isotachophoresis the order of migrating zones represents (in addition to effective mobilities) a further important parameter necessary for qualitative analysis.

Both of the mentioned qualitative parameters can be used successfully only if complete separation and steady-state migration of all zones are achieved at the time of detection. This assumption is, however, not always fulfilled owing to the possible formation of steady-state mixed zones[15,16] and, moreover, the existence of a steady-state is not equivalent to the condition of one zone not influencing another (see ref. 17 for enforced migration).

The aim of this paper is to show how protolytic equilibria influence the migration behaviour of weak bases in cationic isotachophoresis and how migrating strong and weak bases may mutually influence each other as far as the zone order and migration mode are concerned.

THEORETICAL

Single-component sample

The simplest case of cationic isotachophoresis of bases may be represented by the system where between the zone of the leading ion L^+ (strong base) and the terminating zone of H^+ (ref. 11) migrates a single zone of an ion constituent B involving a monohydric base B and its protonated form BH^+. Anion A^- of a weak acid HA serves as the counter ion. Base B migrates in an isotachophoretic configuration if both the front and rear boundary of its zone are self-sharpening. This condition holds if the effective mobility of the leading ion L in the zone of base B, $\bar{u}_{L,B}$, is higher than the effective mobility of base B in its own zone, $\bar{u}_{B,B}$:

$$\bar{u}_{L,B} > \bar{u}_{B,B} \tag{1}$$

and, simultaneously, the effective mobility of base B in the terminating zone, $\bar{u}_{B,H}$, is higher than the effective mobility of H^+, $\bar{u}_{H,H}$ (ref. 18):

$$\bar{u}_{B,H} > \bar{u}_{H,H} \tag{2}$$

(the first and second subscripts designate the substance and the zone, respectively). The effective mobility of a strong base L is equal to its ionic mobility, $\bar{u}_{L,L} = \bar{u}_{L,B} = u_L$.

For a numerical solution of this problem, the values of $\bar{u}_{B,j}$ may be obtained from the equation

$$\bar{u}_{B,j} = u_{BH} \cdot \frac{[H]_j}{[H]_j + K_{BH}} \tag{3}$$

where $K_{BH} = [H] [B]/[BH]$ is the dissociation constant of the protonated form BH^+. The necessary values of pH_H and $\bar{u}_{H,H}$ are calculated by means of relationships given in ref. 18; for the calculation of pH_B see Experimental.

By using conditions 1 and 2, it is possible for an electrolyte system of the above type to demarcate the bases which are able to give isotachophoretic zones, *i.e.*, which can be analysed in this system.

More information can be obtained from a graphical treatment by plotting the values of $\bar{u}_{B,B}$ *versus* pH_B with u_{BH} and K_{BH} as parameters; it can be called the "zone existence diagram" (see below). Fig. 1 shows an example of such a diagram for the system of 0.01 M potassium acetate (leading electrolyte) and H^+ (terminator). The main area of the diagram is marked off by four distinct contour curves:
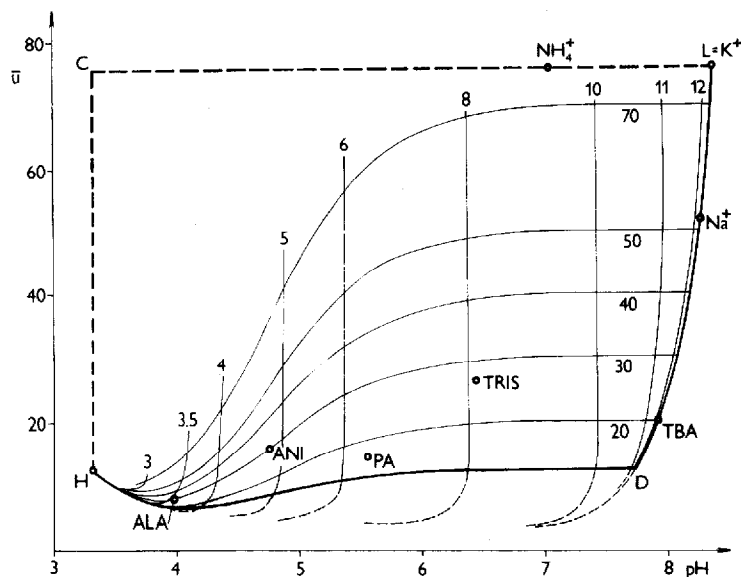

Fig. 1. Zone existence diagram [effective mobilities $\bar{u}$($\times$ 10^{-5} cm²/V · sec) *vs.* pH of zones of bases] for the system 0.01 M potassium acetate (leading electrolyte), H^+ (terminator). Curves 3, 3.5, 4, 5, 6, 8, 10, 11, 12 represent the dependence $\bar{u}_{B,B}$ *vs.* pH_B for the given pK_{BH} as a constant and u_{BH} as the variable parameter; curves 20, 30, 40, 50, 70 represent this dependence for the given constant u_{BH} and variable pK_{BH}. The points designated TRIS, PA, ANI and ALA correspond to zones of tris(hydroxymethyl)aminomethane, papaverine, aniline and β-alanine, respectively. For points D, L, C and H and for further explanation, see text. Constants used for calculation: $u_H = 362.5 \cdot 10^{-5}$, $u_{OH} = 202.5 \cdot 10^{-5}$, $u_{acetate} = 42.4 \cdot 10^{-5}$, $u_K = u_{NH_4} = 76.2 \cdot 10^{-5}$, $u_{Na} = 51.9 \cdot 10^{-5}$, $u_{TBA} = 20.2 \cdot 10^{-5}$, $u_{Tris} = 26.5 \cdot 10^{-5}$, $u_{PA} = 16.5 \cdot 10^{-5}$, $u_{ALA} = u_{ANI} = 30 \cdot 10^{-5}$ cm²/V · sec; pK_{HA}: acetic acid 4.76, ammonium 9.25, Tris 8.1, papaverine 6.4, aniline 4.78, β-alanine 3.55.

(1) The line CL, parallel to the x-axis, represents the limiting case of condition 1, $\bar{u}_{B,B} = u_L$.

(2) The descending curve LD shows the u versus pH dependence for zones of strong (fully protonated) bases BH^+ or cations of strong electrolytes M^+. For such bases, condition 2 has the form $u_i > \bar{u}_{H,H}$, and therefore curve LD ends in the point D (coordinates pH_i, $u_i = \bar{u}_{H,H}$). The point L corresponds to the values pH_L and u_L.

(3) The curve DH represents the limiting case (equality) in condition 2 for weak bases. It can be seen that the permitted values of $\bar{u}_{B,B}$ are even lower than $\bar{u}_{H,H}$. This corresponds to the inversion of effective mobilities (the so-called enforced migration[7,8]) of zones of weak bases migrating in front of the H^+ zone[11]. The coordinates of the point H correspond to the parameters of the terminating zone.

(4) The line HC parallel to the y-axis represents the limiting case $pH_B = pH_H$. It implies that the pH of an isotachophoretically migrating zone is always higher than that of the terminating H^+ zone (pH_H represents the lowest attainable pH in the given system).

Within the area CLDH there are the points $(pH_B, \bar{u}_{B,B}) = f(pK_{BH}, u_{BH})$ corresponding to the existence of zones of bases migrating in the given system in an isotachophoretic configuration. The screen in the diagram is formed by curves representing the dependence in question for constant u_{BH} and pK_{BH} with pK_{BH} and u_{BH}, respectively, as the parameters. This screen allows the direct evaluation of pH_B and the effective mobility $\bar{u}_{B,B}$ of the zone of a base with given u_{BH} and K_{BH}. For illustration, there are depicted points corresponding to zones of ammonium, potassium sodium, tetrabutylammonium (TBA), tris(hydroxymethyl)aminomethane (TRIS), papaverine (PA), aniline (ANI) and β-alanine (ALA) cations. It is obvious that points lying on the left of line HC and on the right of curve LD do not correspond to real zones. Some points lying over line CL and below curve DH (see the dashed elongation of the pK_{BH} curves of the screen) may correspond to real zones migrating, however, in an enforced configuration with another zone of a suitable base (see below).

Fig. 2 shows the zone existence diagram for the system of 0.01 M potassium acetate + 0.01 M acetic acid (leading electrolyte) and H^+ (terminator). It can be seen that if free acid is present in the leading electrolyte the effective mobilities of bases and the H^+ ion become higher and the range of attainable pH values of zones becomes narrower.

Two-component sample

For the investigation of the mutual behaviour of base B and any other base X, their migration order is of decisive importance. In order to determine it, the effective mobilities of these bases must be known not only in their own zones, $\bar{u}_{B,B}$ and $\bar{u}_{X,X}$, but also in the other zones, $\bar{u}_{B,X}$ and $\bar{u}_{X,B}$. The criteria of the migration order can be formulated as follows:

(a) The zone of base X migrates in front of the zone of base B if

$$\bar{u}_{X,B} > \bar{u}_{B,B} \tag{4a}$$
$$\bar{u}_{B,X} < \bar{u}_{X,X} \tag{4b}$$

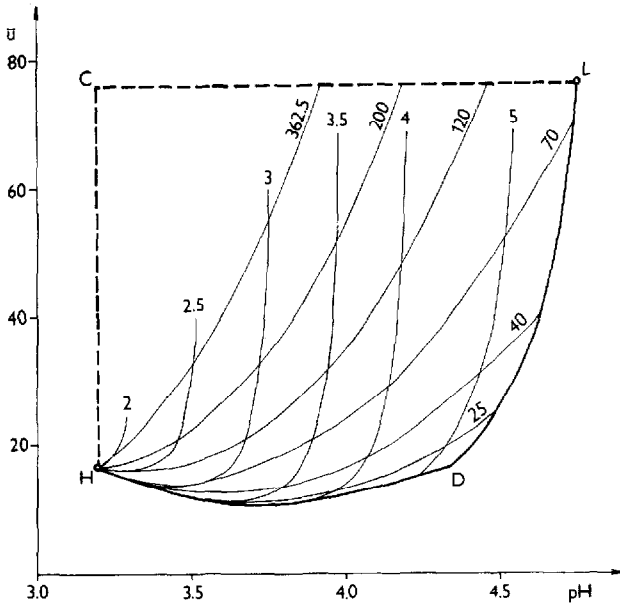*i.e.*, the boundary of the zones X and B shows self-sharpening in both directions.

Fig. 2. Zone existence diagram [effective mobilities $\bar{u}(\times 10^{-5}$ cm^2/V·sec) vs. pH of zones of bases] for the system 0.01 M potassium acetate + 0.01 M acetic acid (leading electrolyte), H$^+$ (terminator). Curves 2, 3, 3.5, 4, 5 represent constant pK_{BH} and variable u_{BH}; curves 25, 40, 70, 120, 200, 362.5 represent constant u_{BH} and variable pK_{BH}. For points D, L, C and H and for further explanation, see text.

(b) The zone of base X migrates behind the zone of base B if

$$\bar{u}_{X,B} < \bar{u}_{B,B} \qquad (5a)$$
$$\bar{u}_{B,X} > \bar{u}_{X,X} \qquad (5b)$$

i.e., the boundary of the zones B and X again shows self-sharpening in both directions.

Moreover, as isotachophoretic migration is assumed, conditions 1 and 2 must hold for both bases. This means that the corresponding points pH$_B$, $\bar{u}_{B,B}$ and pH$_X$, $\bar{u}_{X,X}$ must lie inside the existence area CLDH in their respective common zone existence diagram.

Fig. 3 shows the outline of the diagram from Fig. 1 where a point B is depicted corresponding to the zone of a model base B ($u_{BH} = 30 \cdot 10^{-5}$ cm^2/V·sec, p$K_{BH} = 5$) migrating between the leading zone of 0.01 M potassium acetate and the terminating zone of H$^+$. Curve 1 in Fig. 3 represents the limiting case in conditions 4a and 5a, $\bar{u}_{X,B} = \bar{u}_{B,B}$. Eqn. 3 served for its construction, where the required values of u_{XH} and K_{XH} were calculated from the known values of $\bar{u}_{B,B}$ and pH$_B$; $u_{XH} = \bar{u}_{X,B}(1 + K_{XH}/[H]_B)$. The values obtained were then used for the calculation of $\bar{u}_{X,X}$ and pH$_X$. It holds that in the region above curve 1 condition 4a is fulfilled and in the region below it condition 5a is fulfilled. Curve 2 in Fig. 3 corresponds to limiting cases in conditions 4b and 5b, $\bar{u}_{B,X} = \bar{u}_{X,X}$. Their values were calculated by means of eqn. 3 in the form $\bar{u}_{B,X} = u_{BH}[H]_X/([H]_X + K_{BH})$. It holds for curve 2 that in the region above it condition 4b is fulfilled and below it condition 5b is fulfilled.

It can be said that condition 4 holds only for bases X whose points lie above
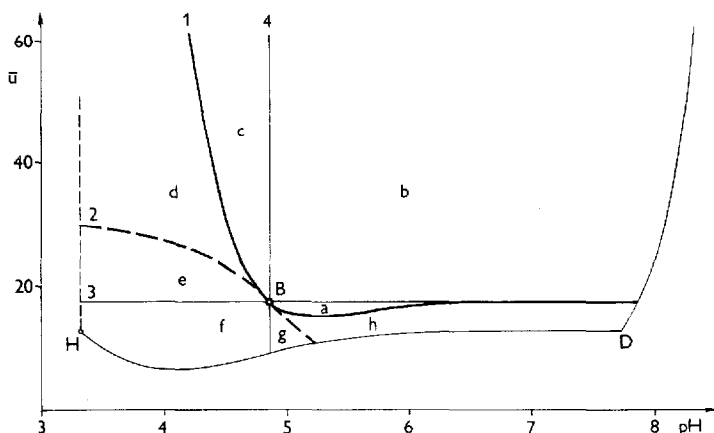
Fig. 3. Zone existence diagram from Fig. 1. Point B corresponds to a zone of base B ($u_{BH} = 30 \cdot 10^{-5}$ cm^2/V · sec, p$K_{BH} = 5$). For curves 1–4, regions (a)–(h) and further explanation, see text.

curve 1 and condition 5 holds only for bases whose points lie below curve 2. As the diagram is constructed in the coordinates pH$_X$–$\bar{u}_{X,X}$ and lines 3 and 4 represent the equations $\bar{u}_{X,X} = \bar{u}_{B,B}$ and pH$_X$ = pH$_B$, respectively, it is clear that the classical[6] idea of an isotachophoretic monotonous stepwise sequence of effective mobilities and pH values is valid only for bases from regions (b) and (f).

For bases X from regions (a), (c), (e) and (g), the correct isotachophoretic migration of the pair of zones B and X is accompanied by two types of inversion:

(i) Inversion of effective mobilities. For the zone of base X with parameters in region (a) in Fig. 3, $\bar{u}_{X,X} < \bar{u}_{B,B}$, i.e., the zone migrates in front of the zone of base B. An analogous case with an inverted migration order of the bases represents the region (e) in Fig. 3.

(ii) Inversion of pH. In the region (c) in Fig. 3, pH$_X$ < pH$_B$, i.e., the zone of a lower pH migrates in front of the zone of a higher pH. Region (g) below curve 2 may be discussed analogously.

Further, from Fig. 3 it can be seen that in addition to the regions of co-existence of separate zones of bases B and X, either above curve 1 (fulfillment of condition 4, the zone of base X migrates in front of the zone of base B) or below curve 2 (fulfillment of condition 5, the zone of base X migrates behind the zone of base B), there are also regions (d) and (h) on the zone existence diagram. From their position with respect to curves 1 and 2 it follows that for the corresponding bases X conditions 4b and 5a are valid, i.e.,

$$\bar{u}_{X,B} < \bar{u}_{B,B}; \; \bar{u}_{B,X} < \bar{u}_{X,X} \tag{6}$$

The physico-chemical meaning of expression 6 is that in the zone of base B (or X) the other base X (or B) is retarded. As both B and X cannot penetrate into the leading or terminating zone, the fulfillment of condition 6 can be explained only by the formation of a steady-state mixed zone of bases B and X. This zone behaves as a zone of a single species with a certain effective mobility.

It is known that two bases B and X form a steady-state mixed zone only if their effective mobilities in this zone are equal, $\bar{u}_{B,mix} = \bar{u}_{X,mix}$. This holds only at one particular value of the pH of the mixed zone, pH_{mix}. From the application of eqn. 3 for $\bar{u}_{B,mix}$ and $\bar{u}_{X,mix}$ the following relationship[15] was obtained:

$$[H]_{mix} = K_{XH} \cdot \frac{1 - (u_{XH}K_{XH}/u_{BH}K_{BH})}{u_{XH}/u_{BH} - 1} \tag{7}$$

It is evident that the value of pH_{mix} corresponds to fixed concentrations of both bases in the mixed zone, $c_{B,mix}$ and $c_{X,mix}$; their values may be calculated by combining the equations of electroneutrality, constant current density and definitions of K_{BH} and K_{XH}.

This theoretical conclusion is significant in practice. If we imagine the zones of bases B and X as if they were in the system alone, in the migration order B in front of X, then (with respect to condition 6) base B starts to penetrate through the rear boundary of its zone into the zone of base X, from which a mixed zone is then formed. This process continues until the parameters of the mixed zone reach the values pH_{mix} (eqn. 7), $c_{X,mix}$ and $c_{B,mix}$, and

$$\bar{u}_{X,B} < \bar{u}_{B,B}; \ \bar{u}_{B,mix} = \bar{u}_{X,mix} \tag{8}$$

(cf., condition 6). The penetration of base B into the mixed zone stops; otherwise, the parameters of the mixed zone would change and $\bar{u}_{B,mix} > \bar{u}_{X,mix}$ (cf., condition 5), and the excess of base B would return into its own zone again. This means that at a sampling ratio $q = n_B/n_X > c_{B,mix}/c_{X,mix}$ a mixed zone is formed in the system containing n_X moles of base X and $n_X c_{B,mix}/c_{X,mix}$ moles of base B. In front of this zone migrates a zone of pure base B containing $n_B - n_X c_{B,mix}/c_{X,mix}$ moles of base B. It is evident that the same considerations apply to the case with the inverted initial migration order of both zones; the same steady-state system is obtained as a result. At a sampling ratio $q = n_B/n_X < c_{B,mix}/c_{X,mix}$ the mixed zone is also formed, accompanied in this instance by a zone containing the excess of base X migrating again in front of the mixed zone.

All the above modes of migration of a pair of bases B and X are listed in tabular form in Fig. 4, together with conditions and a schematic potential-gradient record. The letters a–h correspond to the respective regions in Fig. 3.

So far, we have discussed cases of natural migration only, where either the point corresponding to the zone of a base lies within the existence region CLDH on the zone existence diagram and the base (in a single-component sample arrangement) gives an isotachophoretic zone, or the respective point lies outside the existence region and the base does not migrate isotachophoretically in the given system. However, as will be shown below, points outside the existence region may also correspond to real zones, the migration of which is enforced owing to the presence of a neighbouring zone of a suitable base. Fig. 5 shows an enlarged cut-out of the existence diagram for the system 0.01 M potassium acetate + 0.01 M acetic acid (leading

condition / migration mode	$\bar{u}_{X,B} < \bar{u}_{B,B}$ $\bar{u}_{B,X} > \bar{u}_{X,X}$	$\bar{u}_{X,B} > \bar{u}_{B,B}$ $\bar{u}_{B,X} < \bar{u}_{X,X}$
ü inversion	a. $\bar{u}_{X,X} < \bar{u}_{B,B}$ $pH_X > pH_B$	e. $\bar{u}_{X,X} > \bar{u}_{B,B}$ $pH_X < pH_B$
normal mode	b. $\bar{u}_{X,X} > \bar{u}_{B,B}$ $pH_X > pH_B$	f. $\bar{u}_{X,X} < \bar{u}_{B,B}$ $pH_X < pH_B$
pH inversion	c. $\bar{u}_{X,X} > \bar{u}_{B,B}$ $pH_X < pH_B$	g. $\bar{u}_{X,X} < \bar{u}_{B,B}$ $pH_X > pH_B$
condition / migration mode	$\bar{u}_{X,B} < \bar{u}_{B,B}$ $\bar{u}_{B,X} < \bar{u}_{X,X}$	
steady-state mixed zone (M)	d. $\bar{u}_{X,X} > \bar{u}_{B,B}$ $pH_X < pH_B$	h. $\bar{u}_{X,X} < \bar{u}_{B,B}$ $pH_X > pH_B$

Fig. 4. Possible modes of cationic isotachophoretic migration of two bases.

electrolyte), H^+ (terminator) (see Fig. 2). Within its contours there are (with the same meaning of the symbols as in Figs. 2 and 3) plotted curves 1–4 for a model base B (u_{BH} = $35 \cdot 10^{-5}$ cm^2/V·sec, pK$_{BH}$ = 3.5). It can be seen that base B migrates with a mobility inversion towards the terminator, $\bar{u}_{B,B} < \bar{u}_{H,H}$. In addition to the already known regions (a)–(h) it is possible to designate two additional regions (i) and (j), demarcated by curve DH on the left, by the prolongation of curves LD and 1, respectively, on the right and by curve 3.

Region (i) corresponds to the values of $\bar{u}_{X,X}$ and pH$_X$ of bases X that cannot be terminated directly by the H^+ ion. However, as condition 4a is valid, base X gives an isotachophoretic zone when migrating in front of the zone of base B. Hence the speciality of region (i) is that although the migration of X alone (in front of H) is not realizable, base X migrates in an enforced manner in front of base B. At the same
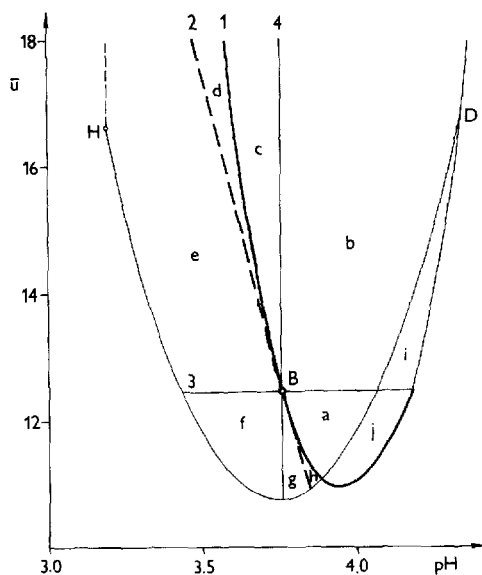
Fig. 5. Part of the zone existence diagram from Fig. 2. Point B corresponds to a zone of base B (u_{BH} = $35 \cdot 10^{-5}$ cm^2/V·sec, pK_{BH} = 3.5). For curves 1–4, regions (a)–(j) and further explanation, see text.

time, base B migrates in front of the terminating H$^+$ naturally, but with an effective mobility inversion. Analogous conclusions can be drawn for region (j) in Fig. 5, where, additionally, bases B and X migrate in a configuration with inversion of effective mobilities (cf., a in Fig. 4).

It is evident that the given theoretical approach based on relationships of type 4 or 5 and on zone existence diagrams allows one to make a general selection of a weak base as the leading or terminating ion. Base L may serve as the leading ion for all bases X migrating in a binary configuration behind the zone of base L (cf., e, f and g in Fig. 4). Similarly, base T may serve as a terminator of all bases X migrating in front of the zone of this base T (cf., a, b and c in Fig. 4). Base X thus gives an isotachophoretic zone migrating between the zones of the leading base L and the terminating base T if

$$\bar{u}_{X,X} < \bar{u}_{L,X} \tag{9a}$$

$$\bar{u}_{X,T} > \bar{u}_{T,T} \tag{9b}$$

In addition, for the pair L, T condition 4 must be fulfilled and for base T the condition of the sharp boundary with the H$^+$ zone must be valid[11].

As an example, Fig. 6 shows the zone existence diagram for a system where base L (u_{LH} = $40 \cdot 10^{-5}$ cm^2/V·sec, pK_{LH} = 8) with a concentration equal to its concentration adjusted behind the zone of 0.01 M potassium acetate (Fig. 1) serves as the leading electrolyte (the counter ion is acetate) and base T (u_{TH} = $30 \cdot 10^{-5}$ cm^2/V·sec, pK_{TH} = 5; cf., Fig. 3) is the terminator. The upper demarcating curve corresponds to equality in condition 9a and the lower demarcating curve corresponds
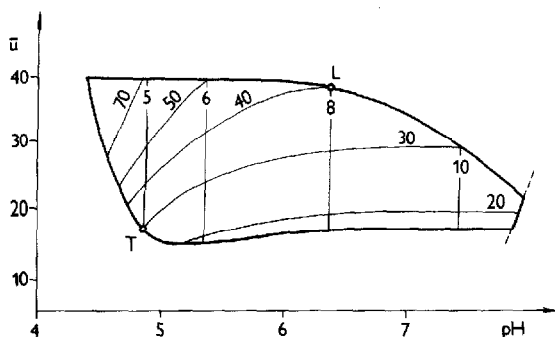
Fig. 6. A cut-out from the zone existence diagram in Fig. 1. Point L corresponds to a zone of base L (leading zone) ($u_{LH} = 40 \cdot 10^{-5}$ cm^2/V · sec, $pK_{LH} = 8$) with the concentration adjusted behind the zone of 0.01 M potassium acetate; point T corresponds to a zone of base T (terminating zone) ($u_{TH} = 30 \cdot 10^{-5}$ cm^2/V · sec, $pK_{TH} = 5$). For curves 5, 6, 8, 10, 20, 30, 40, 50 and 70, see Fig. 1; for explanation, see text.

to equality in condition 9b (this curve is identical with curve 1 in Fig. 3). On the right, the diagram is outlined by a part of the curve corresponding to the migration of strong bases (cf., LD in Fig. 1). The screen in the figure has the same meaning as in Fig. 1. It can be seen that the use of common weak bases as leading and/or terminating ions is possible. However, it permits the analysis of fewer bases than the system with a strong base and H$^+$ as the leading and terminating ion, respectively. From Fig. 6 it is further seen that in the given system L–T two types of migration of a base X with inversion of effective mobilities can be realized:

(i) Inversion towards the leading zone. Here, condition 9 is valid and $\bar{u}_{X,X} >$ $\bar{u}_{L,L}$, i.e., the potential gradient in zone X is lower than in the leading zone L (cf., e in Fig. 4). In the given system, bases with corresponding points lying inside the permitted region and higher than point L migrate in this way.

(ii) Inversion towards the terminator. Here again condition 9 is valid and $\bar{u}_{X,X}$ $< \bar{u}_{T,T}$ (cf., a in Fig. 4). Bases with corresponding points lying inside the permitted region and lower than point T migrate in this way in the given system.

A further important conclusion follows from Fig. 6; a base with the corresponding point lying on the left out of the permitted existence region CLDH and higher than point T gives no isotachophoretic zone, although $\bar{u}_{X,X} > \bar{u}_{T,T}$. The point of the base lies (with respect to zone T) in region (d) (cf., Fig. 4) and condition 8 of the steady-state mixed zone of X and T holds.

Multi-component sample

Two main questions must be answered for a given system and sample: is a steady-state mixed zone formed and what is the migration order of all zones? The answer to both questions is based on relationships of the type $\bar{u}_{i,j} \gtreqless \bar{u}_{j,j}$ testing the direction of migration of substance i in zone j [for numerical purposes in the form sign $(\bar{u}_{i,j} - \bar{u}_{j,j})$], where all possible combinations of bases are considered. Therefore, the pH$_j$ values of all zones of the substances in question (including the leading and terminating zones) and then the $\bar{u}_{i,j}$ values from eqn. 3 must be calculated.

The existence of a mixed zone of two bases i, j is (cf., condition 6) indicated when the equation sign $(\bar{u}_{i,j} - \bar{u}_{j,j})$ + sign $(\bar{u}_{j,i} - \bar{u}_{i,i}) = 0$ is not valid. Analogously, it can be said for a multi-zone system of n zones (including the leading zone and without

the terminating zone if it is formed by H^+) that no pair of its components forms a steady-state mixed zone if

$$\sum_{j=1}^{n-1} \sum_{i=j+1}^{n} [\text{sign }(\bar{u}_{i,j} - \bar{u}_{j,j}) + \text{sign }(\bar{u}_{j,i} - \bar{u}_{i,i})] = 0 \tag{10}$$

To determine the migration order N_i of the zone of substance i, defined as an integral number from the interval $(1, n)$, it is necessary to consider the relationships $\bar{u}_{i,j} \gtreqless \bar{u}_{j,j}$ for all zones j. The sum of the values sign $(\bar{u}_{i,j} - \bar{u}_{j,j})$ for a given species i in all zones j increases two by two from $1 - n$ for the first (leading) zone to $n - 1$ for the last zone. If condition 10 is valid, then for the migration order of the zone of substance i we can write

$$N_i = \tfrac{1}{2}\left[n + 1 - \sum_{j=1}^{n} \text{sign}(\bar{u}_{i,j} - \bar{u}_{j,j}) \right] \tag{11}$$

The H^+ zone is not taken into consideration here as quantities of the type $\bar{u}_{H,j}$ are not defined, and eqns. 10 and 11 cannot be used. If, however, condition 10 is valid, the correct isotachophoretic migration of the system (with respect to the H^+ ions; cf., ref. 11) is guaranteed by the condition $\bar{u}_{n,H} > \bar{u}_{H,H}$.

EXPERIMENTAL

For the calculation of the pH of the zones a Texas Instruments TI-59 calculator was used with a simple program based on the RFQ method introduced by Beckers and co-workers[7,8]. Tabulated values (at 25°C) of dissociation constants[19,20] and ionic mobilities[20] with no corrections were used for the calculations under Theoretical; the ionic mobilities of Tris, aniline, papaverine and β-alanine were calculated according to Jokl's equation[21] with parameters[22] $a = 244.2 \cdot 10^{-5}$ cm^2/V · sec, $b = 4.32 \cdot 10^{-5}$ cm^2/V · sec.

For the calculation of practical systems (see Results and Discussion), the ionic mobilities of anilinium and TBA obtained from isotachophoretic measurements[5] were used ($32.5 \cdot 10^{-5}$ and $19.3 \cdot 10^{-5}$ cm^2/V · sec, respectively). The computing procedure was modified to include corrections of the pK_{HA} of acetic acid and the ionic mobilities of acetate, H^+, K^+ and OH^- to the ionic strength, according to simplified forms of the Debye–Hückel and Onsager equations[23].

The experiments were carried out in an isotachophoretic column made of Perspex with a separation capillary of rectangular cross-section[24]. The potential-gradient detector and the high-voltage supply of stabilized current have been described earlier[5].

All the chemicals used were of analytical-reagent grade (Lachema, Brno, Czechoslovakia).

RESULTS AND DISCUSSION

For the verification of the theoretical conclusions, experiments with model cations were carried out in systems where potassium acetate and acetic acid served as components of the leading electrolyte.
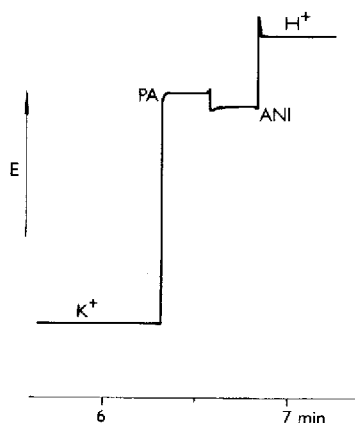
Fig. 7. Isotachopherogram of 1 μl of 0.01 M aniline (ANI) and papaverine (PA) in the system 0.01 M potassium acetate (leading electrolyte), acetic acid (terminator). I = 120 μA.

The case of the inversion of effective mobilities has been described earlier and called enforced isotachophoresis; experimental evidence for various pairs of anions and cations (including H⁺) has been given in a number of papers[7-14]. It is illustrated in Fig. 7 by the pair papaverine (PA)–aniline (ANI). It should be stressed that the zones of PA and ANI do not affect each other and each of them gives the same zone when present in the system alone. Therefore, the term enforced migration is inappropriate here. This term should be reserved for those cases where a substance forms an isotachophoretic zone in the given system only owing to the presence of another zone, and would not form an isotachophoretic zone if it were in the system alone.

According to the theory, such cases may exist in the type of systems discussed. The experimental evidence is given in Fig. 8, which shows the results of analyses using a system with 0.005 M potassium acetate and 0.003 M acetic acid as the leading
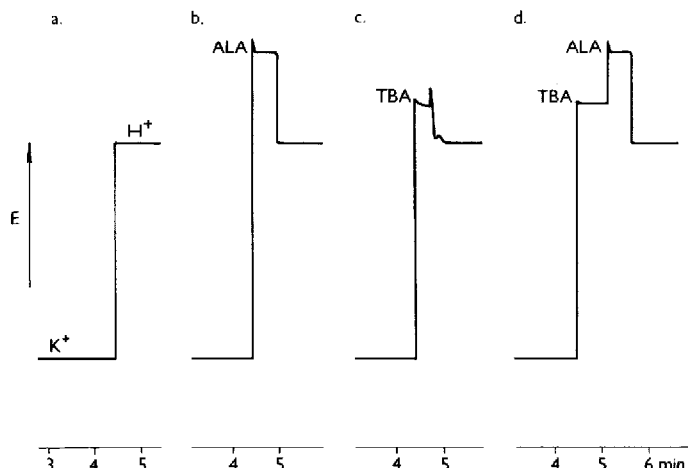

Fig. 8. Isotachopherograms in the system 0.005 M potassium acetate + 0.003 M acetic acid (leading electrolyte), acetic acid (terminator). Sample: 0.3 μl of 0.01 M β-alanine (ALA) (b), 0.7 μl of 0.01 M TBA (c), 0.3 μl of 0.01 M β-alanine and 1.0 μl of 0.01 M TBA (d). I = 100 μA.

electrolyte. Fig. 8b shows the migration of β-alanine (ALA) with a marked inversion of effective mobilities of ALA–H$^+$. Fig. 8c shows the migration of TBA: here $\bar{u}_{H,H} >$ u_{TBA} and the boundary TBA–H$^+$ is not sharp and becomes diffuse in the usual way in zone migration (cf., Fig. 3 in ref. 18). Therefore, isotachophoretic migration of TBA and its isotachophoretic analysis are impossible in the given system. Fig. 8d shows the results of migration of the zone of TBA in front of the zone of ALA. Here, $\bar{u}_{ALA,ALA} <$ $u_{TBA} > \bar{u}_{ALA,TBA}$ (condition 4) and, consequently, TBA gives a quantitatively migrating isotachophoretic zone, the migration of which is enforced by the zone of β-alanine behind it. The quantitative character of migration is confirmed by the calibration graph for TBA, which is linear and passes through the origin. From this experiment it follows that it may be useful in some instances to use a mixture of an acid and a weak base instead of the acid alone as a terminator, where the weak base migrates in front of the H$^+$ zone with a mobility inversion.

Based on the theory presented here, unusual isotachophoretic patterns may be obtained. Fig. 9 shows a record of the analysis of a mixture of aniline and ε-aminocaproic acid in a system with 0.0028 M papaverine acetate as the leading electrolyte. It can be seen that the leading and terminating (H$^+$) ions have the same effective mobility in their own zones; the first sample zone (ANI) shows a $\bar{u}$ inversion towards the leading zone and the second sample zone (EACA) migrates with a mobility inversion towards the terminator. Fig. 10 shows the analysis of aniline in a system with 0.002 M papaverine acetate as the leading electrolyte. Here, all three zones in the system show mutual inversions of effective mobilities. This system shows that even zones arranged in order of decreasing potential gradient (and increasing effective mobilities) may in some instances also form a correct isotachophoretic system.

From the theory, it is further seen that steady-state mixed zones may exist in a defined region of the pH values of the leading electrolyte. At the same time, however, the pH of the mixed zone is constant within this interval; hence the composition of the mixed zone is variable where the concentrations of both bases are linear functions of the concentration of free acid in the leading electrolyte, and the limiting values correspond to zones of pure one and the other base. For a given pH of the leading
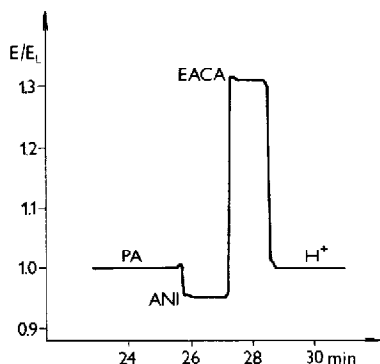

Fig. 9. Isotachopherogram of 1 μl of 0.01 M aniline (ANI) and ε-aminocaproic acid (EACA) in the system 0.0028 M papaverine (PA) acetate (leading electrolyte), acetic acid (terminator). $I = 30$ μA.

Fig. 10. Isotachopherogram of 0.5 μl of 0.01 M aniline (ANI) in the system 0.002 M papaverine (PA) acetate (leading electrolyte), acetic acid (terminator). $I = 120$ μA.
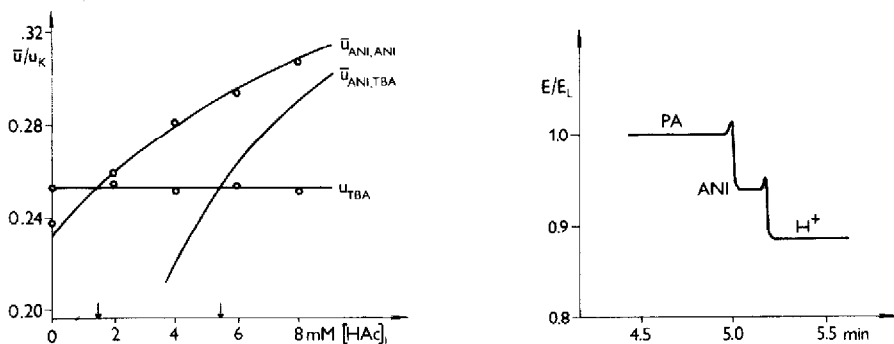
Fig. 11. Calculated curves and experimental values of the dependence of relative (with respect to potassium) effective mobilities of aniline (ANI) and tetrabutylammonium (TBA) on the concentration of acetic acid, $[HAc]_L$, in the leading electrolyte. The system in question is 0.01 M potassium acetate + acetic acid (leading electrolyte), H^+ (terminator).

electrolyte, then, depending on the sampling ratio, the zone of the base being in excess under the given conditions migrates in front of the mixed zone.

To obtain experimental evidence of these phenomena, aniline and tetrabutyl-ammonium (TBA) were used. Fig. 11 shows the calculated curves and experimental points of the dependence of the relative effective mobilities of aniline and TBA on the concentration of acetic acid, $[HAc]_L$, in the leading electrolyte of 0.01 M potassium acetate. It can be seen that the effective mobility of TBA (modelling a strong base) is constant (the points corresponding to the zone of TBA on the zone existence diagrams lie on curve LD; cf., Fig. 1). When considering the behaviour of the zone of
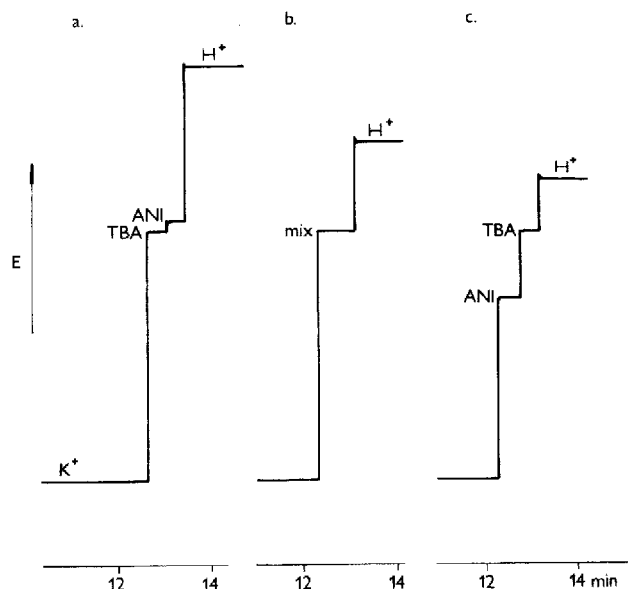

Fig. 12. Isotachopherograms of a mixture of TBA and aniline (ANI) in the systems 0.01 M potassium acetate + (a) 0, (b) 0.004 and (c) 0.008 M acetic acid (leading electrolyte), acetic acid (terminator). Sample: 0.50 μl of 0.01 M TBA and 0.75 μl of 0.01 M ANI. I = (a) 70, (b, c) 75 μA.
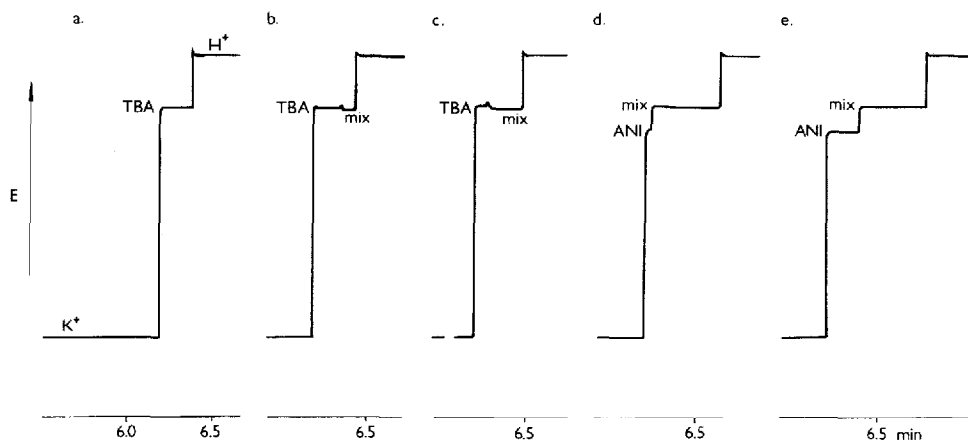
Fig. 13. Isotachopherograms in the system 0.01 M potassium acetate + 0.004 M acetic acid (leading electrolyte), acetic acid (terminator). Sample: 0.5 μl of 0.01 M TBA and (a) 0, (b) 0.25, (c) 0.5, (d) 1.0 and (e) 1.5 μl of 0.01 M aniline. I = 120 μA.

TBA (as base X; cf., Theoretical) towards the zone of aniline (as base B), then in the system with $[HAc]_L$ = 0 (cf., Fig. 1) condition 4 is valid, i.e., $u_{TBA} > \bar{u}_{ANI,ANI}$ and $\bar{u}_{ANI,TBA} < u_{TBA}$, and TBA therefore migrates in front of ANI (case b in Fig. 4). An increase in concentration of acetic acid in the leading electrolyte leads to an increase in $\bar{u}_{ANI}$: at a certain value of $[HAc]_L$ (see the first arrow in Fig. 11) $\bar{u}_{ANI,ANI} = u_{TBA}$ and over this value condition 6 of a steady-state mixed zone is valid, i.e., $u_{TBA} < \bar{u}_{ANI,ANI}$ and $\bar{u}_{ANI,TBA} < u_{TBA}$ (cf., h in Fig. 4). A further increase in $[HAc]_L$ leads to $u_{TBA} = \bar{u}_{ANI,TBA}$ (see the second arrow in Fig. 11) and at even greater values of $[HAc]_L$ condition 5 is valid, $u_{TBA} < \bar{u}_{ANI,ANI}$ and $\bar{u}_{ANI,TBA} > u_{TBA}$, i.e., ANI migrates in front of TBA (g in Fig. 4). It can thus be seen that the mixed zone is formed in a relatively large region of composition (and pH) of the leading electrolyte (in Fig. 11 the interval between the two arrows).

Fig. 12 shows analyses of a mixture of TBA and aniline at three values of $[HAc]_L$. It is seen that in agreement with the theory at $[HAc]_L$ = 0 (Fig. 12a) the migration order is TBA, ANI and at $[HAc]_L$ = 0.008 (Fig. 12c) the migration order is inverted, ANI, TBA. At $[HAc]_L$ = 0.004 (Fig. 12b) both substances form a stable mixed zone; its apparent effective mobility is given by $\bar{u}_{ANI,mix} = \bar{u}_{TBA,mix} = u_{TBA}$, i.e., the potential gradient in the mixed zone is equal here to that of the TBA zone. Fig. 13 shows the dependence of the character of the isotachopherogram[25] on the sampling ratio, $q = n_{ANI}/n_{TBA}$, at the concentration $(HAc)_L$ = 0.004. It can be seen that at low sampling ratios, q = 0.5 or 1 (Fig. 13b and c), $q < c_{ANI,mix}/c_{TBA,mix}$ and the TBA zone of the same potential gradient occurs in front of the mixed zone. In contrast, at q = 2 or 3 (Fig. 13d and e), $q > c_{ANI,mix}/c_{TBA,mix}$ and the ANI zone of a lower potential gradient migrates in front of the mixed zone (cf., Fig. 11).

CONCLUSIONS

Whether a base B does or does not give an isotachophoretic zone in a given system of leading electrolyte and terminator depends not only on the values of the

effective mobilities of the leading ion, base and terminator (parameters of the type $\bar{u}_{i,i}$), but also on the values of the effective mobilities of the given base B in the other zones (parameters of the type $\bar{u}_{i,j}$). For a simple system of a single zone of base B, see conditions 1 and 2.

It is advantageous to construct for a given leading electrolyte the zone existence diagram, in which the values of $\bar{u}_{B,B}$ *versus* pH_B are plotted for u_{BH} and K_{BH} as parameters. This diagram allows one to evaluate simply the parameters of the zone of a base with known u_{BH} and K_{BH} and, moreover, to predict whether the base is actually able to give an isotachophoretic zone in the system in question.

The zone existence diagram also enables one to predict the behaviour of a pair of bases B and X. In the diagram, the curves corresponding to the equations $\bar{u}_{B,X} = \bar{u}_{X,X}$ and $\bar{u}_{X,B} = \bar{u}_{B,B}$ may be constructed for the parametrically given base B, thus dividing the zone existence area into sections where bases X migrate in front of zone B, behind zone B, or form a steady-state mixed zone with base B.

The first two cases, where the bases B and X migrate as two separated individual zones, may be accompanied by two types of inversion. The inversion of effective mobilities means that a zone of a base with lower effective mobility migrates in front of a zone of a base with higher effective mobility (so far, this case has been called enforced isotachophoresis). The inversion of pH means that a zone of a lower pH migrates in front of a zone of a higher pH. In both cases of inversion, the monotonous stepwise order of the values of effective mobilities and pH from the leading electrolyte to the terminator is broken as well as the classical idea about zone order in isotachophoresis.

The last case, *i.e.*, the formation of mixed zones in cationic isotachophoresis, is accompanied by a new phenomenon. With respect to the constant composition and pH of the mixed zone, a zone containing an appropriate excess of one of the bases may occur in front of the mixed zone, its size being dependent on the sampling ratio.

In addition to the above-described cases of independent migration of zones, the zone existence diagram enables one to predict and discuss the case of really enforced migration, where a zone is strongly affected by a neighbouring zone. An example is, *e.g.*, the enforced migration of a strong and slow base (for which condition 2 of the sharp boundary between its zone and the H^+ zone does not hold) in front of the zone of a weak and fast base migrating naturally in front of the H^+ zone with an inversion of effective mobilities.

The described principle of analysis of the mutual behaviour and migration order of zones of two bases may be extended to more complicated systems occuring in multi-component analysis. Here, the testing is based on the use of calculated parameters of the type $\text{sign}(\bar{u}_{i,j} - \bar{u}_{j,j})$. If no mixed zones are formed in the system (*cf.*, eqn. 10), the migration order of a zone may be calculated by eqn. 11.

The precise computer simulation techniques[26,27], when extended to involve also $\bar{u}_{i,j}$ data, may significantly aid in solving such problems.

Obviously, the principles described above based on the use of $\bar{u}_{i,j}$ are of general significance and may be used to describe the migration mode and the zone order in anionic systems, too.

REFERENCES

1 M. Lederer, *An Introduction to Paper Electrophoresis and Related Methods*, Elsevier, Amsterdam, London, New York, 2nd impression, 1957.
2 A. J. P. Martin and F. M. Everaerts, *Proc. Roy. Soc. London, Ser. A*, 316 (1970) 493.
3 F. M. Everaerts, M. Geurts, F. E. P. Mikkers and Th. P. E. M. Verheggen, *J. Chromatogr.*, 119 (1976) 129.
4 T. Haruki and J. Akiyama, *1974 Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy*, Paper No. 420.
5 M. Deml, P. Boček and J. Janák, *J. Chromatogr.*, 109 (1975) 49.
6 H. Haglund, *Sci. Tools*, 17 (1970) 2.
7 J. L. Beckers, *Thesis*, Technological University of Eindhoven, Eindhoven, 1973.
8 F. M. Everaerts, J. L. Beckers and Th. P. E. M. Verheggen, *Isotachophoresis —Theory, Instrumentation and Applications*, Elsevier, Amsterdam, Oxford, New York, 1976.
9 F. E. P. Mikkers, F. M. Everaerts and J. A. F. Peek, *J. Chromatogr.*, 168 (1979) 317.
10 F. Mikkers and F. Everaerts, in F. M. Everaerts (Editor), *Analytical Isotachophoresis (Analytical Chemistry Symposia Series*, Vol. 6), Elsevier, Amsterdam, Oxford, New York, 1981, pp. 1–17.
11 P. Boček, P. Gebauer and M. Deml, *J. Chromatogr.*, 217 (1981) 209.
12 P. Gebauer and P. Boček, *J. Chromatogr.*, 242 (1982) 245.
13 I. Nukatsuka, M. Taga and H. Yoshida, *J. Chromatogr.*, 205 (1981) 95.
14 I. Nukatsuka, M. Taga and H. Yoshida, *Bull. Chem. Soc. Jap.*, 54 (1981) 2629.
15 F. E. P. Mikkers, F. M. Everaerts and J. A. F. Peek, *J. Chromatogr.*, 168 (1979) 293.
16 J. P. M. Wielders and F. M. Everaerts, in B. J. Radola and D. Graesslin (Editors), *Electrofocusing and Isotachophoresis*, Walter de Gruyter, Berlin, New York, 1977, pp. 527–547.
17 P. Gebauer, P. Boček, M. Deml and J. Janák, *J. Chromatogr.*, 199 (1980) 81.
18 P. Boček, P. Gebauer and M. Deml, *J. Chromatogr.*, 219 (1981) 21.
19 L. G. Sillén and A. E. Martell, *Stability Constants of Metal-Ion Complexes*, Special Publication No. 17, Chemical Society, London, 1964.
20 Landolt-Börnstein, *Zahlenwerte und Funktionen aus Physik-Chemie-Astronomie-Geophysik und Technik*, Vol. 2, Part 7, Springer, Berlin, Göttingen, Heidelberg, 6th ed., 1960.
21 V. Jokl, *J. Chromatogr.*, 13 (1964) 451.
22 Y. Kiso and T. Hirokawa, *Chem. Lett.*, (1980) 745.
23 J. Dvořák, J. Koryta and V. Boháčková, *Elektrochemie*, Academia, Prague, 2nd ed., 1975 (in Czech).
24 P. Boček, M. Deml and J. Janák, *J. Chromatogr.*, 106 (1975) 283.
25 P. Boček, *Top. Curr. Chem.*, 95 (1981) 131.
26 T. Hirokawa and Y. Kiso, *J. Chromatogr.*, 242 (1982) 227.
27 T. Hirokawa and Y. Kiso, *J. Chromatogr.*, 252 (1982) 33.