Isotachophoresis: the concepts of resolution, load capacity and separation efficiency. II: Experimental evaluation

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ISOTACHOPHORESIS: THE CONCEPTS OF RESOLUTION, LOAD CAPACITY AND SEPARATION EFFICIENCY

II. EXPERIMENTAL EVALUATION

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SUMMARY

Resolution, load capacity and separation efficiency in isotachophoresis are experimentally evaluated and the results are compared with the theoretically expected values. The theoretical and experimental results show good agreement and confirm the reliability of the transient-state model. The importance of the dimensionless separation number and load capacity for the determination and standardization of experimental performance conforms with theory. It is shown that, in view of these two parameters, the pH of the leading electrolyte is the best rationale for optimization, whereas the pH of the sample has only restricted possibilities. Steady-state configurations in which constituents are not migrating in order of decreasing effective mobilities are shown and discussed.

INTRODUCTION

Resolution in isotachophoresis has been defined as the fractional separated amount of the constituent under consideration[1]. According to this definition, its numerical value may vary between the limiting values of unity and zero. At zero resolution no separation has occurred and the constituent forms an ideally mixed zone with at least one other constituent. Obviously the maximal resolution value of unity should be reached in the shortest time possible and with the most convenient experimental conditions. It follows that the resolution rate must be maximized.

In Part I[1] three rationales and their theoretical background were considered. It was shown that any optimization procedure must act directly on the transient state, in which the sample constituents are separating according to the moving boundary principle. The mixed zones being resolved during the transient state have well defined characteristics governed by the Kohlrausch regulating function concept[2]. By a proper

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choice of operating conditions and operational systems, the mixed zone characteristics that influence the separating power can be well controlled. The result is a steady state in which constituents are migrating in a well defined order with a definite velocity towards a detection system. Using a constant electrical driving current, constituents will migrate with equal velocity through the detector, allowing an easy qualitative and quantitative evaluation[3].

In Part I[1] the three optimization rationales were given as the electrical driving current, the mobility of the counter constituent and the chemical equilibria of the electrolytes. The electrical driving current acts directly on the time needed to resolve a sample. In order to separate a given sample a definite number of coulombs are necessary, but the time interval in which this amount must be delivered is immaterial[2], *i.e.*, the time interval is restricted within its limiting values by diffusional and convective forces.

The time for resolution and the electrical driving current are inversely related, so the latter must be maximized. Neglecting temperature effects, the driving current has no influence on the efficiency of the separation process. It follows that for a given sample the length of the separation compartment is independent of the driving current.

The mobility of the counter constituent acts directly on the transport efficiency. When performing isotachophoretic analyses it is disadvantageous to transport the counter constituent, in which there is no interest, at a relatively high migration rate. In order to obtain a high transport efficiency the ionic mobility of the counter constituent should be as low as possible[3].

The properties of dissociation and complex formation can be used to optimize the separation efficiency. It must be recognized however, that the mobility of the counter constituent has some (marginal) influence on this efficiency. For an optimal separation it has been shown that the ratio of effective mobilities in the mixed state is of decisive importance[1]. Whenever this ratio is unity no separation will occur, as the migration rates, given by the product of the electrical field strength and the effective mobility, in this instance will be identical. Obviously, the mobility ratio must be minimized or maximized depending on the separation configuration[1]. A well known mechanism influencing effective mobilities selectively is given by the dissociation and complex formation equilibria[3,4]. In the former instance the pH can be used in optimization procedures, and until now it has been the most frequently used parameter[3]. For anionic separations optimal conditions will generally be found at low pH, whereas for cationic separations a high pH is preferable.

The direct result of optimal separation efficiency will be a favourable time for resolution and optimal load capacity. The separation efficiency is best expressed by the dimensionless separation number[1], which has its maximal value at unity. This separation number is, neglecting temperature effects, independent of various operating conditions such as the electrical driving current and column geometry. It is, however, strongly affected by the nature of the applied electrolytes and therefore can be used to compare the efficiency of analyses. The load capacity gives direct information on the amount of constituent that can be sampled in a given operational system. Again, this parameter is independent of various operating conditions.

The load capacity can be optimized by following the same rationales as for the separation efficiency[5]. Both parameters can be used to evaluate the experimental performance of isotachophoretic separations.

EXPERIMENTAL

All experiments were performed using the isotachophoretic equipment developed by Everaerts *et al.*[3]. The separation compartment consisted of PTFE narrow-bore tubing with I.D. 0.45, 0.2 or 0.15 mm and corresponding O.D. 0.75, 0.4 and 0.3 mm. The direct constant electrical driving current was obtained from a modified Brandenburg (Thornton Heath, Great Britain) high-voltage power supply. Potential gradient detectors, used in either the potential gradient or the conductance mode, were used for the determination of transient-state and steady-state characteristics. All chemicals used were of pro analisi grade or additionally purified by conventional methods. Operational systems are summarized in Table I. Theoretical calculations were performed with the computerized transient-state model[5] and physico-chemical data were taken from refs. 6, 7 and 8.

TABLE I

OPERATIONAL SYSTEMS

Parameter	*System No.*				
	1	*2*	*3*	*4*	*5*
pH of leading electrolyte	3.60	4.03	4.60	5.04	6.02
Leading constituent	Cl^-	Cl^-	Cl^-	Cl^-	Cl^-
Concentration (M)	0.01	0.01	0.01	0.01	0.01
Counter constituent*	BALA	GABA	EACA	CREAT	HIST
Terminating constituent	C_2H_5COOH	C_2H_5COOH	C_2H_5COOH	C_2H_5COOH	MES**
Concentration (M)	0.005	0.005	0.005	0.005	0.005
Additive to leading electrolyte	0.05% MOWIOL***	0.05% MOWIOL	0.05% MOWIOL	0.05% MOWIOL	0.05% MOWIOL
Temperature	Ambient	Ambient	Ambient	Ambient	Ambient
Electrical driving current (A/cm^2):					
d_t = 0.45 mm	0.0503	0.0503	0.0503	0.0503	0.0503
d_t = 0.20 mm	0.0796	0.0796	0.0796	0.0796	0.0796
d_t = 0.15 mm	0.1415	0.1415	0.1415	0.1415	0.1415

* BALA = β-alanine; GABA = γ-aminobutyric acid; EACA = ε-aminocaproic acid; CREAT = creatinine; HIST = histidine.

** MES = 2-(N-morpholino)ethanesulphonic acid.

*** MOWIOL = polyvinyl alcohol, N-88, Hoechst, Frankfurt, G.F.R.

RESULTS AND DISCUSSION*

Transient-state characteristics can be easily obtained experimentally and several important parameters can be evaluated directly as most are interrelated[1]. For isotachophoretic analyses it is most convenient to use a separation compartment of well defined and constant volume and to apply a constant electrical driving current. Using a fixed point detector and a given operational electrolyte system, all characteristics can be evaluated by injection of known amounts of sample and accurate measurement of all electrical gradient and time events. Because, under these con-

* For symbols and abbreviations, see Part I[1].

ditions, the amount of the leading constituent filling the separation compartment, n_L^{load}, is constant, the first boundary that reaches the detector will always be registered after the same time interval, t_{detfix}:

$$t_{detfix} = n_L^{load} \cdot \frac{F}{I}(1 - r_C) \tag{1}$$

where F is the Faraday constant, I the applied electrical driving current and r_C the ionic mobility of the counter constituent, relative to the leading constituent. Experimentally, the amount of leading constituent can be determined by the injection of a known amount of leading constituent, Δn_L, and measurement of the resulting time delay, Δt, with respect to t_{detfix}:

$$n_L^{load} = t_{detfix} \cdot \frac{\Delta n_L}{\Delta t} \tag{2}$$

Moreover, because for a one-constituent zone the dimensionless separation number, S, is identical with the transport number[9], T, the experimental and theoretical transport efficiency can be compared:

$$T = \frac{F}{I} \cdot \frac{n_L^{load}}{t_{detfix}} = \frac{m_i}{\sum_i m_i} \tag{3}$$

where m_i is the ionic mobility of the constituent i. It should be noted that the transport number of monovalent weakly ionic constituents does not contain effective mobilities, but rather ionic mobilities, because, owing to electroneutrality, the degree of dissociation cancels out. Some experimental results are given in Table II.

TABLE II

CHARACTERISTICS OF THE LEADING ELECTROLYTE

Parameter	*Value*
Leading constituent, chloride	$m_{Cl^-} = -77 \cdot 10^{-5}$ cm²/V · sec
Concentration	$\bar{c}_L^L = -0.01\ M$
Counter constituent, γ-aminobutyric acid	$m_{GABA} = 30 \cdot 10^{-5}$ cm²/V · sec
	$pK_{GABA} = 4.03$
Electrical driving current	$I = 80\ \mu A$
Diameter of separation compartment	$d_i = 0.45$ mm
Appearance of the first boundary	$t_{detfix} = 1112$ sec
Amount of leading constituent sampled	$\Delta n_L = 100$ nmole
Time delay to amount sampled	$\Delta t = 59.2$ sec
Response	$\frac{\Delta n_L}{\Delta t} = 0.592$ nmole/sec
Load of leading constituent	$n_L^{load} = 658$ nmole
Transport number: experimental	$T_{exp} = 0.714$
theoretical	$T_{theor} = 0.720$
Transport efficiency: experimental	$\varepsilon_{exp} = 71\%$
theoretical	$\varepsilon_{theor} = 72\%$

Eqn. 3 is useful, as it provides a practical procedure for the determination of the amount of the leading constituent, once the ionic mobilities and t_{detfix} are known. It also provides a method for the determination of counter-constituent mobilities from experimental results, as

$$r_C = 1 - \frac{1}{T_{exp}} \quad (4)$$

Obviously, a high transport efficiency, due to a low ionic mobility of the counter constituent, is always favourable as it guarantees efficient use of the power applied. For the operational systems given in Table I we can expect transport efficiences between 70 and 75%. The characteristics of a separation process can be evaluated by the injection of known amounts of sample[5]. An example of such a procedure is given in Fig. 1 and relevant parameters are summarized in Table III. The fact that, at a constant load of leading constituent, the first boundary will always be detected at the same time interval, t_{detfix}, is illustrated in Fig. 1 by the resolution line L/A. The low coefficient of variation confirms the excellent performance of the equipment. Injection of a small amount of sample will cause two zones, stacked between the leading constituent L and the terminating constituent T. A sample load of 1.3 μl of the constituent mixture (Fig. 1) where $n_A = 65$ will give a time-based zone length of 124.2 sec for constituent A and detection must be started 1112 sec after injection. The zone length of the second constituent, B, will be 148.1 sec. Other sample loads give

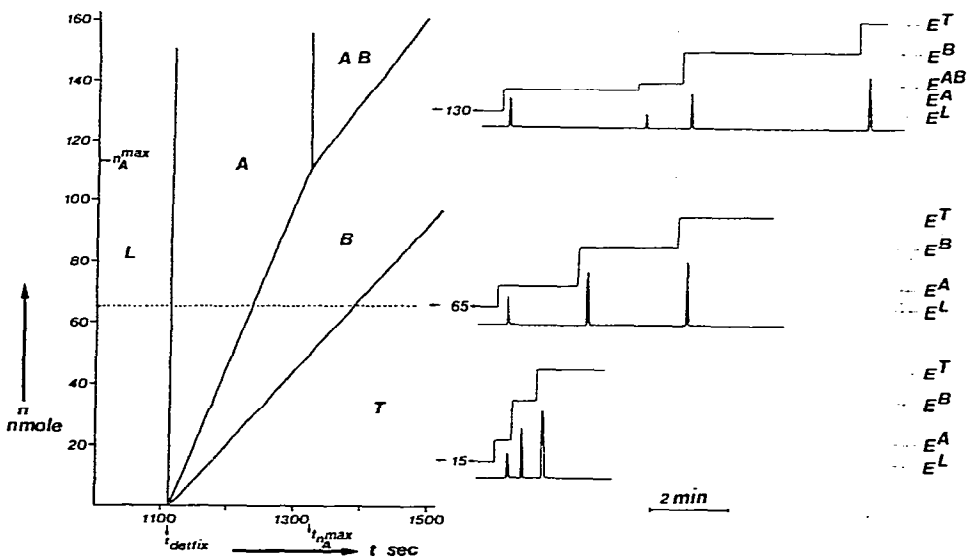

Fig. 1. Resolution lines for a two-constituent mixture. Operational system: Table 1, system No. 2, and Table II. L = chloride; A = formic acid; B = glycolic acid; T = propionic acid; $E^{L,A,B,T}$ = electrical field strength; n = amount sampled.
Sample: $\bar{c}^{sample}_{formate} = -0.05\ M$; $\bar{c}^{sample}_{glycolate} = -0.05\ M$; $pH^{sample} = 3.00$.

TABLE III

RESOLUTION DATA

For operational system see Fig. 1 and Table II. Resolution line: $n = at - b$ (nmole).

Boundary	*No. of determinations*	*a*		*b*		*Coefficient of variation or Correlation coefficient*
		Experimental	*Theoretical*	*Experimental*	*Theoretical*	
L/A	53	0	0	−1112	−1112	0.8%
A/B	13	0.525	0.530	584	590	1.000
B/T	45	0.242	0.251	270	279	1.000
A/AB	6	0	0	−1328	−1317	0.4%
AB/B	6	0.321	0.316	314	312	0.998

Parameter	*Experimental*	*Theoretical*
Load of leading constituent (n_L^{load})	658	647
Maximal sample load (n_A^{max})	113	108
Separation number (S_A)	0.103	0.099
(S_B)	0.103	0.099
Load capacity (C_{load})	0.172	0.167
Separation efficiency (ε, %)	21	20

proportional zone lengths. The characteristics of these steady-state zones have already been discussed extensively and the close agreement of the calculated and experimental resolution lines, *L/A*, *A/B* and *B/T*, indicates the reliability of the calculations.

As the separation compartment has a limited load capacity, at a high load a mixed zone will be detected. The characteristics of these mixed zones are determined by both the leading electrolyte and the sample and are constant with time, as long as they exist. The time interval, $t_{n_A}{}^{max}$, at which the mixed zone will be detected, is again constant, as illustrated in Fig. 1 by the resolution line *A/AB*:

$$t_{n_A}^{max} = t_{detfix} \frac{\bar{m}_L^L E^L}{\bar{m}_B^M E^M} \tag{5}$$

The maximal zone length for the resolved constituent *A*, on a time base, is given by $t_{n_A}{}^{max} - t_{detfix}$. The maximal sample load, n_A^{max}, is given by the intercept of the resolution lines *A/B*, *A/AB* and *AB/B*. For the given pair of constituents, formate and glycolate, the maximal sample load was 113 nmole, which was close to the theoretical value (Table III). From the maximal sample load the load capacity[1], C_{load}, and the dimensionless separation number[1], *S*, can be calculated directly. Optimal column dimensions can be obtained from the load capacity, whereas the dimensionless separation number gives the relationship between amount sampled and electrical driving current or time for resolution. The appropriate procedure is given in Fig. 2.

From the load capacity, C_{load}, of 0.172, it follows that for a sample that contains 5 nmole of both constituents, 29.1 nmole of chloride are necessary. The available volume can now be calculated once the concentration of the leading constituent has been chosen, $\bar{c}_L^L = 0.01$ mole/l. If the inside diameter of the separation compartment is 0.2 mm, the length must be 9.25 cm. Obviously, if we had chosen a higher concen-

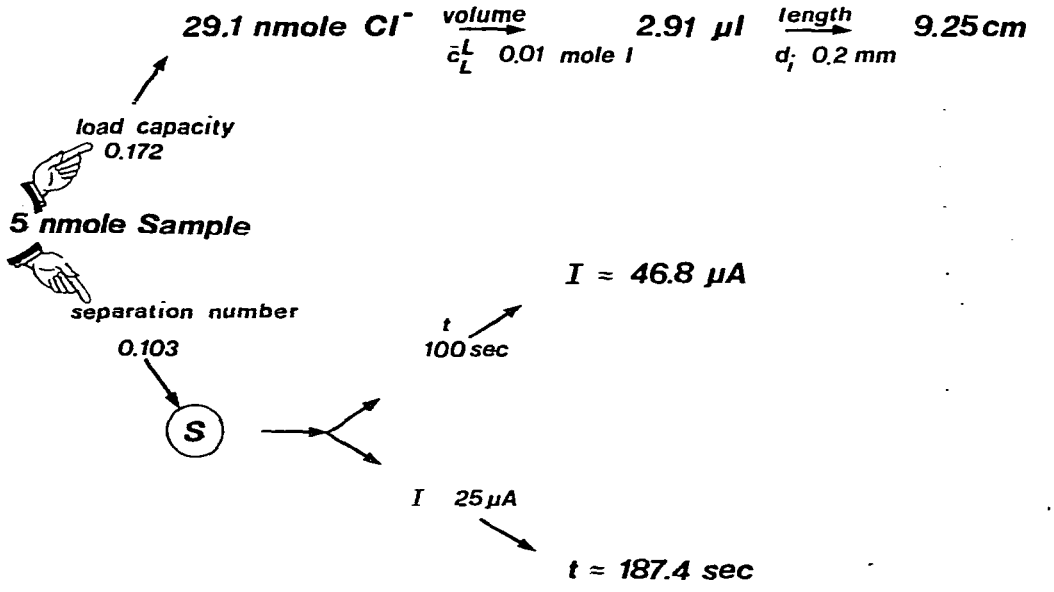

Fig. 2. Column evaluation.

tration of the leading constituent for the same dimensions of the separation compartment, the maximal amount of sample would have been proportionally higher. From the dimensionless separation number, $S = \frac{F}{I} \cdot \frac{n}{t_{res}}$ it follows that for a resolution time of 100 sec, an electrical driving current of 46.8 μA must be applied. At a driving current of 25 μA we can expect a resolution time of 187.4 sec. Although all resolution lines were determined experimentally, the load capacity and the separation number can be obtained with sufficient accuracy from only a few experiments, in which a mixed zone is present. The number of necessary determinations depends largely on the performance of the equipment. A high performance implies that the coefficients of variation in both t_{detfix} and $t_{n_A}{}^{\max}$ are low. It should be emphasized that the column evaluation in Fig. 2 applies to only one specific sample. In general, a sample will show fluctuations in composition and an appropriate safety margin should be considered.

In analytical practice the fluctuations in composition may be due to concentration and/or pH. Once the extreme values of these fluctuations are known, the safety margin can easily be calculated.

In the experimental determinations, not only time events are being registered but also potential gradients. As eqn. 5 contains only one unknown quantity, $\bar{m}_B^M$, the effective mobility of the trailing constituent in the mixed zone can be obtained from the experimental results. For the glycolate constituent in the mixed zone of Fig. 1, it follows that

$$\frac{\bar{m}_{\text{glycolate}}^{\text{Mixed}}}{\bar{m}_{\text{chloride}}^{\text{Leading}}} = \frac{1112}{1328} \cdot 0.481 = 0.403$$

Provided that the ionic mobility of glycolic acid is known, the pH of the mixed zone can be derived. Using the appropriate data and relationships it follows that $pH^M =$

4.28, which is very close to the theoretically expected value of 4.30. From this we can evaluate how much the ratio of effective mobilities in the mixed zone differs from the critical value of unity at which no separation can occur:

$$\frac{\bar{m}^{\text{Mixed}}_{\text{formate}}}{\bar{m}^{\text{Mixed}}_{\text{glycolate}}} = 1.37$$

It follows that this 37% deviation from unity is responsible for the separation of the two sample constituents.

From Fig. 1 and Table III we conclude that even in the presence of a mixed zone, the relationship between the total zone length of the sample and the amount sampled is still linear. Deviations[10,11] from this rule are the result of experimental inaccuracies such as hydrodynamic flow, improper injection and mixing of leading electrolyte with sample and/or terminating electrolyte.

From the separation number in Table III it follows that the separation efficiency, ε, is about 20%. As the separation process is strongly dependent on the pH of both the sample and the leading electrolyte, optimization by choosing suitable electrolytes should be possible. The effect of the pH of the sample has been given in Fig. 3. Owing to the small difference in the dissociation constants of the sample constituents glycolic acid and formic acid ($\Delta pK \approx 0.08$), the effect on the separation efficiency of the pH of the sample is minimal. For the given pair of constituents the theoretical and experimental results show good agreement. When the difference in the

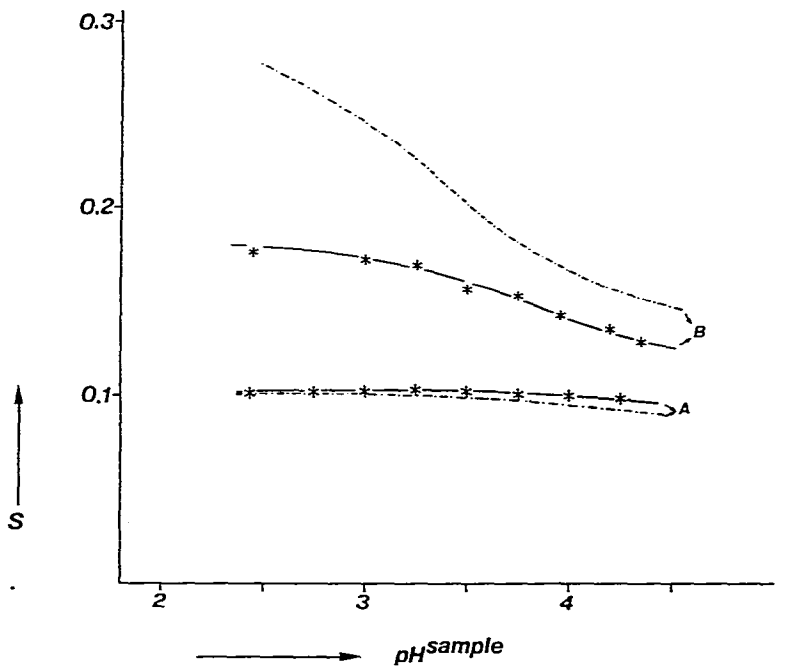

Fig. 3. Influence of the sample pH on the dimensionless separation number. Operational system: Table I, system No. 2. Sample: A = formate–glycolate, equimolar; B = chlorate–formate, equimolar. Solid line and points, experimental values; broken line, theoretical values.

dissociation constants is larger, the effect of the sample pH is much more pronounced, as confirmed by the constituent pair chlorate–formate. In this instance a low pH of the sample clearly favours resolution. Although theoretical considerations suggest a rapid, continuous increase in efficiency, decreasing the pH of the sample, the experimental curve indicates only a moderate increase. In the transient-state model[1] we made no allowance for the influence of a relatively high proton concentration at low pH. Functioning as a mobile counter constituent, protons, at a relatively high concentration, will decrease the efficiency of the separation process. When the constituents have only a small difference in their dissociation constants (formic acid–glycolic acid), the theoretical and experimental results will show only small differences.

When the pK values of the constituents differ substantially, higher pH shifts can be expected and larger deviations result, as confirmed by the constituent pair chlorate–formate.

Irrespective of the numerical discrepancy, for anionic separations a low pH of the sample favours resolution. Appropriate incorporation of the hydrogen and/or hydroxyl constituent into the relevant mathematical formulations is still under investigation, but seems complicated.

In common practice the pH of the sample shows only a small degree of freedom and the most useful optimization parameter is the pH of the leading electrolyte. In most instances a low pH of the leading electrolyte will increase the efficiency of the separation process, dealing with anionic separations. Table IV gives some experimental and theoretical results for the constituents in Fig. 3. From both the theoretical and experimental results it follows that for constituents that have only a small difference in their pK values, the pH of the leading electrolyte is not very useful for optimization. In our theoretical considerations we showed that the ratio of effective constituent mobilities is of importance when considering separability and separation efficiency.

Obviously, the pH of the leading electrolyte has only a minor influence on the mobility ratio when the pK differences are small. For the two samples in Table IV, the ratio of effective mobilities is given in Fig. 4 as a function of the pH of the mixed zone. It can easily be shown that for the limiting values of the mobility ratio it is valid that:

$$10^{(pK_A - pK_B)} \cdot \frac{m_B}{m_A} < \frac{\bar{m}_B}{\bar{m}_A} < \frac{m_B}{m_A} \qquad (6)$$

TABLE IV

INFLUENCE OF THE pH OF THE LEADING ELECTROLYTE ON THE DIMENSIONLESS SEPARATION NUMBER

Parameter		*Constituents*			
		Chlorate–formate		*formate–glycolate*	
Concentration (M)		−0.05,	−0.05	−0.05,	−0.05
pH^{sample}		2.41		2.51	
pH^L	*Counter constituent*	S_{exp}	S_{theor}	S_{exp}	S_{theor}
3.60	BALA	0.259	0.368	0.095	0.099
4.03	GABA	0.179	0.275	0.098	0.100
6.02	HIST	0.075	0.105	0.101	0.092

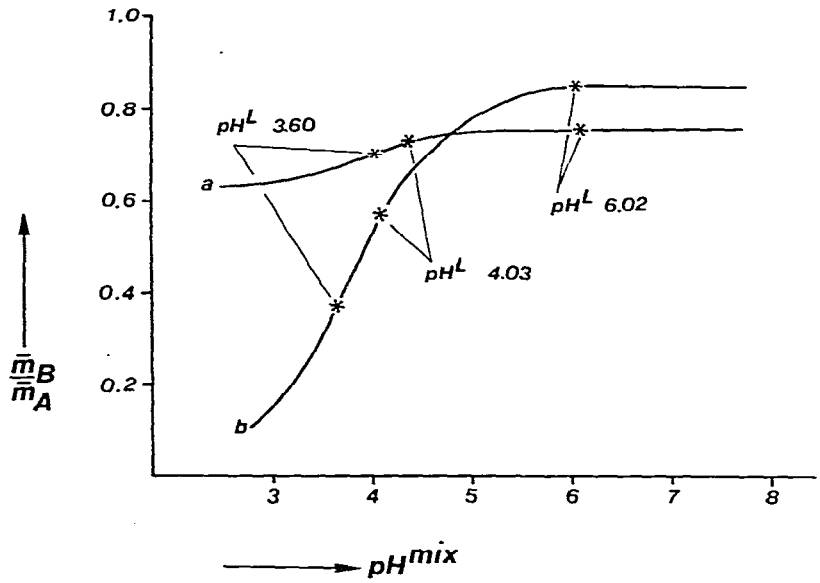

Fig. 4. Influence of the pH of the mixed zone on the ratio of effective constituent mobilities. Operational systems: Table I. Sample: (a) formate (*A*)–glycolate (B); (b) chlorate (*A*)–formate (*B*).

Therefore, for the constituent pair chlorate–formate the ratio of effective mobilities can vary between its maximal value of 0.764 at high pH and its minimal value of 0.635 at low pH. Hence any pH shift, due to either the leading electrolyte or the sample, has hardly any influence on the separation efficiency. Owing to the relatively low mobility of the counter constituent histidine, the separation at $pH^L = 6.02$ has the greater efficiency, although the differences are slight. When differences in pK_A values are more substantial, the rationale for optimization is clearer, as can be seen from the second constituent pair, chlorate–formate, in Table IV and Fig. 4. In this instance the ratio of effective constituent mobilities can vary between zero at low pH and 0.846 at high pH, and therefore the pH can be of great importance. A high pH of the leading electrolyte will cause a high pH of the mixed zone, resulting in an unfavourable ratio of effective constituent mobilities. A low pH of both the leading electrolyte and the sample will result in an optimal ratio and therefore optimal separation efficiency.

From Fig. 4 it can also be seen that for small differences in pK values, the difference in the pH values of the mixed zone and the leading zone is relatively high. When the sample contains stronger acids, this difference is considerably smaller, resulting in a relatively low pH of the mixed zone. Whenever the pH of the leading electrolyte is substantially higher than the pK values of the constituents to be separated, the difference will be small and the constituents will be resolved as ionic species. From the examples given it follows directly that a separation based on pK values is generally more efficient than one based on ionic mobilities.

For anionic straight pairs of constituents, where $m_B < m_A$ and $pK_A < pK_B$, the rationale for optimization is straightforward: low pH of the leading electrolyte and the sample. With anionic reversed pairs[1], where $m_B < m_A$ and $pK_A > pK_B$, this

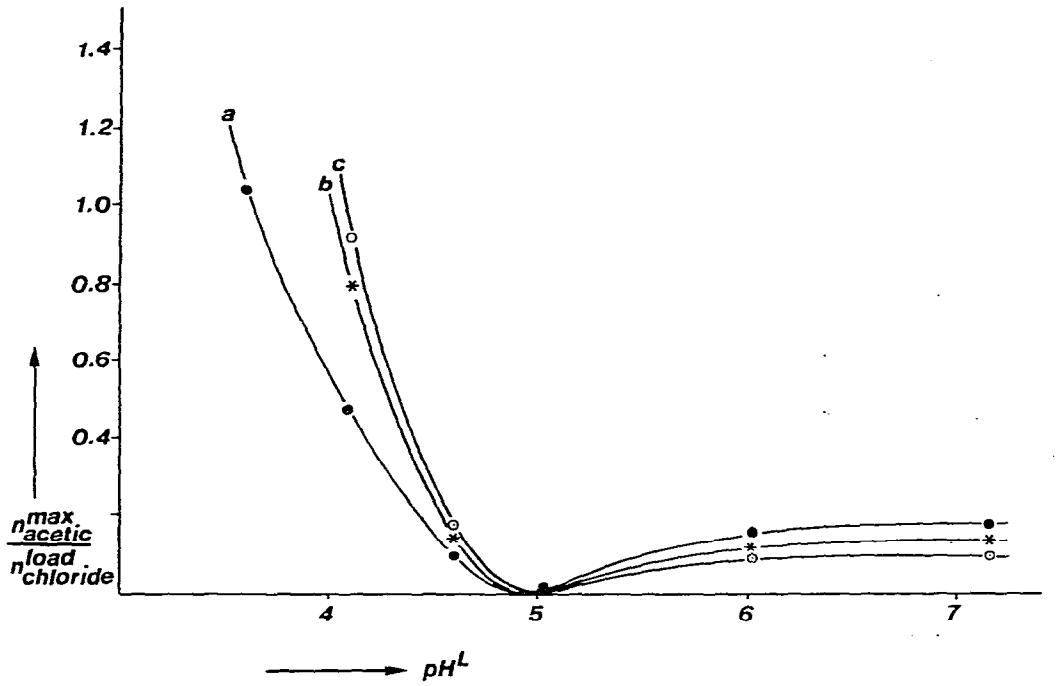

Fig. 5. Influence of the pH of the leading electrolyte on the load capacity for a reversed pair of constituents. Operational systems: Table I. Constituent data: Table V. Sample: acetate–naphthalene-5-sulphonate. (a) $pH^{sample} = 7.0$; (b) $pH^{sample} = 4.75$; (c) $pH^{sample} = 3.00$.

rationale is more complicated. It has been shown that for such pairs a pH will exist, pH^{MO}, at which no separation occurs[1]. Of course, this pH will cause an infinite time for resolution, zero separation number and zero load capacity. Moreover, at this critical pH the order in which the constituents migrate will be reversed. Experimental results concerning the load capacity for a reversed pair are given in Fig. 5 as a function of the pH of the leading electrolyte and the sample. The experimental curves confirm the theoretically predicted behaviour. Using the appropriate data (Table V) and relevant mathematical formulations[1], it follows that the criterion for separation[1] will not be satisfied at a mixed zone pH of 5.19. Obviously, this pH can be generated by numerous combinations of leading electrolytes and sample compositions. Working at the maximal buffering capacity of the common counter constituent, *i.e.*, $\varrho = -2$

TABLE V

DATA FOR A REVERSED PAIR OF SAMPLE CONSTITUENTS

Constituent	*Mobility ($cm^2/V \cdot sec$)*	*pK*	*Concentration (M)*	pH^{sample}
Acetate	$-41 \cdot 10^{-5}$	4.75	−0.005	4.75
Naphthalene-2-sulphonate	$-30 \cdot 10^{-5}$	0	−0.005	4.75

No separation at $pH^{MO} = 5.19$

No separation at $pH^L = 4.98$ ($\varrho = -2$, $m_C = 30 \cdot 10^{-5}$ $cm^2/V \cdot sec$)

or $pH^L = pK_C$, and introducing an acceptable ionic mobility for the counter constituent, $m_C = 30 \cdot 10^{-5}$ cm^2/V·sec, the critical pH of the leading electrolyte, at which no separation occurs, is 4.98.

This was confirmed experimentally by the separation at $pH^L = 5.04$, at which hardly any load capacity was present. At a pH^L higher than the critical value, sample constituents migrate in order of ionic mobilities, and separations can be performed with only moderate efficiency. At low pH^L, however, constituents are migrating in order of their pK values and a much greater efficiency can be obtained, resulting in a high load capacity. For example, the resolution of a 1.5-nmole sample (an absolute amount that can be detected without difficulty) would take about 18 sec, $S = 0.26$, at $pH^L = 4.10$ and $pH^S = 3.00$, whereas the same sample can be resolved in 105 sec, $S = 0.045$, at $pH^L = 7.10$.

The required length of the separation compartment in the former instance is 5.8 times shorter than in the latter. Obviously, for specific samples rigid optimization procedures can be followed, resulting in very short analysis times, small dimensions of the separation compartment and efficient use of the power applied. It must be emphasized, however, that the success of optimization procedures depends largely on the physico-chemical characteristics of the species to be separated and the performance of the equipment. When there are only small differences in ionic mobilities and dissociation constants, optimization procedures are elaborate and result in only a small increase in efficiency. The results from Fig. 5 confirm the predicted behaviour[1] that for anionic reversed pairs a high pH of the leading electrolyte is best, combined with a high pH of the sample, whereas at low pH^L the separation efficiency is favoured by a low sample pH. The theoretical background for this exceptional behaviour has already been extensively discussed. An evaluation of the dual separation phenomenon[1] will be given in a later paper.

Another important conclusion can be drawn from the results shown in Fig. 5. It follows that efficient separations can be achieved whenever the sampling ratio, q (i.e., the concentration of the charged trailing species divided by the concentration of the charged leading species), is small. Biochemical samples often contain substantial amounts of very mobile species such as chloride or perchlorate. Such samples represent typical low "q cases", which can be separated on a relatively short column. The time of analysis, however, will increase substantially in the presence of these mobile species. In very special cases lowering of the sample pH by the addition of, for example, hydrochloric acid will increase the load capacity by the suggested mechanism.

The dimensionless separation number seems to be a reliable quantity for describing the separation performance, as it is independent of operating conditions such as the electrical driving current and column geometry. Table VI gives the observed experimental efficiencies for the same sample under different operating conditions. From these results it follows that, although differences in the separation numbers occur, the overall efficiency is not significantly different. In our theoretical considerations we introduced relative mobilities to suppress the influence of temperature effects. Comparing literature data on ionic mobilities[6–8], it must be concluded that temperature effects are eliminated only partially in this way, as many non-linear effects occur. Moreover, when considering temperature effects, dissociation constants should also be corrected. Mathematical iteration procedures to involve temperature

TABLE VI

INFLUENCE OF THE INSIDE DIAMETER OF THE NARROW BORE TUBE ON THE DIMENSIONLESS SEPARATION NUMBER

Sample: A, formate (−0.05 *M*); B, glycolate (−0.05 *M*); $pH^{sample} = 2.41$. For operational system, see Table I, system No. 2.

Parameter	*Inside diameter (d_i, mm)*		
	0.45	*0.20*	*0.15*
Driving current (I, μA)	80	25	25
Sample load (n_A^{max}, nmole)	87.5	23.5	16.6
(n_B^{max}, nmole)	87.5	23.5	16.6
Time for resolu ion (t_{res}, sec)	1081	1030	703.7
Separation number (S_{theor})	0.100	0.100	0.100
($S_{A,exp}$)	0.098	0.088	0.091
($S_{max,theor}$)	1.000	1.000	1.000
Efficiency (ε, %)	19.6	17.6	18.2

corrections for various physico-chemical parameters can be introduced into the transient-state model with a probable consequent increase in accuracy. There is, however, a lack of reliable data on physico-chemical parameters and the present model is sufficiently reliable, the predicted parameters being confirmed experimentally.

From the theoretical formulations, it follows that the measurement of steady-state effective mobilities can only be used as an indication for experimental separability[1]. Constituents that have equal steady-state effective mobilities can sometimes be separated efficiently. Moreover, it has been shown that enforced isotachophoretic configurations[12] in which a more mobile is migrating behind a less mobile constituent are stable with respect to time. An example is given in Fig. 6.

Several sample constituents confirm the general principle that constituents in isotachophoresis are migrating at equal velocity in order of decreasing effective mobilities. The constituents lactic and mandelic acid (constituents 3 and 4), however, show virtually no difference in effective mobilities, as for their isotachophoretic migration the same electrical gradient seems to be necessary (Fig. 6 and Table VII). From the linear conductance trace it appears that this pair has not resolved during the separation process. The UV trace, however, indicates clearly that the mandelic acid (3) has been resolved from the lactic acid (4) and that the former migrates in front of the latter. The transient-state model[1] reveals that the pH of the mixed zone, from which the pure zones are formed, is just below the critical pH of 4.32 at which no separation occurs. As this is a reversed pair, the mandelic acid will be resolved in front of the lactic acid.

From the data in Table VII it follows that the experimental and theoretical zone characteristics are in good agreement. The minor difference between the transient-state and the steady-state results, X3 (ref. 12), has already been mentioned. The occurrence of a lactic acid ion in the resolved mandelic acid zone will cause a 2.6% deviation from the critical value of unity for the ratio of effective constituent mobilities. Mandelic acid ions, in the resolved lactate zone, would lead to a 1% deviation. From the UV trace it follows that these deviations are large enough to guarantee a sharp separation boundary. The theoretical calculations show a greater difference and for the ratio of effective mobilities in the mixed zone a 2.6% deviation from unity

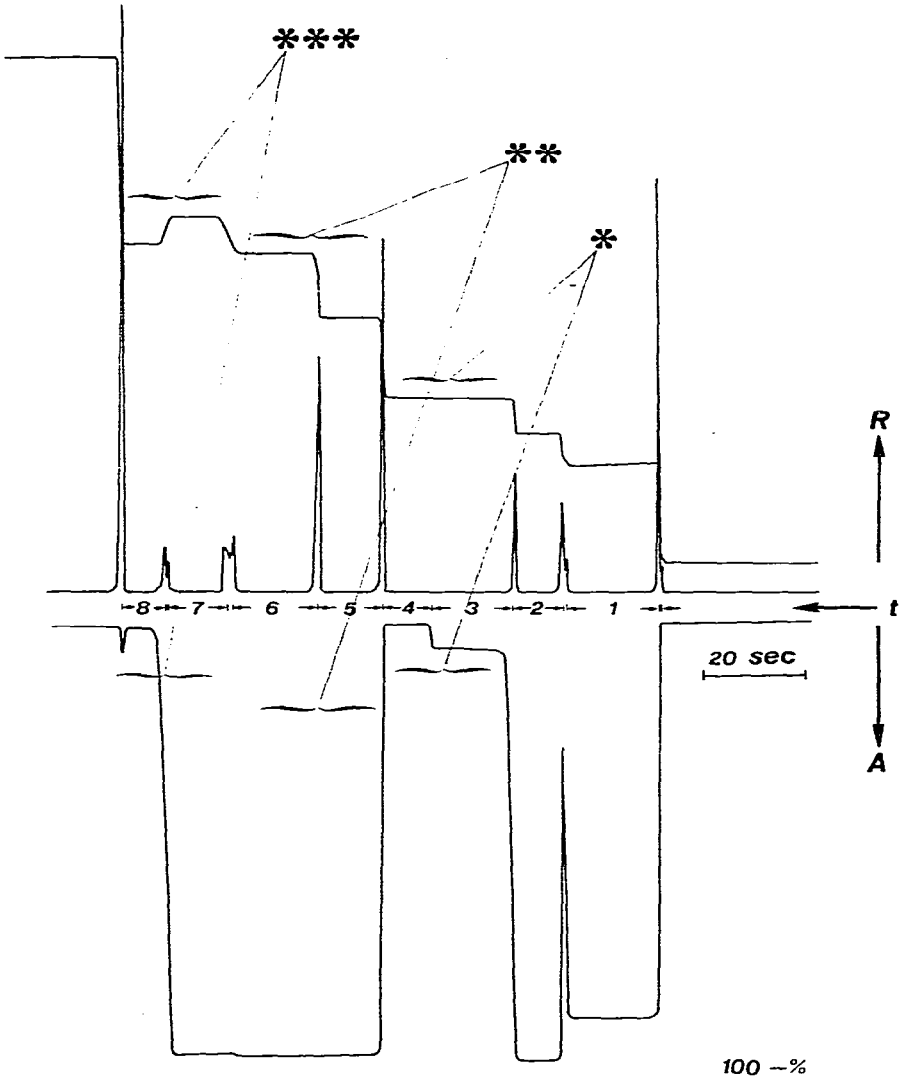

Fig. 6. Isotachophoretic steady-state configurations. Operational system: Table I, system No. 2, $pH^L = 3.95$. R = resistance; A = UV absorption at 254 nm; t = time. 1 = Adenosine-5′-triphosphoric acid (A5TP); 2 = sulphanilic acid; 3 = DL-mandelic acid; 4 = DL-lactic acid; 5 = guanosine-5′-monophosphoric acid (G5MP); 6 = adenosine-3′-monophosphoric acid (A3MP); 7 = adenosine-5′-monophosphoric acid (A5MP); 8 = acetic acid. * A pair of constituents for which conductometric detection indicates no resolution whereas UV detection does; ** a pair of constituents for which UV detection indicates no resolution whereas conductometric detection does; *** a pair of constituents in an enforced isotachophoretic configuration.

was calculated. The experimental separation confirms that this deviation is sufficient to obtain resolution. It must be emphasized, however, that the small deviation results in a low separation efficiency and column overloading can easily occur. Fig. 6 nevertheless indicates clearly that isotachopherograms in which only one detection system

TABLE VII

COMPARISON OF ZONE CHARACTERISTICS

Trans = computerized transient-state model[5]; X3 = computerized steady-state model[12].

Parameter	*Chloride zone, experimental*	*Mandelate zone*			*Lactate zone*		
		Trans	*X3*	*Experimental*	*Trans*	*X3*	*Experimental*
pK	− 2	3.37			3.86		
m (cm^2/V · sec)	$-77 \cdot 10^{-5}$	$-28 \cdot 10^{-5}$			$-33 \cdot 10^{-5}$		
pH	3.95	4.21	4.22	4.25	4.27	4.28	4.29
E^L/E^X	1.00	0.319	0.320	0.322	0.318	0.319	0.322
c_X^X (mM)	−10.00	−6.47	−6.34		−7.16	−7.06	
$\frac{\bar{m}_{lactate}^{mandelate\ zone}}{\bar{m}_{mandelate}^{mandelate\ zone}}$		0.956	0.960	0.974			
$\frac{\bar{m}_{mandelate}^{lactate\ zone}}{\bar{m}_{lacate}^{lactate\ zone}}$					1.027	1.017	1.010
$\frac{\bar{m}_{mandelate}^{mixed\ zone}}{\bar{m}_{lactate}^{mixed\ zone}}$	1.027						

is used must be interpreted with great care. The same applies, of course, when only UV detection is used. From the UV trace in Fig. 6 it would be concluded that the nucleotides G5MP and A5MP have not been resolved. The conductance trace, however, clearly confirms the separation of these two constituents. On most occasions small amounts of impurities, with either UV-absorbing or non-UV-adsorbing properties, will indicate the separation boundary. Moreover, in this particular instance, a difference is visible when the UV results are being traced in the absorbance mode.

The sample constituents A5MP and acetate (constituents 7 and 8) are migrating in an enforced isotachophoretic configuration. The effective mobility of the acetate constituent in its proper zone is higher than that of the nucleotide A5MP in its proper zone, as indicated in Fig. 6 by the lower conductance of zone 7 in comparison with zone 8. For the relative effective mobilities it follows that $\bar{m}_{Acetate}^{Acetate}/\bar{m}_{Chloride}^{Chloride} = 0.212$ and $\bar{m}_{A5MP}^{A5MP}/\bar{m}_{Chloride}^{Chloride} = 0.198$.

The 7% deviation from unity of the mobility ratio ($\bar{m}_{A5MP}^{A5MP}/\bar{m}_{Acetate}^{Acetate} = 0.93$) allows a satisfactory sharpness of the separation boundary between the two constituents. The reason for the stability can be found in the difference in the pH values in the two resolved zones. Using the appropriate relationship it follows that the pH of the acetate zone is 4.57. A nucleotide ion, lost owing to convection or diffusion from its proper zone (7) into the acetate zone, will migrate with an higher effective mobility than that of the acetate constituent. In the nucleotide zone the pH is 4.32, so any acetate ion in the nucleotide zone will migrate with a considerably lower velocity than the nucleotide $\bar{m}_{A5MP}^{A5MP}/\bar{m}_{Acetate}^{A5MP} = 1.37$.

Hence the self-restoring capabilities of the separation boundary allow the enforced isotachophoretic configuration to be stable with respect to time. It should be noted, however, that enforced isotachophoretic configurations will not be encountered frequently in practice.

From both our previous theoretical considerations[1] and the experimental evaluation presented here, it follows that through optimization a considerable increase in separation efficiency and load capacity and a decrease in time for resolution can be obtained. It must be emphasized that the success of such a procedure depends largely on the nature of the sample. We restricted our theoretical and experimental studies mainly to two constituent samples but the same optimization rationales hold, to a lesser extent, for multi-constituent samples[5]. For very complex mixtures, in which multi-component information must be obtained, optimization can sometimes be elaborate and difficult. Analyses in more than one operational system are inevitable. Moreover, in one-component analyses of multi-constituent samples a considerable amount of effort is put into the separation of constituents of little interest. Column-switching techniques[13] may prove useful here.

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