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ANALYTICAL ISOTACHOPHORESIS

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SUMMARY

A survey is given of the latest research on isotachophoresis, especially instrumentation aspects and the application of analytical isotachophoresis. A complete scheme for the linear conductivity detector, to be used with the set of micro-sensing electrodes in direct contact with the electrolytes, is given, and a circuit for a potential gradient detector is also shown.

Possible applications of analytical isotachophoresis are considered, and isotachopherograms are given in order to show the way in which information can be deduced from the apparatus described. Arbitrarily chosen examples include small anionic and cationic ionic species, amino acids, peptides and proteins. Analyses in water and deuterium oxide are compared in order to demonstrate the differences in these very similar solvents. A new pump for a counter flow of electrolyte is briefly described.

INTRODUCTION

About 13 years ago, the first succesful experiments with analytical isotachophoresis (known then as displacement electrophoresis) were performed in our laboratory[1]. There is now much interest in this technique, in spite of the fact that only a small number of workers, mainly in Europe, is working on its evaluation. The physical nature of isotachophoresis has been re-evaluated elegantly by several workers. From the start of isotachophoresis, however, there was mainly a need for an analytical (qualitative and quantitative) model and several theoretical approaches have been made[2–4], resulting in different computer programs. These have been shown to be in good agreement with one another and with the experimental data. No doubt certain aspects will have to be reconsidered, but at the moment the theoretical models provide a reliable basis for the experimental work.

The development of a reliable detection system was important for the further evaluation of isotachophoresis. The early, very reliable, but low-resolution, thermometric detector has been replaced several years ago by better high-resolution detector systems, *viz.*, conductivity, potential gradient and UV absorption detectors. No doubt other detectors can and will be developed, although at the moment there is no urgent need for them. Reliability and reproducibility are the essential features of the detectors

chosen, and we shall therefore present in this paper some relevant details of the detection systems used in our equipment.

Excellent power supplies and other auxiliary equipment have been commercially available for many years. Injection blocks, injection valves, electrode compartments and capillary tubing are the result of skilful design and very accurate machining. The flexibility of isotachophoretic equipment is important, and we shall therefore give details of the equipment we have developed especially for analytical isotachophoresis.

The main requirements in analytical isotachophoresis can be stated as follows.

(i) Isotachophoretic experiments need to be carried out under uniform conditions and hence the analytical results will have a general accessibility for interpretation. Therefore, we shall describe some new operational systems that were especially selected for their optimal characteristics and wide applicability.

(ii) In isotachophoresis, the reliability and accuracy are merely a function of the detection systems and other operating conditions. Thus, the chemicals used in the operational systems should be of at least pro analysi grade and if necessary further purified by recrystallization, distillation or ion exchange. The detection systems should have no drift, noise or flutter. The operating conditions, *e.g.*, current density and temperature, should be stated precisely.

(iii) The time of analysis is dependent mainly on the operating conditions, the operational system and the sample involved, and may vary from 2 to 90 min. The lower time limit has restricted applicability, while an average time of analysis of 10–15 min is appropriate for most analytical problems.

The initial great expectations of the use of a counter flow of electrolyte are now more modest, because it has been shown that the disturbance of the zone profiles cannot be neglected and is almost impossible to depress. A new means of regulating the counter flow of electrolyte will be given, because a counter flow of electrolyte can be applied succesfully if the differences in concentration between the ionic species of the sample are great and the differences in effective mobility are sufficient.

OPERATIONAL SYSTEMS

Tables I–V list operational systems for anionic and cationic separations, these systems being used in the analyses described later. Special attention should be paid to the purity of the chemicals, especially if experiments with a counter flow of electrolyte are considered[5–7]. Recognizing the moving-boundary influence of impurities in the electrolytes, both qualitative and quantitative information are lost to a great extent, in spite of the use of correction factors[8]. For this reason, the electrolyte systems applied must be carefully controlled for impurities and, once the level of impurities has been established, one can decide whether it is acceptable to work with the selected operational system or not[9]. From our experience, we know that most chemicals need to be purified before they can be applied in isotachophoretic experiments[10].

If terminators are sought with smaller effective mobilities, if a choice can be made ionic species should be selected that have a low effective mobility because of the pK_a value. The pH of the terminating electrolyte may play an important role, while its concentration may also influence the final result in many instances.

Although the displacing concentrations of consecutive zones will be adjusted

TABLE I

OPERATIONAL SYSTEM AT pH 7.5 SUITABLE FOR ANIONIC SEPARATIONS

Solvent: H_2O and D_2O. Electric current (μA): *Ca.* 50–100. Temperature: 22°. I.D. (capillary): 0.45 mm.

	Electrolyte	
	Leading	*Terminating*
Anion	Chloride	MES*
Concentration	0.01 *N*	*Ca.* 0.01 *N*
Counter ion	Tris	Tris
pH	7.5	*Ca.* 7.0
Additive	0.05% Polyvinyl alcohol (Mowiol)	None

* Purified by recrystallization.

TABLE II

OPERATIONAL SYSTEM AT pH 6 SUITABLE FOR ANIONIC SEPARATIONS

Solvent: H_2O and D_2O. Electric current (μA): *Ca.* 50–100. Temperature: 22°. I.D. (capillary): 0.45 mm.

	Electrolyte	
	Leading	*Terminating*
Anion	Chloride	MES*
Concentration	0.01 *N*	*Ca.* 0.01 *N*
Counter ion	Histidine	Tris
pH	6	*Ca.* 6
Additive	0.05% Polyvinyl alcohol (Mowiol)	None

* Purified by recrystallization.

TABLE III

OPERATIONAL SYSTEM AT pH 4.5 SUITABLE FOR ANIONIC SEPARATIONS

Solvent: H_2O and D_2O. Electric current (μA): *Ca.* 50–100. Temperature: 22°. I.D. (capillary): 0.45 mm.

	Electrolyte	
	Leading	*Terminating*
Anion	Chloride	MES*
Concentration	0.01 *N*	*Ca.* 0.01 *N*
Counter ion	ε-Aminocaproic acid	Tris
pH	4.5	*Ca.* 6
Additive	0.05% Polyvinyl alcohol (Mowiol)	None

* Purified by recrystallization.

TABLE IV

OPERATIONAL SYSTEM AT pH 3 SUITABLE FOR ANIONIC SEPARATIONS

Solvent: H_2O and D_2O. Electric current (μA): *Ca.* 50–100. Temperature: 22°. I.D. (capillary): 0.45 mm.

	Electrolyte	
	Leading	*Terminating*
Anion	Chloride	*E.g.*, acetate, propionate
Concentration	0.01 *N*	*Ca.* 0.01 *N*
Counter ion	β-Alanine*	Tris
pH	3	*Ca.* 5
Additive	0.05% Polyvinyl alcohol (Mowiol)	None

* Purified by recrystallization from methanol–water; the crystals are washed with acetone.

TABLE V

OPERATIONAL SYSTEM AT pH 5 SUITABLE FOR CATIONIC SEPARATIONS

Solvent: H_2O and D_2O. Electric current (μA): *Ca.* 50–100. Temperature: 22°. I.D. (capillary): 0.45 mm.

	Electrolyte	
	Leading	*Terminating*
Cation	K^+	Tris
Concentration	0.01 *N*	*Ca.* 0.01 *N*
Counter ion	Acetate	Acetate
pH	5	*Ca.* 5
Additive	0.05% Polyvinyl alcohol (Mowiol)	None

according to the isotachophoretic principle, a considerable amount of sample can be lost if the pH or the concentration of the terminator is chosen wrongly. To a lesser extent, the same applies to the pH and concentration of the sample. The result may be that ionic species of the sample are partially lost, mixed zones can be expected, irreproducible results are obtained and the point of injection is critical[11]. Therefore, the pH and concentration should be chosen according to the isotachophoretic requirements, governed by the conditions of the leading electrolyte. Also, the sample must not have an extremely high concentration and a large difference in pH in comparison with the pH of the leading electrolyte. If the operating conditions are well chosen, an easily reproducible result will be obtained ($\sigma < 1\%$) and the point of injection is no longer critical.

It should be mentioned that the operational systems given in Tables I–V can be optimized for special applications, *e.g.*, by changing the concentration of the leading ion or the pH of the leading electrolyte. The leading ions chosen are commercially available in a highly pure form, chemically stable, with a high effective mobility, independent of pH.

The counter ion, and hence the pH of the leading electrolyte in which the analysis is to be carried out, is chosen for its optimal buffering capacity, small effective

mobility, chemical stability and purity. It should be noted that in some operational systems, different from those given in Tables I–V, the leading ion (*e.g.*, acetate in anionic separations at "high" pH) is sufficiently mobile, while in other operational systems it is applied as a terminator (*e.g.*, acetate in anionic separations at "low" pH) or as a counter ion (*e.g.*, acetate in Table V). For special applications, the pH of the leading electrolyte may be varied or even another counter ion may be chosen[12]. As a general rule, we can give the following guidelines for the pH of the leading electrolyte:

For anionic separations:

$$pK_C - 0.5 < pH_L < pK_C + 0.5 \quad (1)$$

For cationic separations:

$$pK_C + 0.5 > pH_L > pK_C - 0.5 \quad (2)$$

where pK_C is the pK_a value of the counter ion and pH_L is the pH of the leading electrolyte.

An example of a leading ion other than chloride is given in Fig. 12, where the separation of some peptides is shown. From the linear traces of both the conductivity detector and the UV absorption detector, it can be seen that an ion that is more mobile than the leading ion is present. In this particular instance, the sample contained chloride ions, arising from the peptide Gly-Gly·HCl. It should be noted that the qualitative and quantitative information for the ionic species within the isotachophoretic system is not influenced by the fact that the sample contained an ion with an effective mobility higher than that of the leading ion; this component will, from the start of the analysis, pass the first separation boundary and migrate "zone electrophoretically", under well defined conditions, through the leading electrolyte. Hence the conditions of the leading electrolyte behind the mixed zone of chloride and leading ion are identical with the conditions of the leading electrolyte in front of this mixed zone[8]. Obviously, this is a consequence of the fact that the Kohlrausch regulating function concept in any electrophoretic system cannot be overruled by the electrophoretic separation process. It also means that the mixed zone is adapted to the conditions of the leading electrolyte, which applies to all other zones moving with equal speed. This can be seen from the linear traces of both the conductivity detector and the UV absorption detector.

The current density is very important, because it determines the time of analysis, the temperature and the sharpness of the boundary profiles. Problems with the temperature distribution can be expected if too high current densities are applied. However, if the conditions as given in Tables I–V, are followed, the influence of temperature is not deleterious and reproducible analyses can be achieved. Subsequent experiments will show whether more attention needs to be paid to thermostating the narrow-bore tube and the detectors, because the influence of the different temperatures of the various zones on the effective mobilities, activity coefficients, pK_a values of the ionic species and even on the profile of the zones cannot be overlooked. It must be kept in mind, however, that in many instances an increase in temperature from the leading electrolyte towards terminating electrolyte can influence the separation in a positive way. The advantage of temperature programming has not yet been studied.

INSTRUMENTATION

Fig. 1 shows the isotachophoretic equipment developed in the Department of Instrumental Analysis of Eindhoven University of Technology. The equipment is provided with an injection block[12] and a six-way valve[12], which gives the possibility of introducing the sample by means of a microlitre syringe or sandwiched between the leading and terminating electrolyte via a sample tap. The counter electrode compartment is provided with a semipermeable membrane (transparent cellulose polyacetate) and a septum for introducing the counter flow of electrolyte with a pumping

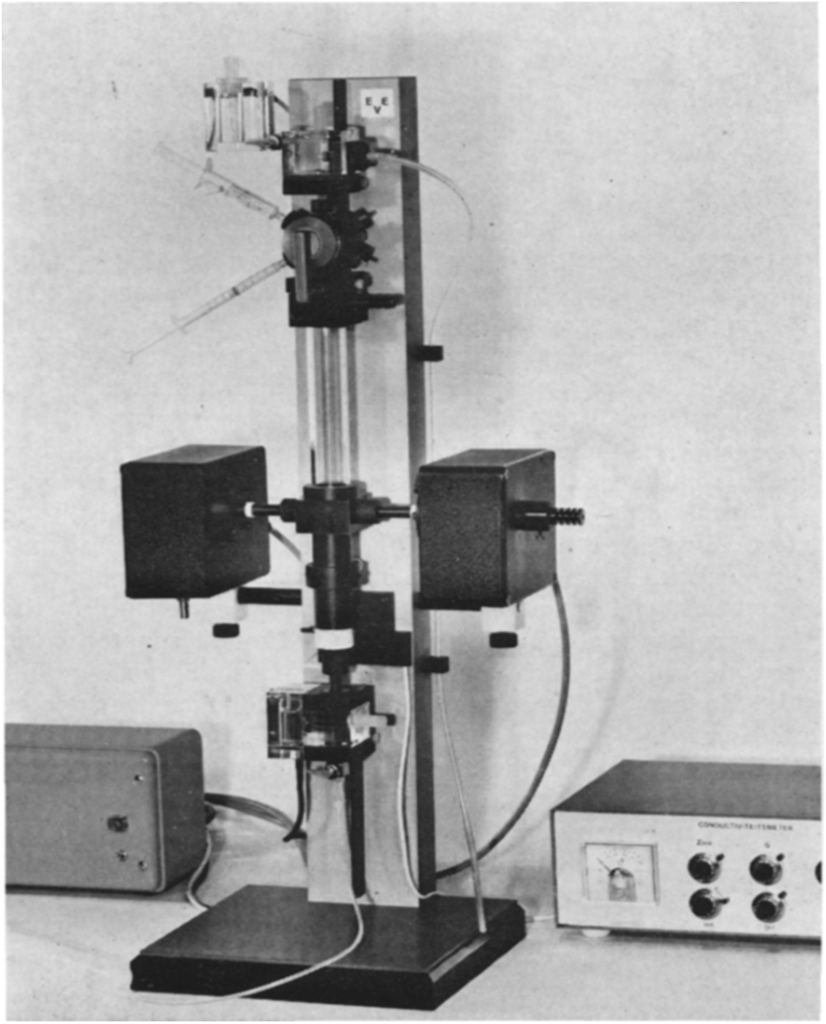

Fig. 1. Instrument for analytical isotachophoresis developed in the Department of Instrumental Analysis of Eindhoven University of Technology. UV absorption, a.c. conductivity and potential gradient detectors can be used. The sample can be introduced via a microlitre syringe and with a six-way sample valve. A counter flow of electrolyte can be applied to increase the length available for separation.

mechanism, which is generally regulated. The UV absorption detector[12], conductivity detector[12] and potential gradient detector[12] can be used. Because the UV absorption detector makes use of a well known principle, is commercially available, and the UV source (a low-pressure mercury tube in a high-frequency electric field) is also commercially available, it need not be considered in detail here. We shall consider only the potential gradient detector ("d.c. conductivity detector") and the a.c.-conductivity detector, both of which have an output signal that is proportional to the ohmic resistance inside the measuring probe[12]. For the measurement of the potential gradient, the micro-sensing electrodes must be mounted axially, while for the measurement of the conductivity via the a.c. conductivity detector, a probe can be used in which the micro-sensing electrodes are mounted equiplanar.

In the book by Everaerts *et al.*[12], much attention is paid to electrode reactions that occur if no precautions are taken. Briefly, we recommend that the measuring electrodes should be thin (approximately 10 μm) in order to prevent electrode reactions. For good contact of the electrolytes inside the measuring probe with the micro-sensing electrodes and to decrease the electroendosmotic flow, surfactants must be added to the electrolytes[12].

The conductivity detector (a.c. method)

In order to measure the conductivity (resistance) of an electrolyte, in which a potential of about 6 kV towards earth is present, good galvanic insulation is necessary between the sensing electrodes and the electronic circuitry (*i.e.*, the conductimeter) at low potential. This can be achieved by measuring the conductivity with an a.c. current that passes through a transformer with two separated coils. All types of electrical leak currents must be prevented. Even a leak current of 10^{-9} A via the sensing electrodes will have a major influence on the measurement of the conductivity. This was shown particularly in a paper which deals with the coating of electrodes[13]. We found that the conductivity of isotachophoretic zones could be determined optimally by a probe in which the micro-sensing electrodes were constructed equiplanar. In order to prevent an electric current from flowing from one sensing electrode towards the other if the electrodes are mounted badly so that a potential difference exists between the electrodes by the driving current via the transformer, a capacitor is inserted in series with the transformer. The conductivities of consecutive zones are not defined by the electric driving current, assuming that temperature effects can be neglected.

This conductimeter (Fig. 2) is the result of our latest research and gives a linear response as a function of the resistance to be measured. If the ohmic resistance of both coils of the transformer used for galvanic separation can be neglected and the coupling of both coils is assumed to be unity, the transformer can be considered as an ideal transformer containing in parallel a resistor (R_v) and a coil (L). The losses in iron and copper are responsible for the magnitude of R_v. If the material of the core is not saturated, then R_v will be constant.

The capacitor (C) and the coil (L) together form a resonance circuit:

$$\omega_r = \frac{1}{\sqrt{LC}} \tag{3}$$

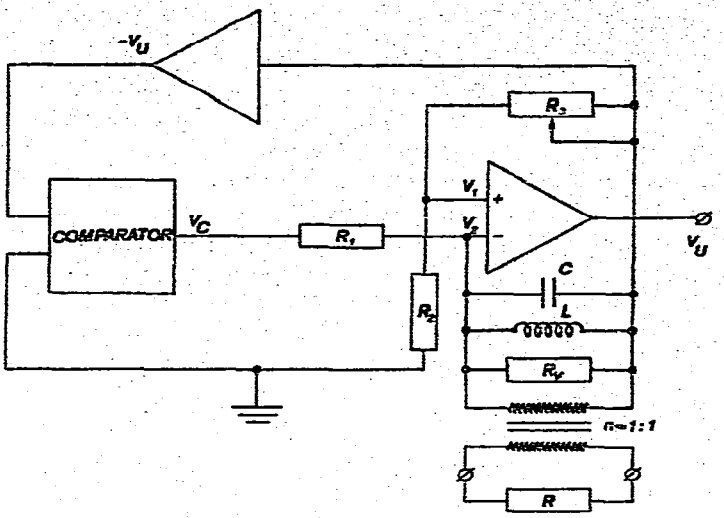

Fig. 2. Schematic diagram of the principle used for the a.c. conductivity detector.

If the ratio of the number of turns is unity, the impedance (z) of the circuit between the inverting input and output of the operational amplifier of those signals which have a frequency ω_r is well defined:

$$z = \frac{R_v R}{R_v + R} \tag{4}$$

where R is the unknown resistance, for example between the micro-sensing electrodes of the conductivity cell.

If $v_C = V_C \cos \omega_r t$ (v = a.c. voltage; V = d.c. voltage) and we assume that the amplification of the operational amplifier is infinity and that all input currents are equal to zero, we can write

$$v_1 = v_2 \tag{5}$$

$$\frac{R_2}{R_2 + R_3} \cdot v_U = v_C + (v_U - v_C) \cdot \frac{R_1}{R_1 + R_v R\,(R_v + R)^{-1}} \tag{6}$$

If

$$\frac{R_2}{R_3} = \frac{R_1}{R_v} \tag{7}$$

then

$$R_2(R_2 + R_3)^{-1} = R_1(R_1 + R_v)^{-1} \tag{8}$$

We can now write

$$\left[\frac{R_1}{R_1+R_v} - \frac{1}{\left(1+\frac{R_v}{R_1}\right)\left(\frac{R}{R_v+R}\right)}\right] v_U = \left[1 - \frac{1}{\left(1+\frac{R_v}{R_1}\right)\left(\frac{R}{R_v+R}\right)}\right] v_C \tag{9}$$

and

$$\left[\frac{R_1}{R_1+R_v} - 1 + \left(\frac{R_v}{R_1+R_v}\right)\left(\frac{R}{R_v+R}\right)\right] v_U = \frac{R_v}{R_1}\left(\frac{R}{R_v+R}\right) v_C \tag{10}$$

or

$$\frac{R_v}{R_1+R_v}\left(\frac{R}{R_v+R} - 1\right) v_U = \frac{R_v}{R_1}\left(\frac{R}{R_v+R}\right) v_C \tag{11}$$

Now,

$$\frac{-R_1 R_v}{R_1+R_v} \cdot v_U = R\, v_C \tag{12}$$

and therefore

$$v_U = V_U \cos \omega_r t = -\frac{R_1+R_v}{R_1 R_v} \cdot R\, V_C \cos \omega_r t \tag{13}$$

We can conclude that v_C is constant if $\frac{R_2}{R_3} = \frac{R_1}{R_v}$ and the amplitude of v_U is proportional to R. After rectification and smoothing of v_U, the resulting potential is also proportional to R.

In order to keep the frequency of v_C equal to the resonance frequency ω_r, a comparator is applied, which generates v_C. This comparator is controlled by v_U, a square-wave voltage. In all the equations given, we have considered only the first harmonic of this square-wave voltage. The higher harmonics are suppressed by the circuitry applied, assuming that R is not too small. These higher harmonics can be neglected in this instance.

The circuit as finally applied is shown in Fig. 3. The circuitry was developed for two ranges, 10 kΩ–1 MΩ and 1 MΩ–10 MΩ.

IC_2 is the operational amplifier, as already discussed above. IC_3 rectifies the voltage v_U in a single-phase manner. The μA meter measures the average value of this rectified signal. By means of IC_5, a pre-adjusted voltage can be added to this signal, which is then smoothed and amplified by IC_4. The smoothing capacitor is chosen such that the time constant involved in the amplification of IC_4 is equal to a maximum of 0.1 sec. The potential recorder used in our laboratory in combination with the conductimeter also has a time constant of approximately 0.1 sec.

Both the d.c. voltage and the amplification can be adjusted by means of two potentiometers (P_3 and P_4), which are supplied with a set of Multi-dials. The lowest position of both of these dials agrees with an output voltage of IC_5 and an amplification of IC_4 of zero. The circuit with IC_1 is the comparator. The capacitor of 1 μF and the resistor of 1000 kΩ are included so as to ensure that the oscillation of the oscillator formed by IC_1, IC_2 and IC_3 is always guaranteed.

The resistor of 47 kΩ in series with the capacitor of 47 pF prevents an undesirable oscillation of IC_1 during triggering. This circuitry for resistance determination was developed for use with the micro-sensing electrodes. The volume of the conductivity cell is approximately 1.6 nl. The output voltage of IC_1 is attenuated 11 or 110 times, depending on the switch "Range". If the switch "Range" is in the open

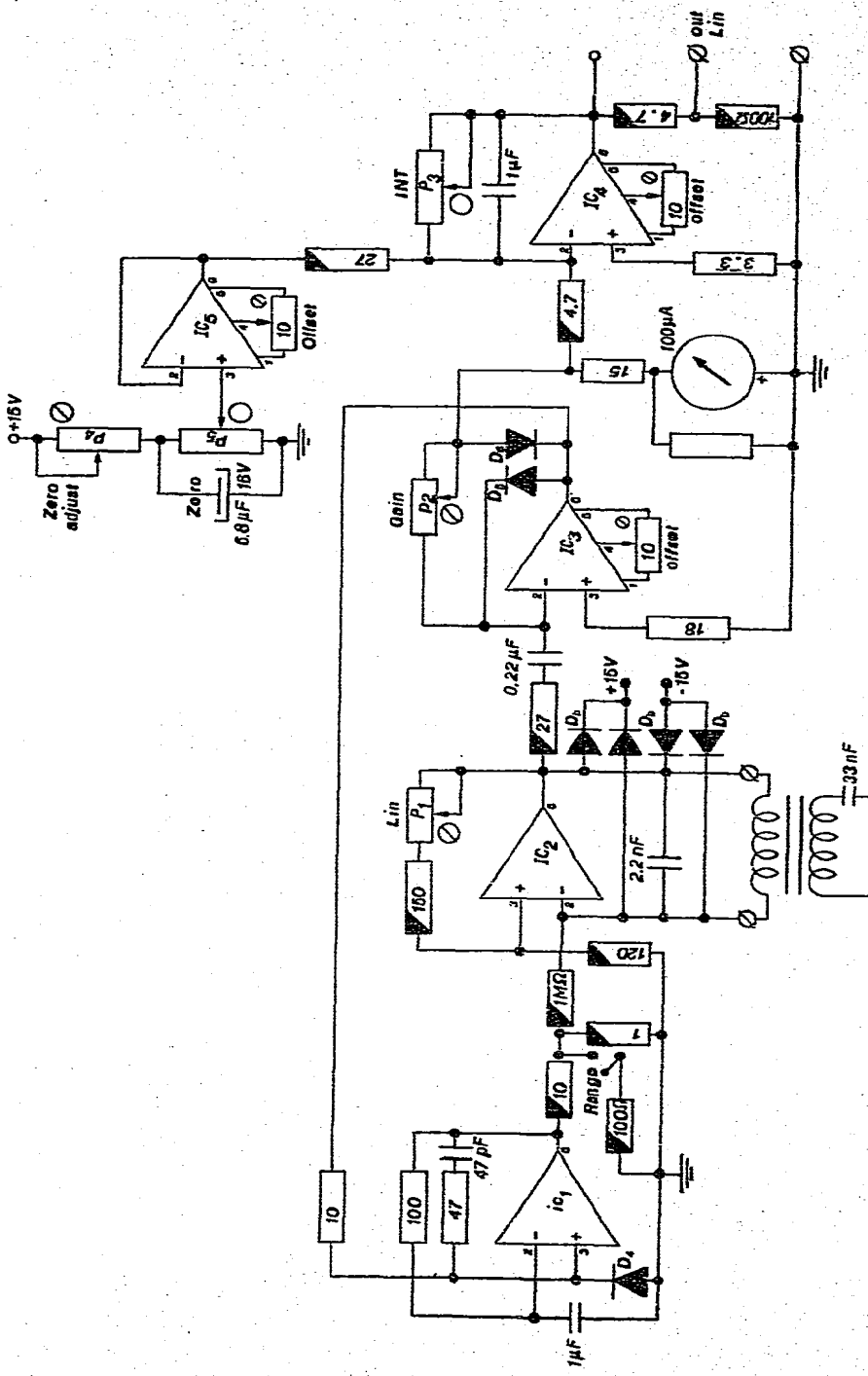

Fig. 3. The a.c. conductivity detector. The metal film resistances all are 1%, 1/8 W; $ic_1 = \mu A\ 709$; IC_2–$IC_5 = \mu A\ 741$; D_b, D_4, D_5 and $D_6 = 1N4148$, 1N914; P_1, P_2 and $P_3 = 100\text{-k}\Omega$, ten-turn potentiometer; $P_4 = 10\text{-k}\Omega$, ten-turn potentiometer; $P_5 = 50\text{-k}\Omega$, ten-turn potentiometer; the transformer is made of 2×1000 turns of Cu enamel, 0.1 mm; the potcore used is P36/22.3B7 or 3H1, $\mu_e = 2030$, with an air gap of 0.2 mm. Resistances are given in $\text{k}\Omega$, unless stated otherwise.

position, the resistance can be measured between 10 kΩ and 1 MΩ; if it is closed, resistances can be measured between 1 and 10 MΩ, but less accurate than in the open position.

The proportions and construction of the transformer are mainly determined by this. If the inductance of the primary coil is too high, the quality of the resonance circuit is too low, which is particularly inconvenient if small resistances have to be measured. The measurement of these resistances is no longer accurate, because the square-wave voltage from IC_1 is filtered badly. If the inductance of the primary coil is too small, the transformer is saturated if the resistance between the micro-sensing electrodes is high. Consequently, the voltage over the primary coil is small if the resistance between the micro-sensing electrodes is small. A high capacitance of the capacitor results in rapid saturation of the primary coil, but low values of this capacitance make the influence of parasitic capacitances too large.

From many possibilities, a capacitor of 2.2 nF, a self-induction of the primary coil of about 800 mH and a ratio of the number of turns of unity were chosen. Under these conditions, the resonance frequency is *ca.* 4000 Hz and the quality is about 1 if the resistance to be measured is of the order of 20 kΩ. This value is acceptable for a sufficiently accurate measurement of the resistance. The quality is dependent on the quality of the capacitor used, and a mica capacitor is recommended because the dielectric losses are small.

It should be noted that the circuit does not work satisfactorily for resistances below 1 kΩ, as the coupling of the two coils can no longer be assumed to be unity. If one wishes to measure a resistance that is, for example, a factor A smaller, the number of turns of the secondary coil must decrease by a factor $\sqrt{A}$. The number of turns of the primary coil remains unchanged. The core material is P36/22.3B7 or 3H1, μ_e (permeability) = 2030. This potcore is provided with a gap for applying a foil of insulating material between the two parts in order to limit the temperature drift of the inductance.

A P33/22 potcore, $\mu_e = 220$, which is provided with an air gap, can also be used. The micro-sensing electrodes can have a maximum potential of *ca.* 6 kV towards earth, otherwise the leak current towards earth is too high. For this purpose, the secondary coil must be well insulated, *e.g.*, by constructing both the primary and secondary coils in a housing made of PTFE. In our equipment, this transformer, which separates galvanically the sensing electrodes from the circuitry at low potential, was mounted on the electrophoretic equipment itself, about 3 cm from the conductivity probe.

Even if a well insulated cable is available, a length of about 1 m is enough to influence the recording because of the parasitic capacitance that results from it. For continuous recording of the resistance, the output of "Lin" is connected with a potential recorder with a sensitivity of 100 mV. If the switch "Range" is in the 1 MΩ position, the resistances to be measured can be determined by the equation

$$\frac{R}{1\ \text{M}\Omega} = \frac{V}{100\ \text{mV}} \cdot \frac{0.1}{\text{Int}} + \frac{\text{Zero}}{1} \tag{14}$$

where R is the resistance to be measured, V is the output voltage of "Lin" and "Int" and "Zero" indicates the ratio between the real value and the maximum value of the Multi-dial connected to the potentiometers "Int" and "Zero", respectively.

For the determination of resistances higher than 1 MΩ, the switch "Range" is set in the second position. In a similar way, the resistance can be determined by replacing 1 MΩ in eqn. 14 by 10 MΩ. The potentiometers "Zero" and "Lin" must have such a position that during the analysis the output voltage on "Lin" always remains between 0 and 100 mV.

All of the results presented in this paper in the various operational systems were measured with the circuit described here. At the level of 1 MΩ the accuracy of the resistance determination with this linear circuitry is better than 2 kΩ, while at the level of 10 MΩ, the accuracy is better than 30 kΩ. If the ambient temperature increases by 10°, the difference in the resistance determination at the level of 1 MΩ is less than 1 kΩ and at the level of 10 MΩ it is less than 20 kΩ.

The stepwise trace obtained during an isotachophoretic analysis when the zones pass the conductimeter can easily be interpreted to give qualitative and quantitative information, because the steps are sharp enough. This is in contrast to thermometric recording. Two main reasons can be given why a differentiator is applied: if small zones are present, stacked between the others, they can be detected much more easily by an electronic differentiator, and if electronic devices are available and used to measure the time interval between two successive peaks, a pulse is needed for the printer.

The accuracy of time recording is better than 0.1 sec, which represents an accuracy of $5 \cdot 10^{-9}$ g-equiv./l, assuming an electric driving current of 70 μA and a concentration of the leading electrolyte of 10^{-2} g-equiv./l.

The potential gradient detector (d.c.–a.c. converter)

Fig. 4 shows an electronic circuit ("d.c.–a.c. converter") that can be used in combination with the conductimeter discussed above. The conductimeter was developed for resistance determinations between 50 kΩ and 10 MΩ, although a fairly linear response can be obtained between 10 and 50 kΩ.

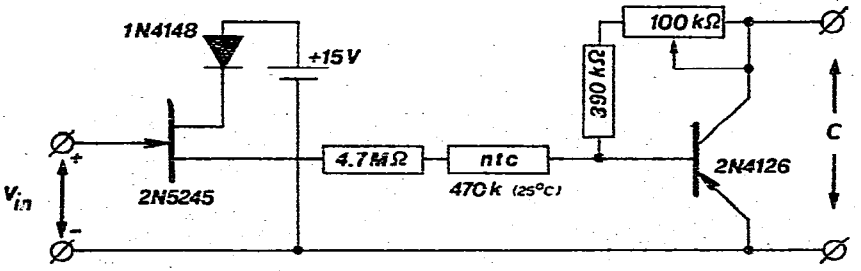

Fig. 4. The d.c.–a.c. converter.

With the converter, a maximum potential difference of 10 V can be measured between two points, which has a maximum common mode potential of 6 kV with respect to earth. The impedance between the two points at the input of the circuit shown in Fig. 4 is greater than 10^{12} Ω, and the common mode impedance is greater than 10^{15} Ω. The junction-field effect transitor (FET) is applied as a source follower. This junction-FET is supplied by a battery. This battery can be replaced with an electronic circuit, but in order to minimize both the leak current towards earth and

the parasitic capacitance towards earth, a battery is applied. A further advantage of using a battery is that the supply current of the circuit is very small ($< 3\ \mu A$). The diode protects the electronic circuit against incorrect connections of the battery and against a high positive input voltage (*e.g.*, if a gas bubble is present at the micro-sensing electrodes). If V_{in} increases or decreases, the LC circuit in the conductivity measuring circuit is more or less damped. The output signal of V_{in} can be determined via the output signal of the conductivity measuring circuit. With the adjusting potentiometer (100 kΩ), the circuit shown in Fig. 4 can be calibrated. The ntc thermistor is included in order to reduce the temperature drift, and is mounted in the neighbourhood of the pnp transistor.

If we make allowance for the value of the "Offset", V_{in} is given by the equation,

$$\frac{V_{in}}{10\ V} = \frac{V}{0.1\ V} \cdot \frac{2}{Int} + \frac{Zero - Offset}{5} \tag{15}$$

where V is the voltage on the potential recorder and Int and Zero are the positions of the dials "Int" and "Zero". The μA meter indicates 50 μA if $V_{in} = 0$ V and 100 μA if $V_{in} = 10$ V.

The accuracy in measuring V_{in} is better than 50 mV. If the ambient temperature changes from 10° to 35°, the difference in the measured value of V_{in}, which of course is not changed during this procedure, is less than 20 mV. There is a linear relationship between the input signal of the d.c.–a.c. convertor and the electric current applied.

The membrane pump for experiments with a counter flow of electrolyte

For the regulation of the counter flow of electrolyte, a membrane pump (Fig. 5) was constructed[12]. In the electrolysis cell of the membrane pump, an amount of gas can be produced that is regulated via signals from the current-stabilized power supply. The gas produced displaces a pre-stressed thin rubber membrane so that leading electrolyte flows out of the specially constructed compartment into the capillary tube. The circuitry for the regulation of the counter flow of electrolyte has been discussed extensively elsewhere[12].

APPLICATIONS

Anionic and cationic separations

The qualitative and quantitative determination of low-molecular-weight ionic species has been dealt with in many papers. In a forthcoming paper we shall present more relevant data, collected in the various operational systems (Tables I–V) with water and deuterium oxide as the solvents. To demonstrate the range of applications of isotachophoresis, in this paper some interesting examples are considered. The choice of the solvent is very important in electrophoretic separations, and hence also for separations by isotachophoresis[12].

Figs. 6 and 7 show some separations in water and deuterium oxide. These solvents have slightly different physico-chemical properties, resulting in different electrophoretic behaviour. In the isotachopherograms in Fig. 6, the separation of nitrate and sulphate in water and deuterium oxide is shown. Under the operating

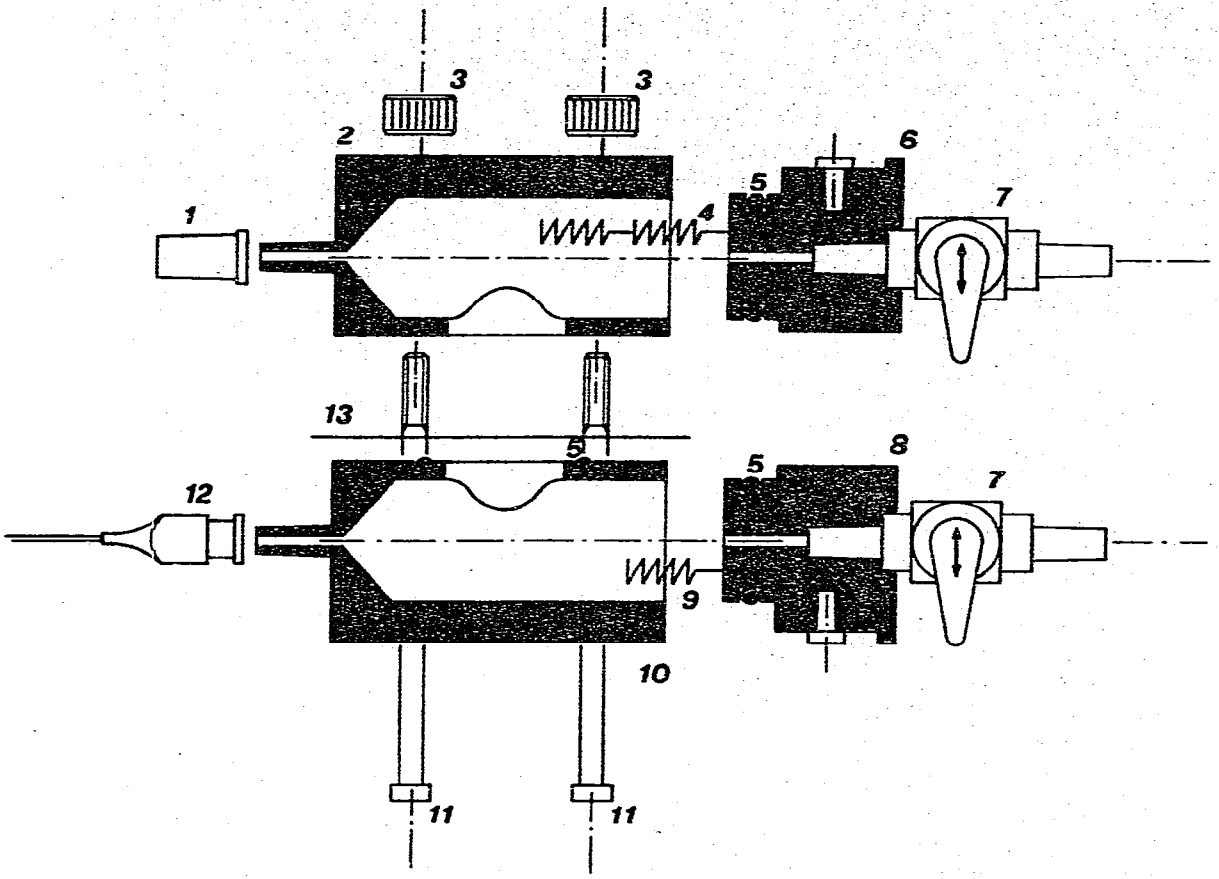

Fig. 5. The membrane pump for isotachophoretic analyses with a counter flow of electrolyte. 1 = Cap for closing the electrolysis chamber; 2 = electrolysis chamber; 3 = nuts for mounting components 2 and 10; 4 = electrodes connected with the regulation circuit; 5 = rubber O-ring; 6 = cap for closing the electrolysis chamber; 7 = PTFE-lined Hamilton two-way valve (1MM1); 8 = cap for closing the chamber filled with leading electrolyte; 9 = electrode to be used if experiments are to be carried out without the use of a semipermeable membrane; 10 = chamber filled with leading electrolyte; 11 = bolts for mounting components 2 and 10; 12 = stainless-steel needle; 13 = pre-stressed thin rubber membrane.

conditions chosen (the concentration of the leading anion is particularly important), complete separation of the two ions could not be expected. This was confirmed by both the UV absorption and the conductivity detector when water was used as the solvent. By using deuterium oxide, complete and reproducible separation of sulphate and nitrate could be achieved. Fig. 6 also shows that a change in conductivity of the various zones (also the leading electrolyte zone) is obtained if water or deuterium oxide is used as the solvent.

In Table VI the step heights of the various constituents of the anionic test mixture (see Fig. 16) obtained in water and deuterium oxide are given. Several effects may give rise to such conductivity and mobility changes. Firstly, there is the effect on the ionic mobility of the different ionic species, as an overall result of differences

Fig. 6. Isotachopherograms for the separation of sulphate and nitrate in the operational system listed in Table II. The positions of all dials of the linear conductivity detector were not changed; the experiments were carried out in water and deuterium oxide. R = Increasing resistance; A = increasing UV absorption; t = increasing time; $I = 80\,\mu A$. 1 = Chloride; 2 = nitrate; 3 = sulphate; 4 = acetate.

in solvation, relaxation, etc. Ionic mobilities are lower in deuterium oxide than in water. The second effect is due mainly to the difference in the pH scale. This, of course, will influence the equilibria of the various ionic constituents. The effective mobilities of the ionic constituents with a relatively high pK_a value (the pH of the operational system applied is 6) will therefore show the influence of the difference in the pH or pD scale. This is clearly visible in the step height difference for morpholinoethanesulphonic acid (MES). Differences can also be expected if cationic species are separated with water and deuterium oxide as the solvents, as shown in Fig. 7. In cationic separations, the effect of the extended pD scale will be seen for constituents with low pK_a values, *e.g.*, β-alanine. Again, the strong ions show only the effect of solvation differences. By means of isotachophoresis, useful information can be obtained for studying interactions of solvents, solutes, etc.

Reaction kinetics

When studying reaction kinetics, one is mostly interested in a reliable, rapid analytical method that gives qualitative and quantitative results for most of the components involved. For ionic and many non-ionic reaction constituents, isotacho-

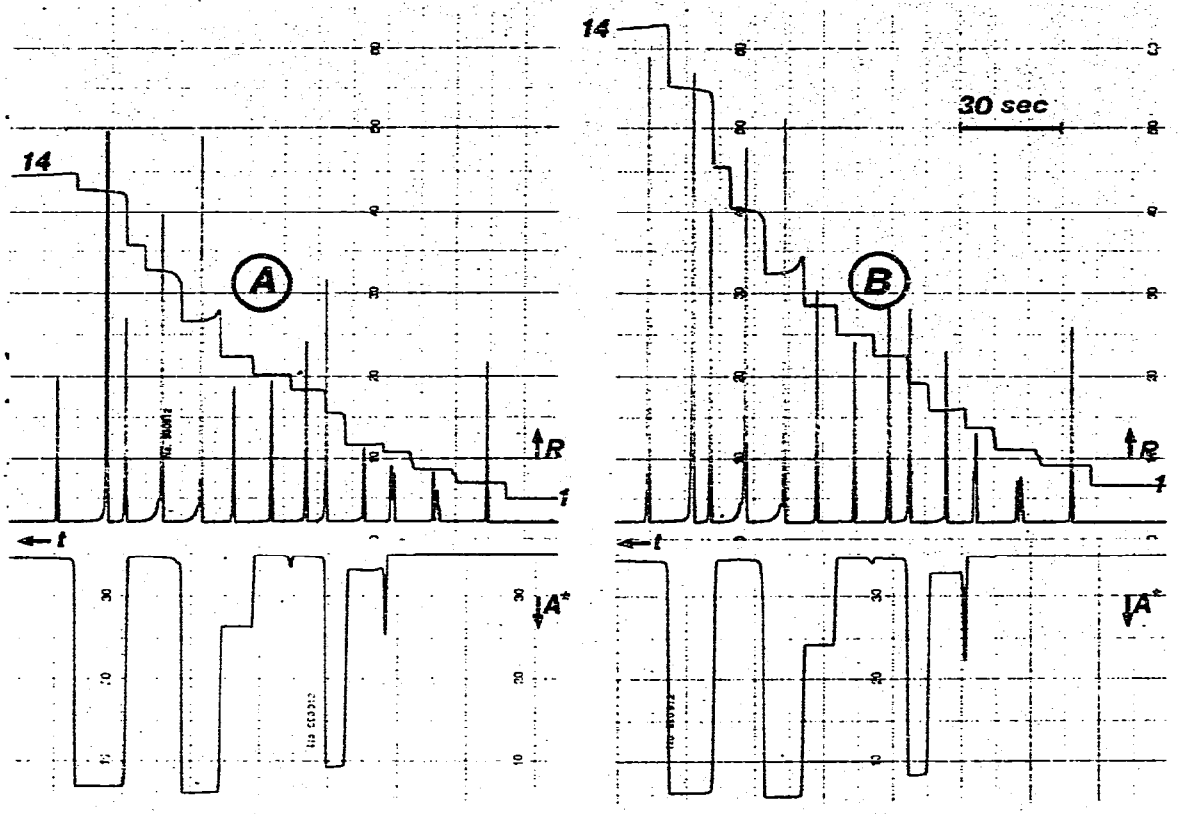

Fig. 7. Isotachopherogram for the separation of a standard mixture of cations in water (A) and deuterium oxide (B) carried out in the operational system listed in Table V. Peaks in sequence from 1 to 14: 1 = K^+ (leading ion); 2 = Ba^{2+}; 3 = Na^+; 4 = $(CH_3)_4N^+$; 5 = Pb^{2+}; 6 = Girard reagent P^+; 7 = $Tris^+$; 8 = histidine$^+$; 9 = creatinine$^+$; 10 = benzidine^{2+}; 11 = ε-aminocaproate$^+$; 12 = γ-aminobutyrate$^+$; 13 = aminophenazone$^+$; 14 = β-alanine$^+$ (terminating ion). R = Increasing resistance; A^* = increasing UV absorption; t = increasing time; i = 100 μA.

phoresis can be used for this purpose. Fig. 8 shows an example of such an analysis, carried out routinely in our laboratory.

The isotachopherogram shows the reaction products obtained by the homogeneous oxidation of glucose in aqueous solution. If isotachopherograms are derived as a function of the reaction time, clearly visible ionic components are formed and disappear again. The reaction procedure can easily be followed by isotachophoresis, giving reliable results for the most important components involved in the reaction. The isotachopherogram shows the reaction mixture in the end-phase of the glucose oxidation. In the first stage of the process, gluconic acid is the major component, which is decomposed to several other organic acids of which, under proper selected conditions, glutaric acid is the most important. In any phase of the process, an accurate and rapid scale-up of all the acids could be made, giving reliable kinetic data.

It should be obvious that even non-ionic compounds can be subjected to isotachophoresis, provided that they can be converted into ionic forms. If, for instance,

TABLE VI

COMPARISON OF RELATIVE STEP HEIGHTS (h) OF SOME ANIONS (SEE ALSO FIG. 16) IN WATER AND DEUTERIUM OXIDE

The electric driving current was stabilized at 80 μA. The values have a reproducibility better than 4%.

Ionic species	$100 \cdot \frac{h_{\text{ionic species}}}{h_{\text{chlorate}}}$	
	H_2O	D_2O
Chloride	0	0
Sulphate	52	60
Chlorate	100	100
Chromate	138	144
Malonate	206	230
Pyrazole-3,5-dicarboxylate	284	323
Adipate	390	451
Acetate	486	553
β-Chloropropionate	612	701
Benzoate	712	828
Naphthalene-2-sulphonate	796	925
Glutamate	917	1069
Enanthate	976	1141
Benzyl-*dl*-aspartate	1120	1306
Morpholinoethanesulphonate	1533	1921

the transport processes in an artifical kidney are to be investigated, many important compounds can be studied *e.g.*, uric acid, creatinine and urea. Urea, which is a non-ionic compound, can easily be converted into ammonium ions by means of urease, and it can then easily be determined in this form by means of isotachophoresis. In fact, all enzymatic reactions can easily be studied by isotachophoresis[12]. With no problems, even in different operational systems, ATP, ADP, AMP, NAD, NADH, NADP and NADPH can be separated.

Pharmaceutical

Many raw materials and products in the pharmaceutical field contain ionic constituents that can easily be analyzed by means of isotachophoresis. Psycho-stimulants such as amphetamines, chlorophentermines, antidepressants such as phenelzine, vitamins, peptides and hormones in many instances can be determined quantitatively and qualitatively with few problems. Figs. 9 and 10 show two simple, arbitrarily chosen examples.

Fig. 9 shows isotachopherograms from the oxidation of Aspirin. As shown in Fig. 9A, the "pure" product contains a considerable amount of phosphate. After oxidation of the Aspirin, by introducing air into a hot solution of the drug, the presence of phosphate, salicylate, acetylsalicylate and acetate is clearly visible. Fig. 9B shows, moreover, that at pH 6 a stable mixed zone is formed between phosphate and salicylate. This means that, if a UV absorption detector had not been available, a false interpretation could have been made. The mixed zone, in this particular instance, can easily be resolved by changing the pH of the leading electrolyte, as can be seen in Fig. 9C. The degradation products of Aspirin in this isotachopherogram are clearly

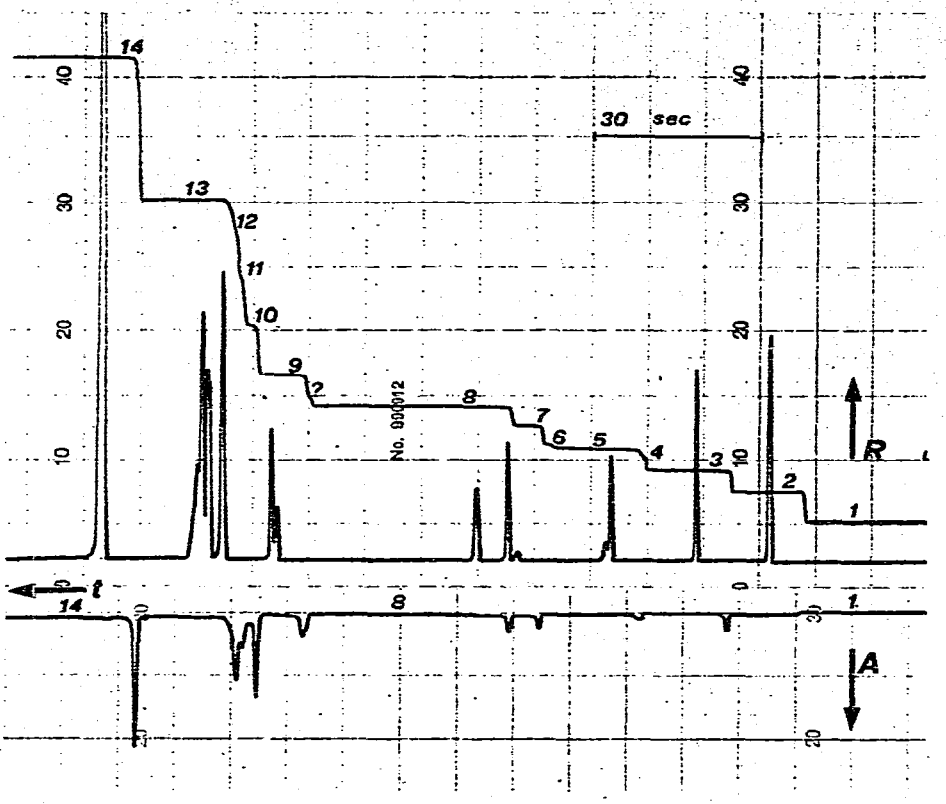

Fig. 8. Isotachopherogram for the separation of a reaction mixture after the homogeneous oxidation of glucose in aqueous solution. 1 = Chloride; 2 = oxalate; 3 = tartronate; 4 = malonate; 5 = tartrate; 6 = formate; 7 = succinate; 8 = glucarate; 9 = glycolate and acetate; 10 = glycerinate; 11 = laevulinate; 12 = arabinate; 13 = gluconate; 14 = morpholinoethanesulphonate. R = Increasing resistance; A = increasing UV absorption; t = increasing time. The analysis is carried out in the operational system listed in Table II. $i = 80\,\mu A$.

visible. It should be emphasized, however, that if the components of the mixed zone are known, all relevant details for a complete quantitative and qualitative analysis can also easily be achieved from this mixed zone[12].

Fig. 10 shows an example of the separation of dexamethazone sodium phosphate, carried out in the operational system at pH 6.

Amino acids, peptides and proteins

The separation of amino acids by isotachophoresis has been investigated by several workers[14–16]. It was found that many amino acids can be analyzed as anions. The pH of the leading electrolyte must be fairly high, however (*ca.* 9), which may cause disturbances due to carbon dioxide from air, if no precautions are taken[16].

An example of the separation of a mixture of amino acids is shown in Fig. 11, 5-bromo-2,4-dihydroxybenzoate (0.004 M) being used as the leading ion and L-lysine as the counter ion.

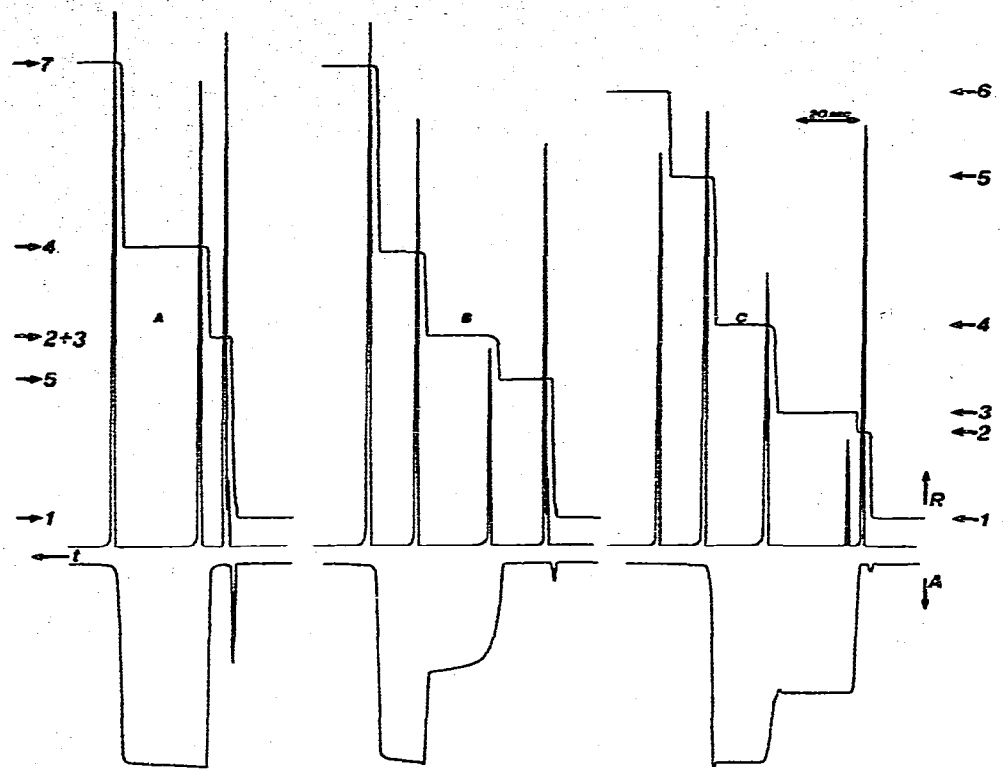

Fig. 9. Isotachopherograms for the separation of the reaction products of the oxidation of Aspirin: (A) before and (B), (C) after the oxidation. Experiments in (A) and (B) were carried out in the operational system at pH 6 (Table II); experiment in (C) was performed in the operational system at pH 3.2 (Table IV). 1 = Chloride; 2 = phosphate; 3 = salicylate; 4 = acetylsalicylate; 5 = acetate; 6 = propionate (terminating ion); 7 = morpholinoethanesulphonic acid (terminating ion). R = Increasing resistance; A = increasing UV absorption; t = increasing time; $I = 80\,\mu A$.

In the book by Everaerts *et al.*[12], more information is given on which amino acids can be separated and which cannot. The more basic amino acids can easily be separated in the operational system listed in Table V. Extreme pH values, of both the leading and terminating electrolytes, must be avoided, because they lead easily to irreproducible results[12].

The addition of aldehydes, which easily form Shiff's bases with amino acids, in order to influence both the effective mobility and the pK values of these ampholytes cannot be recommended because the reproducibility is poor and disturbances may arise from the corresponding acids of the aldehydes, which are not so stable[12]. Further studies are being made in order to obtain a greater analytical differentiation in the analysis of amino acids.

A separation of an arbitrarily chosen sample of peptides is shown in Fig. 12. Similar comments for the analysis of peptides as for the analysis of amino acids can be made. Many of the substances of lower molecular weight can be analyzed iso-

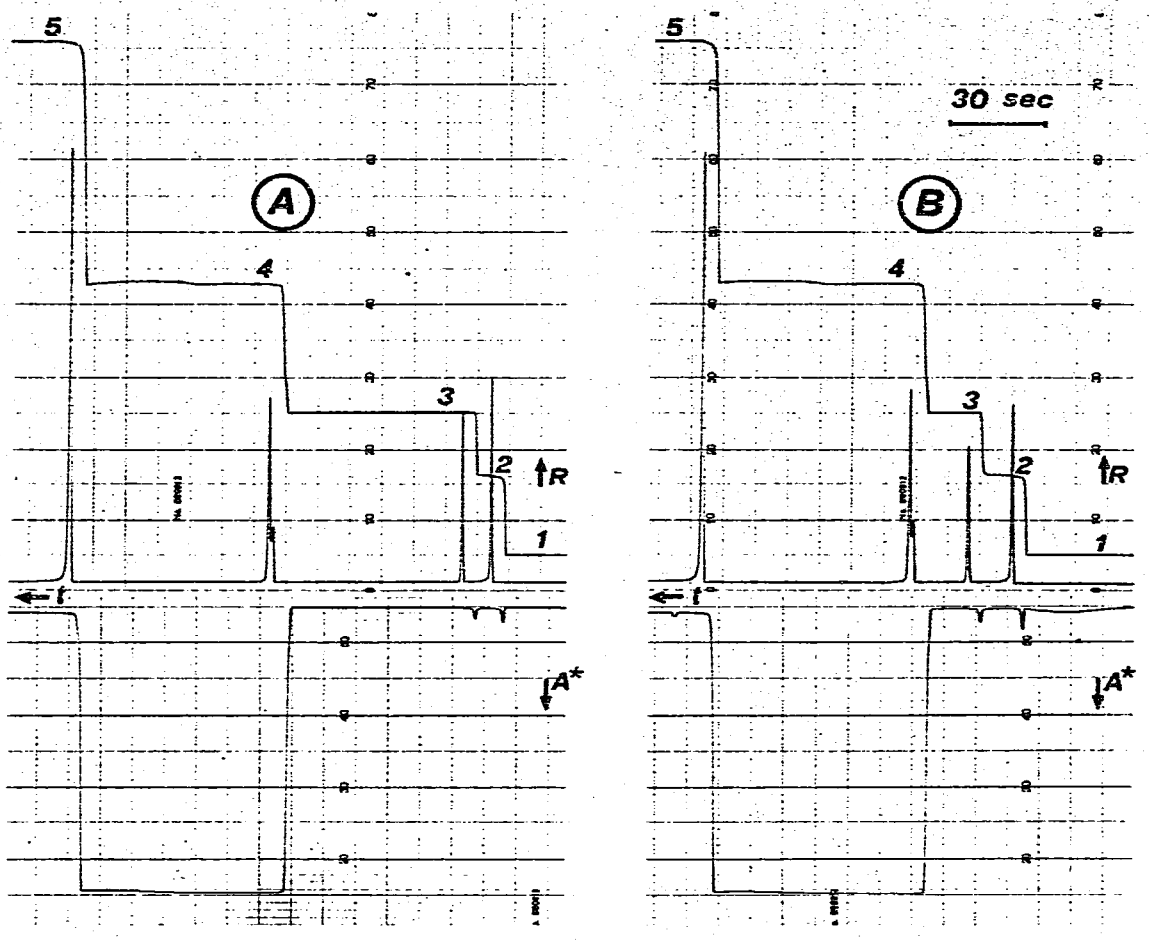

Fig. 10. Isotachopherogram for dexamethazone sodium phosphate in the operational system at pH 6 (Table II) with N-(2-acetamido)-2-aminoethanesulphonic acid (ACES) as the terminator, (A) before and (B) after a purification step. 1 = Chloride; 2 = pyrophosphate; 3 = orthophosphate; 4 = dexamethazone sodium phosphate; 5 = ACES (terminator). A^* = Increasing UV absorption; R = increasing resistance; t = increasing time; $i = 80\,\mu A$.

tachophoretically (qualitatively and quantitatively) without problems due to solubility and stabilization. In general, no additions to the sample need to be made.

Fig. 12 also shows that by changing the leading ion (in this instance from chloride to 5-bromo-2,4-dihydroxybenzoate), only part of the sample can be monitored. In this instance we were not interested in the chloride ion and possibly other ions with an effective mobility higher than that of the leading ion. Chloride ions, as mentioned above, move in front of the first separation boundary and do not change the composition of the leading electrolyte while they migrate through it. By UV absorption of the 5-bromo-2,4-dihydroxybenzoate the presence of the chloride ion is visualized, although in this particular analysis we were not interested in the qualitative and quantitative analysis of the chloride ions. If chloride is used as the leading ion,

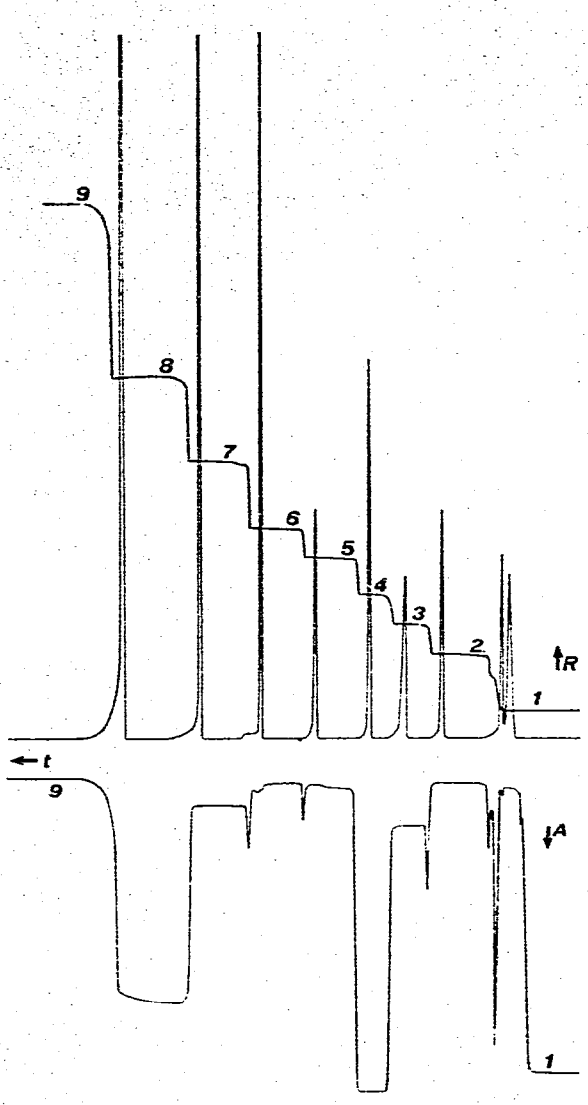

Fig. 11. Isotachopherogram of the separation of some amino acids in an operational system at pH 9.2 with 5-bromo-2,4-dihydroxybenzoate (0.004 *M*) as the leading ion and L-lysine as the counter ion. Note carbonate, moving in front of L-Asp, clearly visible in the linear trace of the UV absorption detector. The carbonate has an effective mobility in this system comparable with that of the leading ion. 1 = 5-Bromo-2,4-dihydroxybenzoate; 2 = L-Asp; 3 = L-Cys; 4 = I_2-L-Tyr; 5 = L-Asn; 6 = L-Ser; 7 = L-Phe; 8 = DL-Trp; 9 = β-Ala. *R* = Increasing resistance; A = increasing UV absorption; *t* = increasing time; *I* = 80 μA.

the presence of chloride can be detected only by measuring the retardation of the appearance of the first peak (zone boundary). An accurate injection must also be made, however.

As shown above, the isotachophoresis of low-molecular-weight substances is obviously a practical and reliable analytical method. When dealing with high-molecular-weight substances, such as proteins, some points need to be considered:

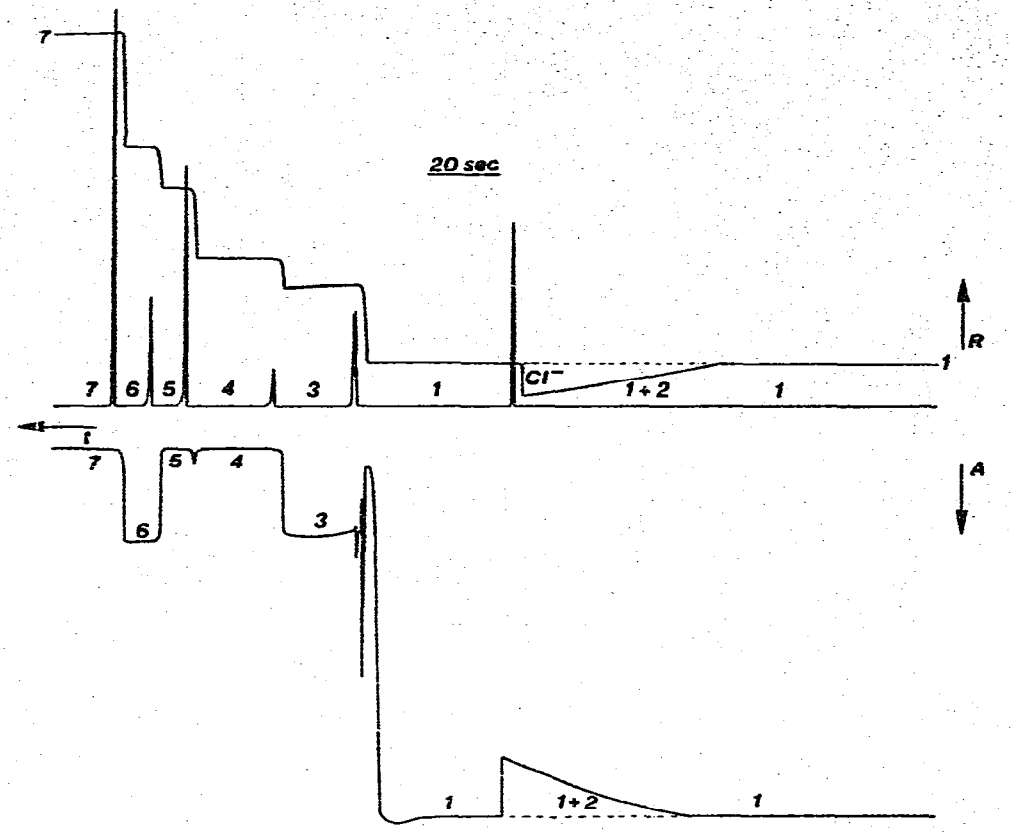

Fig. 12. Isotachopherogram for the separation of some peptides in an operational system at pH 9.0 with 5-bromo-2,4-dihydroxybenzoate (0.04 *M*) as the leading ion and L-lysine as the counter ion. 1 = 5-Bromo-2,4-dihydroxybenzoate; 2 = Cl^-; 3 = gluthathione; 4 = Gly-Gly; 5 = Gly-Gly-Gly-Gly; 6 = Leu-Tyr; 7 = α-Ala. *R* = Increasing resistance; *A* = increasing UV absorption; *t* = increasing time; $I = 100\ \mu A$.

(i) An isotachophoretically moving protein zone will contain, in addition to the protein itself, only the system counter ion. Many proteins, however, need to be stabilized by ionic constituents of lower molecular weight.

(ii) Most proteins have a very low effective mobility, which results in relatively high electric gradients and hence heat production that cannot be neglected.

(iii) The mass concentration (high mass to charge ratio) in the different protein zones is usually high. Problems can be expected with the solubility of the proteins.

It must be stressed that these problems cannot always be solved by effective cooling (thermostating) or anticonvective stabilization. On the other hand, reproducible information can be collected, *e.g.*, of the separation of serum proteins, even if these proteins are analyzed purely by isotachophoresis[12,17]. The information obtained, however, is poor. The simultaneous injection of ampholytes with a small molecular weight, *e.g.*, Ampholines (LKB, Stockholm, Sweden), which dilute and space the various protein zones, gave reproducible isotachopherograms, although these were difficult to interpret. Without the use of specific reactions, it is difficult to con-

clude if a separation of mainly proteins is achieved, or if a separation of the degradation products of the sample components is also obtained. For some of the above reasons, we shall show here the separation of pepsin (Merck, Darmstadt, G.F.R.; 2500 FIP-U/g, 35,000 *E*/g). Fig. 13A shows the analysis of pepsin in the operational system at pH 4.5 with chloride (0.01 *N*) as the leading ion, histidine and ε-aminocaproic acid (molar ratio 1:1) as the counter ions and N-(2-acetamido)-2-aminoethanesulphonic acid (ACES) as the terminator. The current was stabilized at 70 μA. A 0.5-μl volume of a 2% solution of pepsin in water was injected. Fig. 13B shows the analysis of the thermal denaturation product(s) of pepsin. All other conditions were as in Fig. 13A. It is clear that a difference is obtained, although the isotachopherogram shown in Fig. 13A still may contain denaturation product(s) of the sample.

Figs. 14 and 15 illustrate the influence of low-molecular-weight ampholytes. Fig. 14 shows a separation of Ampholines (pH ranges 2.5–4 and 4–4.5, both 2%, w/w). A 0.5-μl volume was injected in the operational system used for the separation of the pepsin described above (Fig. 13A). In Fig. 15, 0.5 μl of pepsin (2%, w/w) was

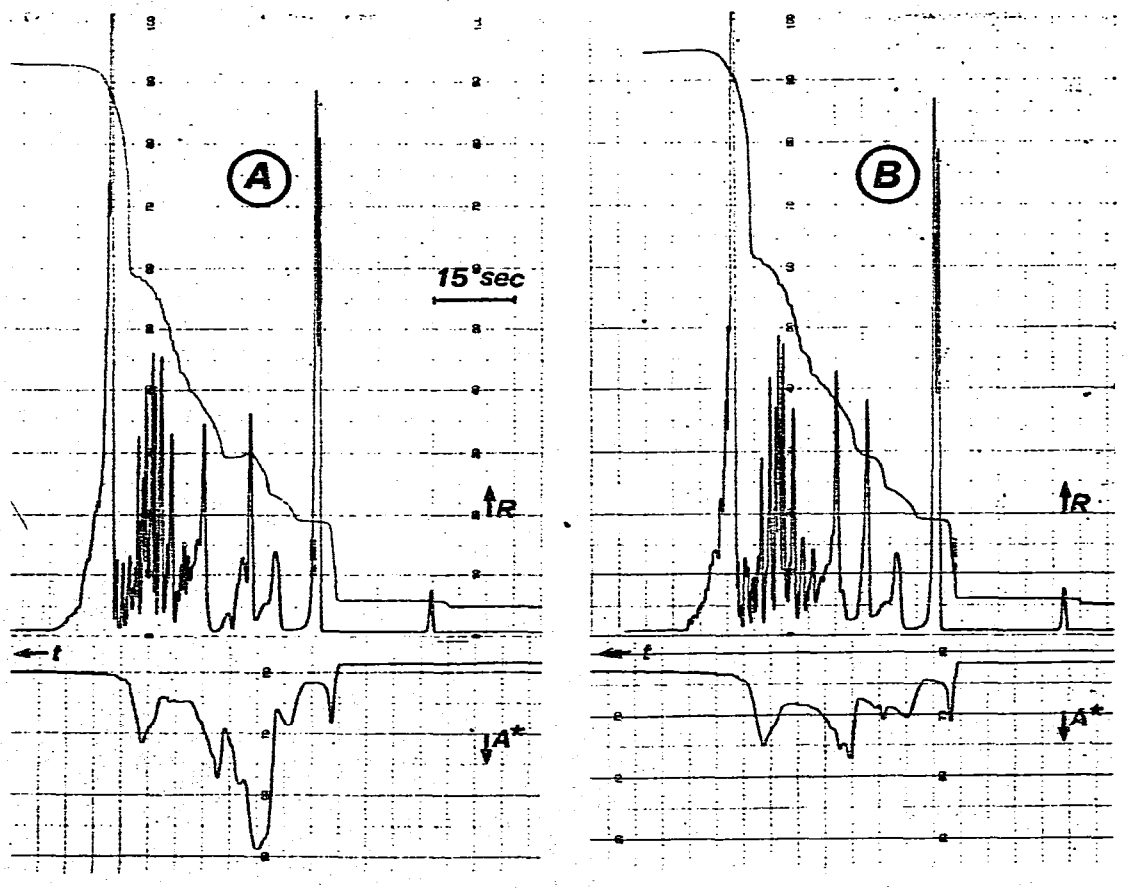

Fig. 13. Separation of pepsin (0.5 μl of a 2%, w/w, solution), (A) before and (B) after thermal degradation. *R* = Increasing resistance; *A** = increasing UV absorption; *t* = increasing time. For operational conditions, see text.

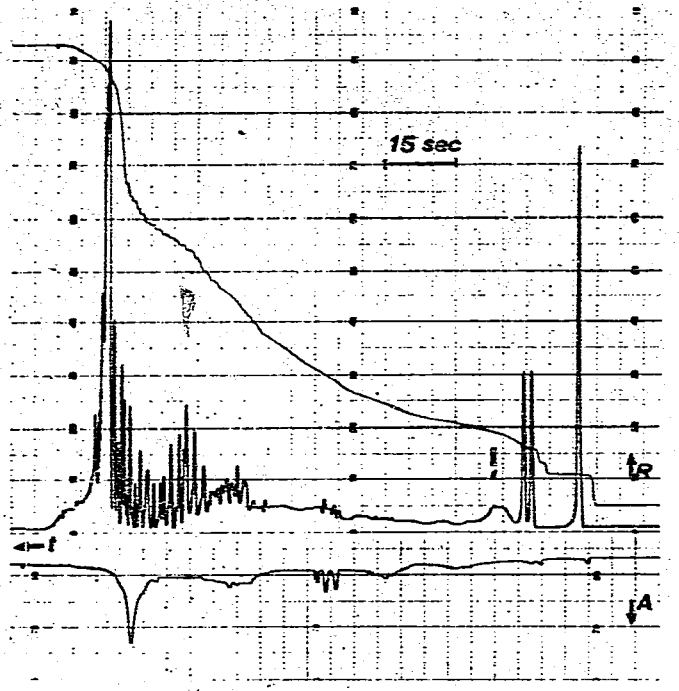

Fig. 14. Separation of a mixture of Ampholines in the pH ranges 2.5–4 and 4–4.5. Both mixtures are 2% (w/w), mixed in the ratio 1:1. A 0.5-μl volume was injected. Other conditions as in Fig. 13. R = Increasing resistance; A = increasing UV absorption; t = increasing time.

injected simultaneously with the ampholytes, again in the operational system as used for the separation shown in Fig. 13A. In order to be able to give a proper interpretation of Figs. 13–15, much more research needs to be carried out, although the data obtained already have practical value. Futurous experiments will show if, for a complete reproducible analysis, it is possible to create a suitable gradient with a spacer and carrier function for high-molecular-weight substances, which is constant in length and constant in inclination. This means that the composition of the mixture (*e.g.*, Ampholines) must be of constant quality.

Counter flow of electrolyte

It has been shown experimentally[12] that a counter flow of electrolyte has an important influence on the sharpness of the profiles. While initially, with a counter flow of electrolyte of less than 30%, a sharpening of the profiles is obtained[12], with a 100% counter flow of electrolyte (which stops the zones), many zones are re-mixed if the differences in effective mobilities between the ionic species moving in consecutive zones are too small[12]. An experiment is shown below, however, in which a successful counter flow of electrolyte was applied.

In Fig. 16, two isotachopherograms are shown, with and without a counter flow of electrolyte, performed in the operational system at pH 6 (Table II). In (a), too much of the sample was introduced to expect a complete separation in the length available for separation. Many mixed zones can be seen, especially where the effective

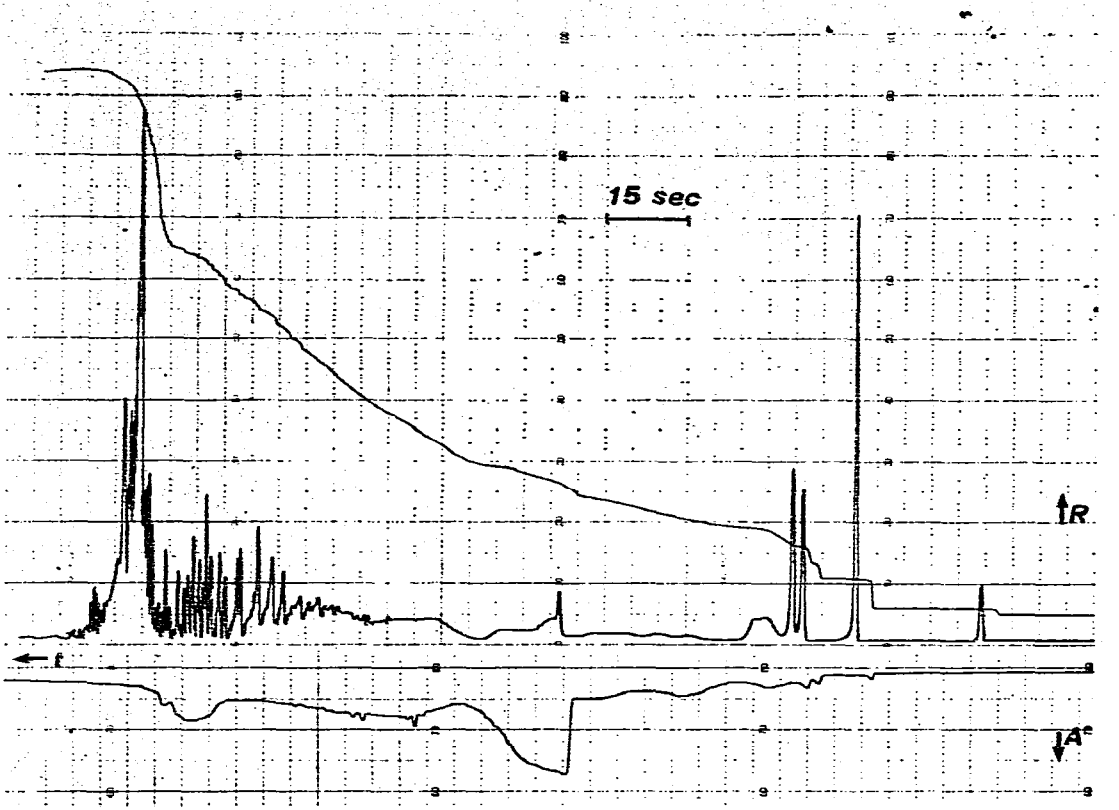

Fig. 15. Separation of pepsin (0.5 μl of a 2%, w/w, solution) in the ampholyte gradient as shown in Fig. 14. All other conditions as in Fig. 13. R = Increasing resistance; A = increasing UV absorption; t = increasing time.

mobility is large. The zones with a low effective mobility all have a longer time of analysis and thus a complete separation can be achieved more easily. If a counter flow of electrolyte is applied (b), the mixed zones disappear. It should be noted that the zones due to the impurities of the chemicals used in the operational systems are enlarged.

CONCLUSION

Isotachophoresis is a separation technique with a high resolution and a broad field of applicability. Reproducible qualitative and quantitative analyses can be performed. To be able to describe more precisely the separation process theoretically, more data on pK values and effective mobilities, at the concentrations and temperatures used, need to be measured accurately. Moreover, chemicals for isotachophoretic analyses that are purer and more stable than those now commercially available must be produced. The effective mobility of the counter ions must receive particular at-

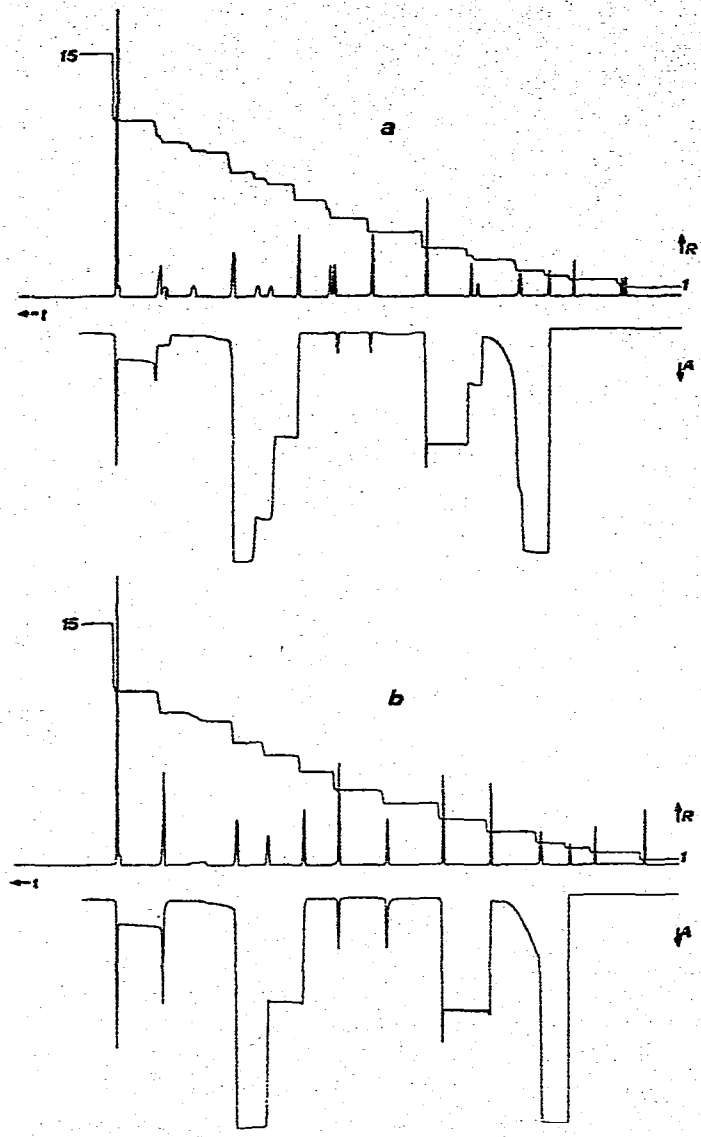

Fig. 16. Isotachopherogram for the separation of a standard mixture of anions in the operational system at pH 6 (Table II), (a) without and (b) with a counter flow of electrolyte. Peaks in (b) in sequence from 1 to 15: 1 = chloride; 2 = sulphate; 3 = chlorate; 4 = chromate; 5 = malonate; 6 = pyrazole-3,5-dicarboxylate; 7 = adipate; 8 = acetate; 9 = β-chloropropionate; 10 = benzoate; 11 = naphthalene-2-sulphonate; 12 = glutamate; 13 = enanthate; 14 = benzyl-*dl*-aspartate; 15 = morpholinoethanesulphonate. R = Increasing resistance; A = increasing UV absorption; t = increasing time; $I = 70\ \mu A$. It can clearly be seen that the zones of the impurities commonly present in the chemicals applied are enriched by the counter flow of electrolyte. In Table VI, the step heights of this mixture in water and deuterium oxide are compared.

tention. Analyses carried out in non-aqueous solvents and in their mixtures with, *e.g.*, water, must also be investigated, because these will greatly extend the field of application.

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