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ISOTACHOPHORESIS

APPLICATIONS IN THE BIOCHEMICAL FIELD

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SUMMARY

A few possibilities are discussed in which isotachophoresis can be used by workers more concerned with applications than with a detailed interpretation of the results.

Analyses are shown of enzymatic reactions and of fruit juices. Some operational systems that can be used in isotachophoretic experiments are outlined, and the combination of high-resolution conductometric and UV detection is considered.

INTRODUCTION

Theoretical aspects of isotachophoresis have been dealt with earlier[1-4], and therefore only some possible applications are presented in this paper.

Although some phenomena, especially as a result of analyses being carried out in narrow-bore tubes, still require satisfactory interpretation, the analyses show such a reproducibility that applications can already be made by those workers who are more concerned with application work than with a detailed interpretation of the results.

In this paper, in addition to the presentation of possible applications of two high-resolution detectors, both mounted on the same capillary, the specific UV detector and the general conductivity detector are also considered. The isotachopherograms obtained with these detectors can be analyzed far more accurately and rapidly than if only one type of detector is applied.

Isotachophoresis has proved to be of particular value if small ions are involved, *e.g.*, in the analysis of fruit juices and the study of enzymatic reactions. Research is being continued in the field of macromolecules.

EXPERIMENTAL AND RESULTS

The analyses were performed in a narrow-bore PTFE tube with an I.D. of 0.45 mm and an O.D. of 0.7 mm, fixed between two electrode compartments.

Because in isotachophoresis three different electrolytes (*i.e.*, the leading electro-

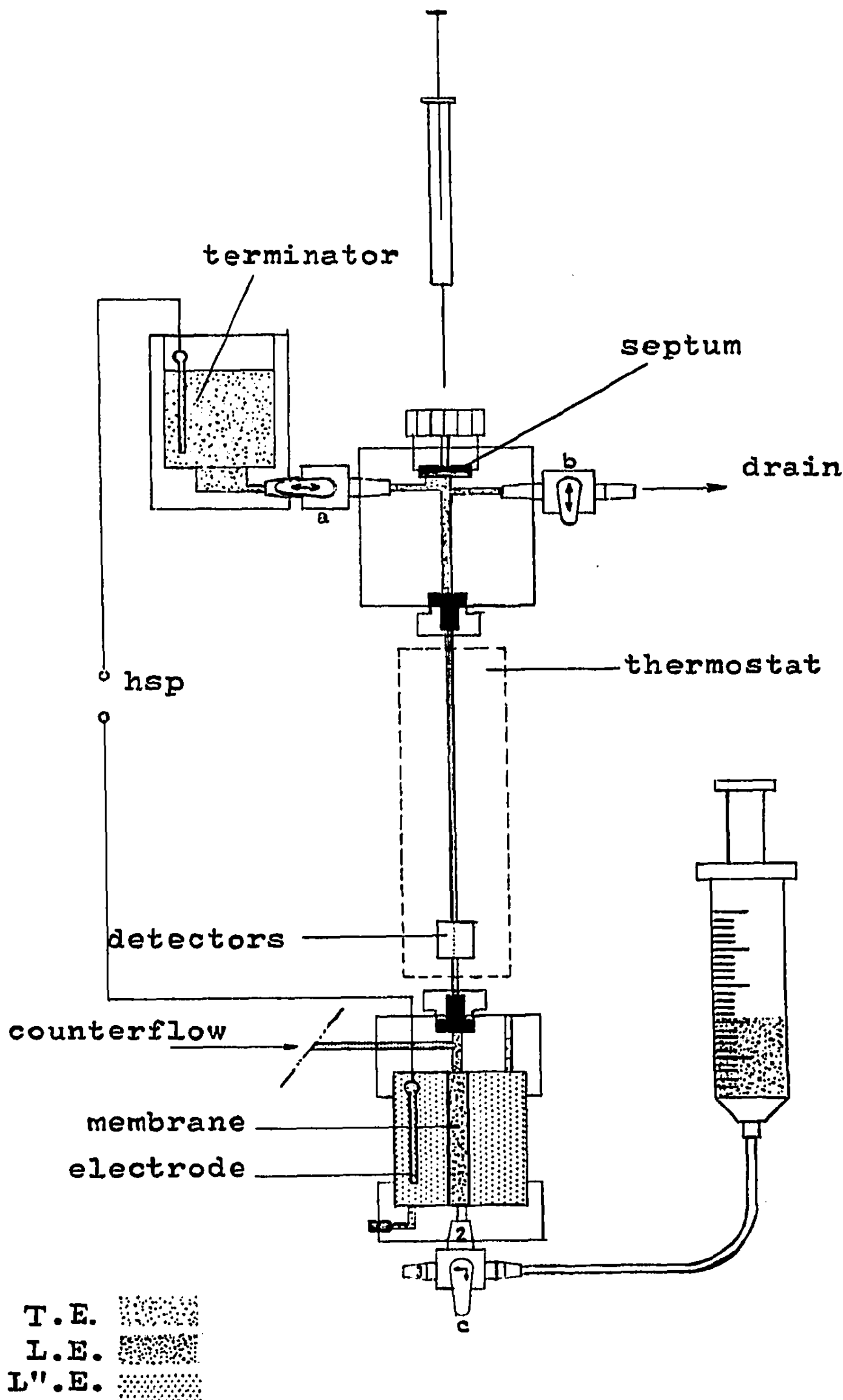

Fig. 1. Schematic diagram of the isotachophoretic equipment. hsp = Current-stabilized power supply (30 kV); T.E. = terminating electrolyte; L.E. = leading electrolyte; L''.E. = leading electrolyte possibly enriched with some buffer.

lyte (L.E.), the intermediate electrolyte (I.E.) or sample and the terminating electrolyte (T.E.)) are applied, the equipment was constructed so that one electrode compartment was filled with the terminating electrolyte, and this compartment was open to the air (Fig. 1). The counter-electrode compartment consisted of a semi-permeable membrane of cellulose acetate, and in this compartment the leading electrolyte was present as in the narrow-bore tube. A device (injection block) has been developed in order to introduce the sample (I.E.) on to the boundary between the leading electrolyte and the terminating electrolyte. By this means, zones that are not easily mixed can be expected between the very mobile leading ion and the mobile ions from the I.E. A UV detector (with a 0.2-mm slit, operating at 256 nm) was mounted 15 cm from the injection point. (It should be noted that the length for the separation of a given sample is determined by the difference in concentrations and the difference in mobilities of the ionic constituents of the I.E. Furthermore, the possibility of the application of a counter-flow of electrolyte[5,6] influences the choice of length of the narrow-bore tube necessary for the separation of the I.E.) About a 2-cm length of the narrow-bore tube separated this UV detector from the conductivity detector (4 kHz and 1 kHz; ref. 7). In a forthcoming paper, this instrument will be discussed in detail.

All chemicals used were of analytical grade (E. Merck, Darmstadt, G.F.R.), unless stated otherwise.

In order to obtain optimal sharpness of the zone boundaries and an adequate suppression of possible electrode reactions (bright platinum was used for the electrode material), the addition of about 0.05% of surface-active compounds was necessary. In addition to the suppression of the electrode reactions, by increasing the overpotential, the surfactants may also increase the viscosity in the vicinity of the wall and thus suppress electroendosmosis. From measurements with thermocouples mounted on the outside of the narrow-bore tube, we observed that the increase in temperature obtained after the passage of a zone was greater when an additive was used, which implies that the heat transfer coefficient must be increased by the addition of surface-active agents. If more heat is transported to the wall, the temperature difference between the inside of the narrow-bore tube and the wall is smaller.

Both the suppression of electroendosmosis and the increase in the heat transfer coefficient[8] flatten the parabolic profile, and the more the sample zones are situated towards the rear, the greater is this influence. As a result, the detection of the zone boundaries becomes sharper, although the real sharpness may not be improved (this effect is discussed later). The effects of the addition of different surface-active agents are discussed in a related paper[9].

In some experiments, the conductometer cell was coated with 1-aminoanthracene in order to make the detector sensitive to the presence of singly and doubly charged ions when the frequency of the measuring signal was varied; this is discussed elsewhere[9].

Because the selection of the solvent (aqueous or non-aqueous) and pH (affecting the effective mobilities) gives the possibility of a range of conditions, a combination of such conditions can be used in the operational system. Four examples of operational systems are given in Tables I–IV. With these systems, analyses were performed at pH 6.6, 6, 4.48 and 3.88, respectively. A different pH can be chosen however; in general, the concentration of the anion, in anion analyses, must be kept

TABLE I

OPERATIONAL SYSTEM FOR ISOTACHOPHORETIC ANALYSES AT pH 6.6

Electrolyte	*Property*	*Condition*
General	Solvent	Water
	Stabilizing agent	None
	Electric current	70–100 μA
	Temperature	22°
Leading electrolyte (L.E.)	Conc. anion	0.01 *M* HCl (p.a. grade)
	Conc. buffer	Ethylenediamine, adjusted to pH 6.6*
	Additive	0.05% polyvinyl alcohol
Intermediate electrolyte (I.E.)	Ionic strength	Adjusted approximately to the ionic strength of the leading electrolyte
	pH	Adjusted approximately to the pH of the leading electrolyte
	Additive	None
Terminating electrolyte (T.E.)	Conc. anion	*ca.* 0.01 *N***
	pH	$\leqslant$ 6.6
	Additive	None

* The buffer is purified by running it over an anion exchanger.

** The terminating ion is selected for its low mobility (effective). In many experiments, glutamic acid (p.a. grade) was found to be slow enough.

constant, while the concentration of the cation (counter-ion) must be adapted to the pH required. Because in anion analyses the pH increases towards the terminator side, again as a general rule, the pH may be varied according to the condition $\text{p}K + 0.5 > \text{pH} > \text{p}K - 1$.

TABLE II

OPERATIONAL SYSTEM FOR ISOTACHOPHORETIC ANALYSES AT pH 6

Electrolyte	*Property*	*Condition*
General	Solvent	Water
	Stabilizing agent	None
	Electric current	70–100 μA
	Temperature	22°
Leading electrolyte (L.E.)	Conc. anion	0.01 *M* HCl (p.a. grade)
	Conc. buffer	0.02 *M* histidine
	Additive	0.05% polyvinyl alcohol
Intermediate electrolyte (I.E.)	Ionic strength	Adjusted approximately to the ionic strength of the leading electrolyte
	pH	Adjusted approximately to the pH of the leading electrolyte
	Additive	None
Terminating electrolyte (T.E.)	Conc anion	*ca.* 0.01 *N**
	pH	$\leqslant$ 6
	Additive	None

* The terminating ion is selected for its low mobility (effective). In many experiments, glutamic acid (p.a.) was found to be slow enough.

TABLE III

OPERATIONAL SYSTEM FOR ISOTACHOPHORETIC ANALYSES AT pH 4.48

Electrolyte	*Property*	*Condition*
General	Solvent	Water
	Stabilizing agent	None
	Electric current	70–100 μA
	Temperature	22°
Leading electrolyte (L.E.)	Conc. anion	0.01 *M* HCl (p.a. grade)
	Conc. buffer	Aniline, adjusted to pH 4.48*
	Additive	0.05% polyvinyl alcohol
Intermediate electrolyte (I.E.)	Ionic strength	Adjusted approximately to the ionic strength of the leading electrolyte
	pH	Adjusted approximately to the pH of the leading electrolyte
	Additive	None
Terminating electrolyte (T.E.)	Conc. anion	*ca.* 0.01 *N***
	pH	⩽ 4.48
	Additive	None

* The buffer is purified by running it over an anion exchanger.

** The terminating ion is selected for its low mobility (effective). In many experiments, pivalic acid or caproic acid (both purified) was found to be slow enough.

For the operational system, where histidine is used as a buffering counter-ion (pH = 6), counter-ions could also be taken such as β-picoline and pyridine. There are several reasons why histidine was selected: it is much simpler to work with histidine with respect to the purity, smell, the poisonous properties of pyridine and

TABLE IV

OPERATIONAL SYSTEM FOR ISOTACHOPHORETIC ANALYSES AT pH 3.88

Electrolyte	*Property*	*Condition*
General	Solvent	Water
	Stabilizing agent	None
	Electric current	*ca.* 40 μA
	Temperature	22°
Leading electrolyte (L.E.)	Conc. anion	0.01 *M* HCl (p.a. grade)
	Conc. buffer	β-Alanine, adjusted to pH 3.88*
	Additive	0.05% polyvinyl alcohol
Intermediate electrolyte (I.E.)	Ionic strength	Adjusted approximately to the ionic strength of the leading electrolyte
	pH	Adjusted approximately to the pH of the leading electrolyte
	Additive	None
Terminating electrolyte (T.E.)	Conc. anion	*ca.* 0.01 *N***
	pH	⩽ 3.88
	Additive	None

* The buffer is purified by precipitating the β-alanine with ethanol (p.a. grade) from a saturated solution of β-alanine in double-distilled water.

** The terminating ion is selected for its low mobility (effective). In many experiments, caproic acid (purified) was found to be slow enough.

β-picoline and the fact that histidine is a solid; the UV absorption of histidine is negligible; and the more mobile the components chosen are, the more electricity is transported by these ions. This results in higher conductivities of the zones and less resolving power. Thus, longer narrow-bore tubes need to be used for similar mixtures to be separated, which increases the time required for analysis. Also, the sharpness of a zone boundary is influenced by the choice of a mobile or less mobile counter-ion, because the self-correction of the zone boundaries is a function of the potential gradients present in the consecutive zones.

Because the narrow-bore tubes used have large stabilizing effects for small ions, if one side is closed with a semi-permeable membrane, no stabilizing agent (*e.g.*, agarose or polyacrylamide) need be applied.

Fruit juices

The separation of the ionic components of fruit juices is of interest and a reliable and rapid analysis is often required. The application of other high-resolution techniques, *e.g.*, gas chromatography, often needs complicated sample pre-treatments, and other methods fail owing to the lack of sensitive detectors, if many components need to be analyzed simultaneously.

An example of the isotachophoretic separation of anions in the juice of cherries is shown in Fig. 2. Two isotachopherograms are shown, both obtained when comparable amounts of juice were injected and the operational system in both instances was similar (operational system of Table IV). The components found to be present were not only determined by checking them with the pure components in one operational system (Table IV), but all qualitative information was collected in different operational systems (Tables I–III) (also called separation by pK values).

The juice giving the left-hand trace was of primary quality, made of the pulp, whereas the juice giving the right-hand trace was obtained by cooking the stones with some remaining pulp on them. Attention is drawn particularly to the large difference in the concentrations of citric and lactic acids. In the primary quality juice, citric acid was the main constituent and lactic acid could only just be detected, whereas in the second quality juice the opposite was found. It was also found unexpectedly that detectable and comparable amounts of vitamin C were found in both juices.

The combination of the two high-sensitivity detectors (Fig. 3) made the interpretation much easier. Because they are mounted in a fixed position, the zones of the intermediate electrolyte must appear with a constant retardation. And if it does not, it can be concluded that the steady state has not yet been reached. The specificity of the detector can clearly be seen from these isotachopherograms. From the conductometric detector, general information can be deduced.

In this work, an investigation was made to establish whether nitrate or sulphate was present. These zones cannot be made visible with the UV detector, and the separation by pK values also does not help, because neither ion is affected by changes in pH. The use of a different concentration is a possibility (at a concentration of 1 mM, sulphate moves more quickly than chloride), but much more information needs to be collected in the field of low concentrations before this method can be applied. In order to elucidate whether sulphate or nitrate was present, the electrodes were coated

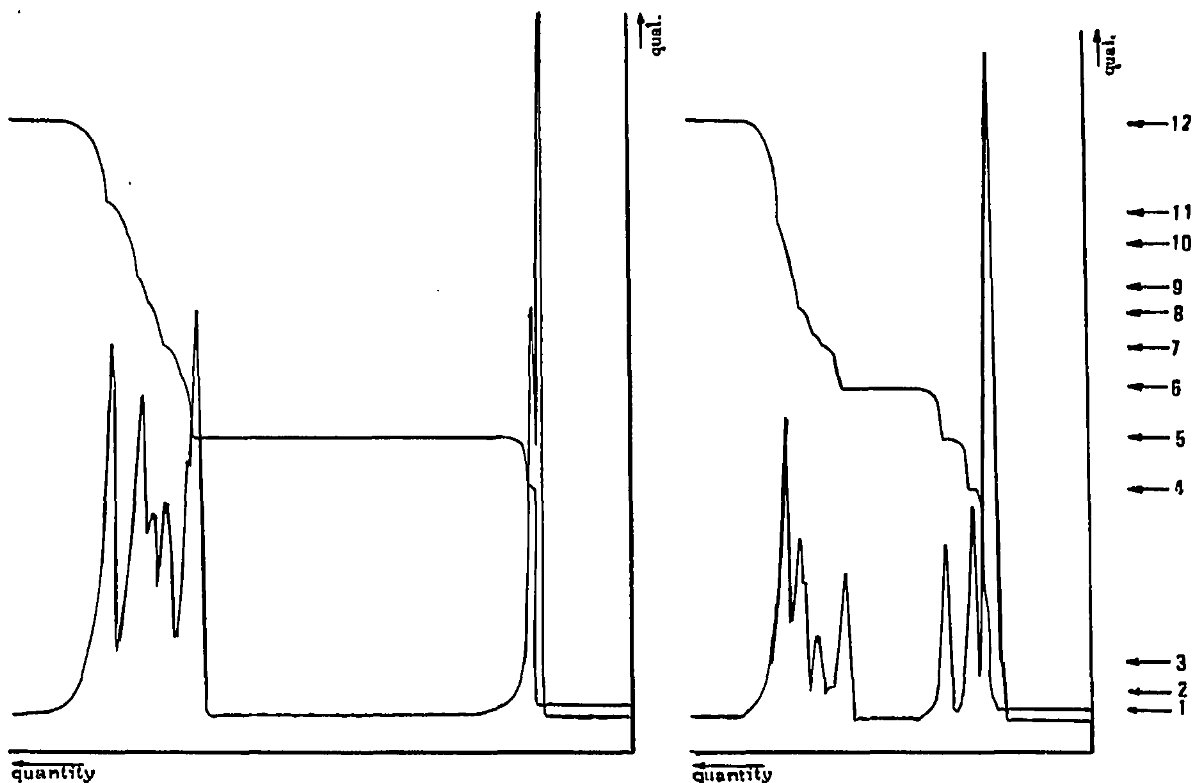

Fig. 2. Isotachopherogram of the separation of the juice of cherries. The left-hand isotachopherogram shows an analysis of the juice prepared from the pulp (primary quality) and the right-hand isotachopherogram shows an analysis of the juice of similar cherries, but prepared after cooking the stones with the remaining pulp (second quality). 1 = Chloride; 2 = sulphate; 3 = unidentified; 4 = phosphate; 5 = citrate; 6 = lactate; 7 = quinate; 8 = unidentified; 9 = benzoate; 10 = unidentified; 11 = vitamin C; 12 = capronate (terminator). The juice (concentrate) was diluted 50 times; the injected volume was 0.7 μl. The current was stabilized at 70 μA; the initial voltage was 3 kV and the final voltage was 6 kV. The time for analysis was 10 min; the recorder speed 6 cm/min. For full qualitative determination of the components involved, all operational systems were applied (listed in Tables I–IV). The isotachopherogram shown was obtained using the operational system detailed in Table IV.

with 1-aminoanthracene[9] so that they became sensitive to the presence of singly and doubly charged ions when the frequency of the measuring signal was varied. From these experiments, it was concluded that sulphate was present in the juice[9].

Extra information can be deduced if a combination of a conductivity and a UV detector is used. If zones are present that consist of unseparated ions, which show different UV absorption*, the presence of these zones will not be detected by the conductivity detector, but a signal different from that for pure components is obtained from the UV detector. (If the mixed zone consists of singly and doubly charged ions, the conductometer can give specific information if the electrodes are

* In isotachophoretic analyses, if the steady state has been reached, all zones move together with constant speed and calculable decreasing concentrations of the ionic constituents.

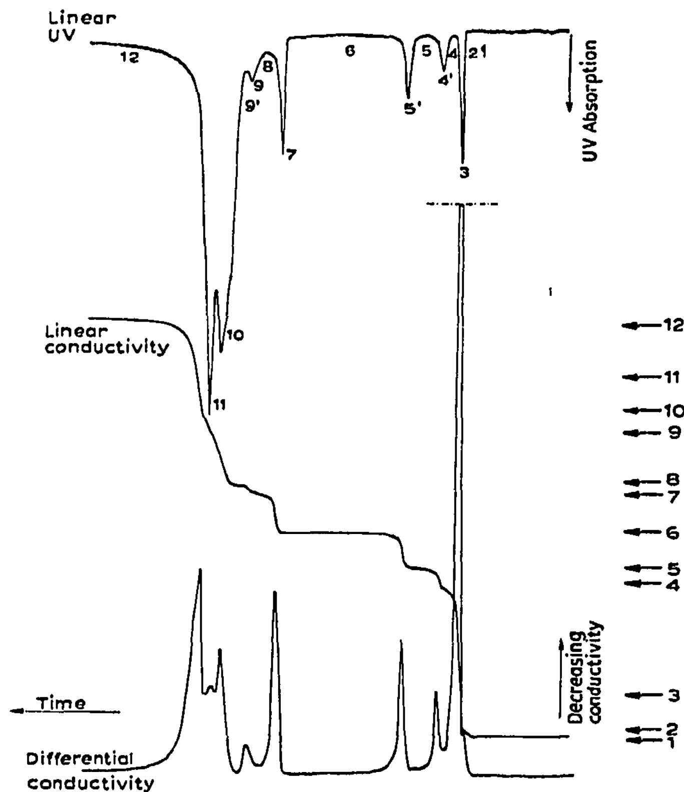

Fig. 3. Isotachopherogram of the separation of the juice of cherries, already shown in Fig. 2 (right). The pH of the operational system (as listed in Table IV) was 4 instead of 3.88. At least three more components could be detected in this operational system (4′, 5′ and 9′) with the UV detector.

coated.) If the mixed zones consist of weak acids or bases and the ionic species have different pK values, the ions can be separated by changing the pH in the direction of the pK values. It must be borne in mind, however, that the pH in mixed zones is not the same as the pH in the zones of the pure components. This may affect the results, especially if components are present of which the UV absorption is strongly influenced by variations in pH.

More information on the anion analyses of juices of different origin and on the cation analyses of these juices was given by Vos[10].

Enzymatic reactions

The course of enzymatic reactions can be followed by measuring the change in the concentrations of *e.g.* NAD or NADP. One of these components must participate in the enzymatic reaction in order to be able to follow the reaction due to the difference in absorption of dihydropyridine and pyridine at 260 and 340 nm. In some enzymatic reactions, however, these components are not present, and in such cases these reactions must be followed by utilizing other reactions in which NAD or NADP again participates. Also, other components are sometimes involved that absorb at 260 or 340 nm and cause interference.

In this work a reaction was studied for which it is normally necessary to utilize another reaction in order to be able to follow the first[4,11]. Again, the isotachopherograms are obtained from a combination of a conductometric and a UV detector. The reaction studied was

Because both NAD and NADP are absent, this reaction is normally followed by utilizing the reaction

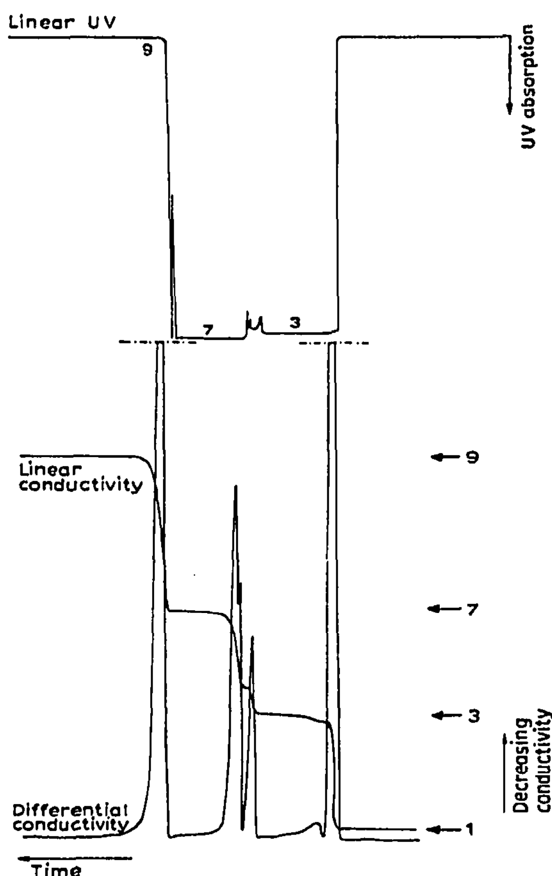

Fig. 4. Isotachopherogram of a reaction mixture composed of ATP and NADP to be applied in the enzymatic conversion of glucose by hexokinase from yeast and followed by the enzymatic reaction catalysed by glucose-6-phosphate dehydrogenase from yeast. The NADP consisted of at least four UV-absorbing and two non-absorbing impurities. Two impurities were faster moving than NADP, possibly phosphate and ATP. Three impurities, all UV-absorbing, were slower moving and could not be characterized. The purity was about 80–90%. The ATP consisted of two UV-absorbing components, both moving more slowly than ATP. For further details, see the text and Fig. 7.

By using isotachophoresis, these reactions can be studied separately or a combination can be taken, because all ionic components can be separated in the operational system given in Table IV, and isotachopherograms were obtained with glucose, ATP and NADPH as reagents.

Glucose and ATP were of analytical grade (E. Merck, Darmstadt, G.F.R.) and all other chemicals were of biochemical purity (Boehringer, Mannheim, G.F.R.).

In Fig. 4, the reaction mixture is shown before the addition of the enzymes hexokinase and glucose-6-phosphate dehydrogenase. A 1-μl volume of a mixture of 0.0067 *M* glucose, 0.0067 *M* ATP and 0.0067 *M* $NADP^+$ was injected. Both the UV and conductometric detection showed impurities, usually present in biochemicals, even when chemicals of analytical grade were applied. Although these impurities disturbed the isotachophoretic pattern, the broad zones of ATP and $NADP^+$ were still fully reproducible and recognizable. Moreover, when using the UV detector, these impurities may be essential because they may mark the passage of a zone boundary[12]. Caproic acid was used as the terminating electrolyte.

To 3 ml of the reaction mixture, 10 μl of hexokinase (*ca.* 14 i.u.) and 10 μl of glucose-6-phosphate dehydrogenase (*ca.*35 i.u.) were added. The pH was maintained at 3.8 in order to prohibit any enzymatic reaction, which enabled us to check if any possible impurity had been added that might have obscured the result. In Fig. 5, an isotachopherogram of this mixture is shown; 1 μl of the reaction mixture was injected. From Fig. 5, it can be seen that the introduction of the enzymes increased

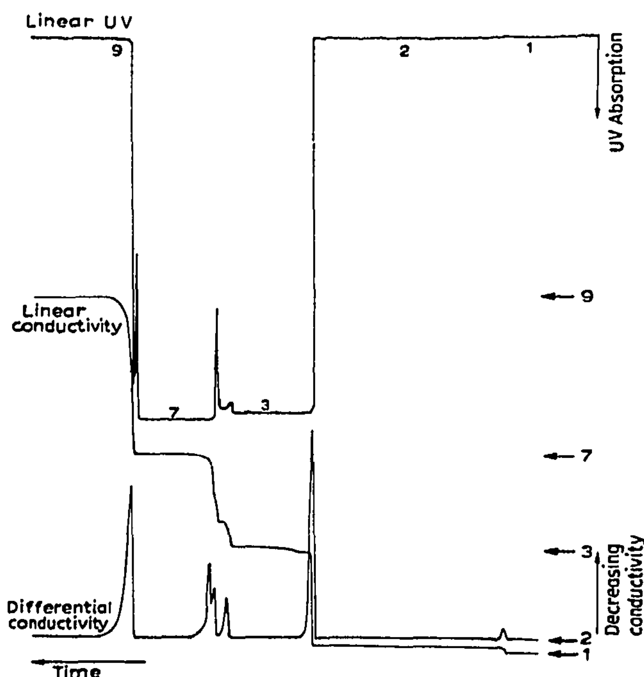

Fig. 5. The reaction mixture shown in Fig. 4 after the addition of the enzymes hexokinase and glucose-6-phosphate dehydrogenase. The amounts of impurities increased and clearly visible are the zones of ATP and NADP. For further details, see the text and Fig. 7.

the amount of impurities. The sulphate, detected by the conductometric detector, originated from the ammonium sulphate added to the enzyme mixture as a stabilizer. If only one enzyme is added, this zone can be taken as a standard for the addition of the enzyme.

In Fig. 6, the isotachopherogram of the analysis is shown, the pH from the reaction mixture with the enzymes being increased to 7.6 by the addition of Tris. A 1-μl volume of the reaction mixture was injected. It must be remembered that the pH of the operational system used is 3.88, and therefore the injection was directly into the leading electrolyte and the reaction was stopped immediately.

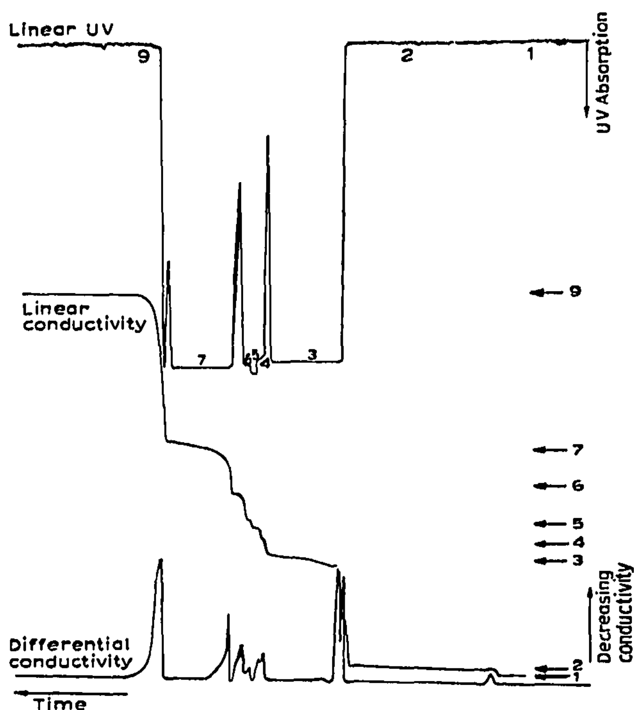

Fig. 6. The initial phase of the conversion is shown, a sample of the reaction mixture being analyzed directly after the increase of the pH, by addition of Tris, up to 7.6. (The reaction mixture as shown in Fig. 5 is not directly applicable because the pH is 3.5.) Although 6-phosphogluconate already can be detected, the amount of glucose-6-phosphate is too low to make detection possible under the conditions chosen. For further details see text and Fig. 7.

In Fig. 7, the isotachopherogram of the separation is shown for a reaction mixture kept for 1 h at 22°. It was possible, using both UV and conductometric detection, to observe the conversion of ATP into ADP and NADP into NADPH. The reaction products were also visible.

The time for analysis of these isotachophoretic experiments was 10 min; the recorder paper speed was 2 cm/min.

The study is being continued in order to establish the impurities found in the

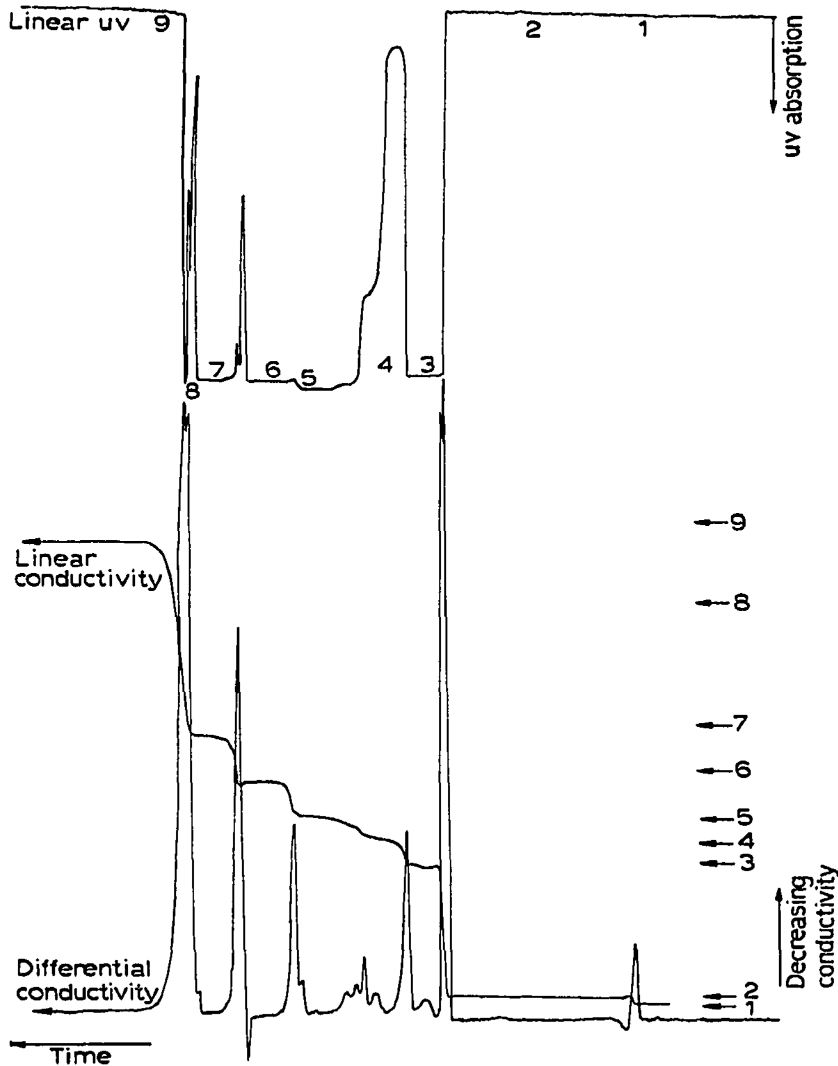

Fig. 7. Enzymatic conversion of glucose by hexokinase from yeast, followed by the enzymatic conversion of glucose-6-phosphate by glucose-6-phosphate dehydrogenase from yeast. 1 = Chloride; 2 = sulphate; 3 = ATP; 4 = 6-phosphoglyconate; 5 = NADPH; 6 = ADP; 7 = NADP; 8 = glucose-6-phosphate; 9 = caproate (terminator). Many components are detected as well by the UV detector as by the conductivity detector, but have not yet been characterized.

reaction constituents and to find which other components are formed during the reaction.

Resolution

Beckers and Everaerts[13] introduced a test mixture for the operational system listed in Table II. In order to make possible a comparison between the two detectors considered in the present work, an isotachopherogram recorded simultaneously by

these two detectors is shown in Fig. 8. Although the zones of adipate, acetate and β-chloropropionate do not show any UV absorption, these zones are marked by impurities present in the chemicals of which the sample mixture is composed. In the

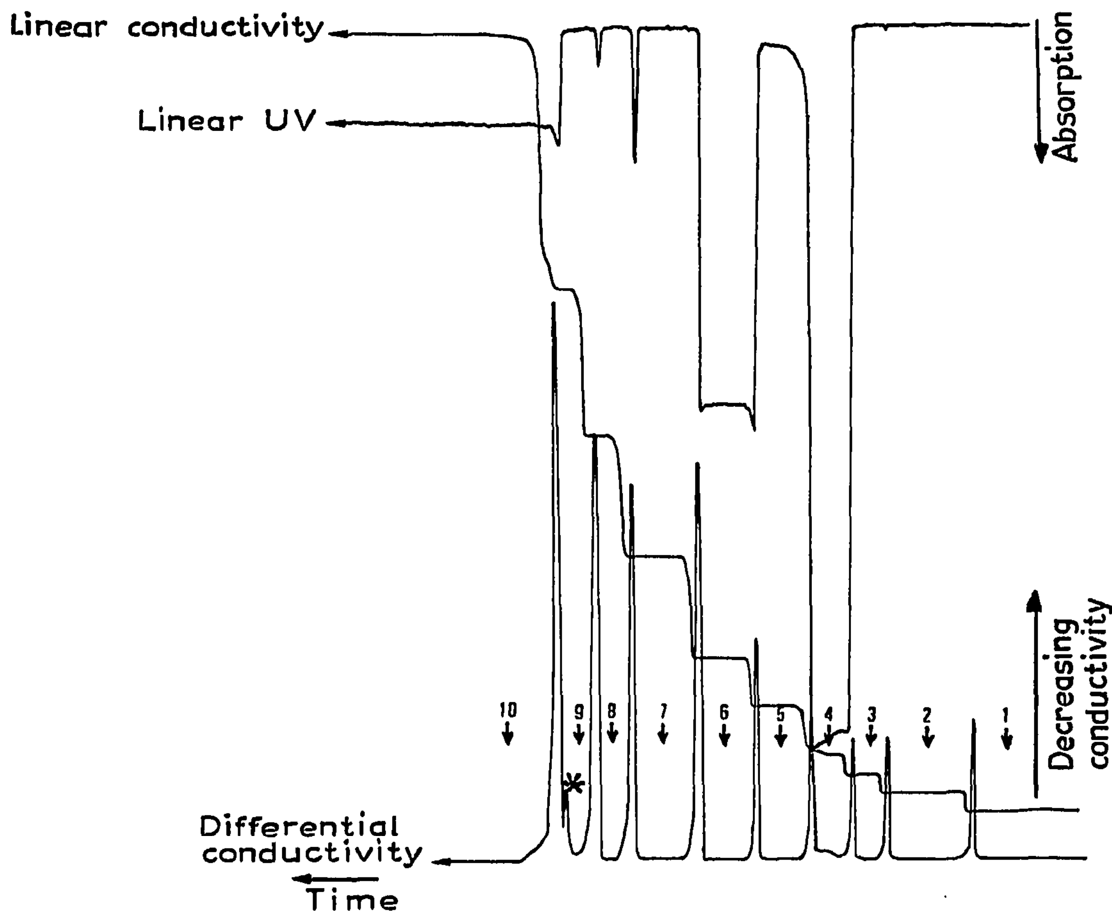

Fig. 8. Results for the test mixture[13] for the operational system listed in Table II. The isotachopherogram is shown in order to make possible a comparison between the UV detector and the conductivity detector. 1 = Chloride; 2 = sulphate; 3 = chlorate; 4 = chromate (UV absorbing); 5 = malonate; 6 = pyrazole 3,5 dicarboxylate (UV absorbing); 7 = adipate; 8 = acetate; 9 = β-chloropropionate; 10 = phenylacetate (UV absorbing). The component marked with an asterisk is an unidentified component of the sample of adipate (produced by BDH, Poole, Great Britain). At least four more impurities are detected by the UV detector.

isotachopherogram shown in Fig. 8, at least four impurities are present. For qualitative work, the information deduced from the UV detector is poor.

A possible explanation will be given of why these impurities can sometimes be observed so clearly by the UV detector but very poorly by the conductivity detector, which has a comparable resolving power. As already discussed, the more the zones

of the intermediate electrolyte are located towards the rear, the more pronounced is the parabolic profile, owing to the temperature difference between the centre of the tube and the wall of the narrow-bore tube. The UV absorbing impurities are located between the zones of interest. The more the zones show a parabolic shape, the longer is the length of the tube actually (Fig. 9 a, c) occupied by this impurity. The real zone

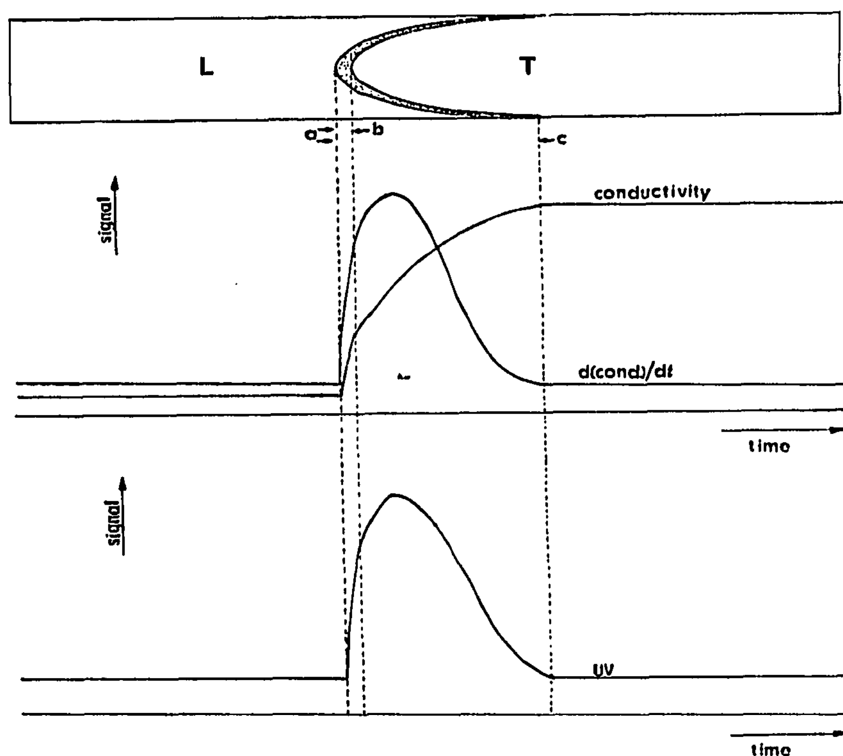

Fig. 9. Schematic explanation of the detection of small amounts of UV-absorbing impurities. ab = Real length of the zone of UV-absorbing impurity; ac = zone length as recorded by the UV detector; L = leading electrolyte; T = terminating electrolyte. Although the differential trace of the conductivity detector and the linear trace of the UV detector appear similar, their origin and interpretation are totally different; with the conductivity detector, the UV-absorbing impurity is only just apparent.

length may be only a few micrometres (Fig. 9 a, b). This means that the zone length, as detected, is determined by the parabolic shape of the boundary transition and not by the boundary sharpness. Also, the conductometric detector may determine a parabolic profile as a diffuse zone.

The test mixture, as shown in Fig. 8, can be separated in about 3 min. The length of the capillary in these experiments was only a few centimetres, which was found to be long enough for a complete separation. For further improvement in the technique, more attention should be paid to effective thermostatic control. Also, treatment of the wall[8], even in capillary systems, may be important.

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