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SEPARATION OF SOME TYPICAL KREBS CYCLE ACIDS BY HIGH-SPEED ISOTACHOPHORESIS

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SUMMARY

In order to perform a required separation, an experimental procedure for the selection of suitable operating conditions is suggested and discussed. It is based on measurements of the dependences of the relative effective mobilities of the components under investigation on the pH of the leading electrolyte. The procedure was applied to a set of typical Krebs cycle acids and the values of the relative effective mobilities measured are given in tables and graphs. A pH of 3.8 was selected as the most suitable. At this value, the acids investigated were successfully separated in less than 4 min using 0.011 *M* hydrochloric acid + β-alanine as the leading electrolyte.

INTRODUCTION

The tricarboxylic acid cycle is a key stage in the metabolic pathway that is characteristic of the aerobic oxidative degradation of carbohydrates, lipids and proteins in most biological systems on the one hand, and on the other hand it is characteristic of the biosynthesis of cellular material from simple carbon sources under anaerobic conditions. The analysis of such a complex mixture of keto and hydroxy acids is very difficult. The decisive factors are speed, sensitivity and the resolving power of the selected analytical method. It is logical that most separation methods have been tested and certain positive results have been obtained by using both paper electrophoresis[1–4] and thin-layer (*e.g.*, ref. 5), ion-exchange (*e.g.*, ref. 6) and recently also gas[7–9] and liquid[10,11] chromatography.

Gas chromatography has been shown to be the most promising method as far as resolving power and sensitivity are concerned. However, even by this method, the most important acids of the metabolic cycle mentioned cannot be completely separated. The fact that the acids, which are mostly found in aqueous solutions in practice, must be converted into sufficiently volatile water-soluble esters, remains the main drawback of gas chromatography. The derivative formation proceeds[7] to a various degree with acids (90–100% with dimethyl esters and 50–55% with methyl and tri-

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methyl esters) and with hydroxy acids the formation of several different derivatives from one acid often occurs.

Therefore, further methods have been investigated, even at the cost of lower sensitivity and increased expense, such as high-performance liquid chromatography[11]. The separation time varies from 30 to 50 min at best and it will probably not be possible to decrease it to below 20 min without a substantial loss of resolution.

Isotachophoresis, which has shown possibilities for the separation of important ionic species, among others, and also of some acids from the Krebs cycle[12–14], has attracted a great deal of attention recently. Using the possibilities that are provided by high-speed analytical isotachophoresis[15] for both quantitative[16] and qualitative[17] processing of the records obtained in an isotachophoretic separation, we applied our approach also to more complex mixtures of the acids typical of the Krebs cycle. We obtained results that are promising as far as speed, resolving power and sensitivity are concerned. Also, aqueous solutions of these acids can be used directly in the analysis.

Analysis of the problem

The successful application of isotachophoresis to the separation of the acids under investigation requires the selection of operating conditions such that the acids differ sufficiently in their effective mobilities. The pH of the leading electrolyte is the main factor that permits the effective mobilities of the anions of weak acids to be changed over a wide range. The selection of the operating conditions then consists in the selection of the pH of the leading electrolyte and the selection of a suitable terminating electrolyte[18,19]. If all of the required data on the dissociation constants of the components and the absolute mobilities of their ionic species are known ,then the effective mobilities of the components studied in their zones can be determined for the given pH of the leading electrolyte by means of routine computer programs[20].

If the required data are not known, which is common in practice, particularly for biochemically interesting substances, suitable operating conditions must be established experimentally. The procedure that has been published[21] is based on plotting the heights of the steps of the components being investigated, read from the isotachophoretic record, against the pH of the leading electrolyte. Although this procedure determines the sequence of the zones for a given selected composition of the leading electrolyte, the values of the step heights do not, however, represent the mobilities directly and, in addition, the data measured at different pH values of the leading electrolyte cannot be compared in a simple way.

The procedure based on the relative mobilities seems to be used to advantage in isotachophoresis in order to compare directly the mobilities at different pH values. Using this procedure, the mobilities of the components studied are related to the mobility of a suitable reference compound separated under the same conditions, the mobility of which is virtually independent of pH. For instance, the chloride anion is a suitable reference component for anions[1].

In isotachophoresis, we have already reported[17] the use of the relative mobilities in connection with the direct experimental determination of the relative mobilities from the record of the separation obtained by using a gradient detector and applying these values to the identification of the components investigated in their zones. This procedure can obviously also be used for the selection of the optimal separation con-

ditions. If the chloride anion is selected as a reference component and its mobility is represented by u_{Cl}, and if the mobility of the component, i, which is separated simultaneously, is represented by u_i, then

$$E_i u_i = E_{Cl} u_{Cl} = v = \text{constant}$$

where E_i and E_{Cl} are the electric gradients in the relevant zones and v is the velocity of migration of the zone. If the values of the electric gradients are recorded on the isotachopherogram in the form of step heights, h_i and h_{Cl}, then

$$u_i/u_{Cl} = h_{Cl}/h_i$$

Tables or graphs of the dependences $h_{Cl}/h_i = f(pH)$ can then be constructed directly. The ratio h_{Cl}/h_i gives directly the relative effective mobilities of component i and the values measured at different pH values can then be compared. Operating conditions for performing the required separation can then easily be selected.

EXPERIMENTAL

A monolithic block of organic glass (5 × 4 × 30 cm), in which electrode compartments, an injection device, control valves, connecting channels and a separation capillary are built, is the basic part of an analytical isotachophoreograph. The capillary is formed by a flat groove with a rectangular cross-section (0.2 × 1 × 200 mm) in the lower part of the wall of the monolithic block covered with a PTFE foil pressed to the whole body with a metallic thermostated plate. The temperature of the plate was 295°K. Detection was carried out by sensing the electric gradients in the zones separated by means of platinum contacts (platinum wires of 0.05 mm O.D., 0.05 mm apart in the longitudinal direction) penetrating into the capillary groove at a distance of about 16 cm from the injection compartment. A detailed description can be found elsewhere[15,17].

A high-voltage power supply provided d.c. current controllable up to 400 μA at a maximum of 16 kV. The detection device consisted of a voltmeter with a high input resistance, which, at the same time, insulated electrically a high voltage section of the measuring circuits connected to a detection cell from the circuits serving for the connection of a recorder. The recorder used was a Perkin-Elmer Model 1969. The d.c. current power supply and the detection device were of our own construction and their descriptions can be found elsewhere[17].

The chemicals used were of analytical-reagent grade, supplied by Lachema (Brno, Czechoslovakia). The solutions of most of the components studied are unstable and substantial changes in their compositions occur within 2–4 days at laboratory temperature, due either to decomposition or to the activity of microorganisms. Therefore, the standard solutions and the leading and terminating electrolytes were kept in a refrigerator at 273°K. The solutions kept under such conditions lasted longer, but several solutions kept even in this way (*e.g.*, isocitric acid, oxaloacetic acid and urotropine) became visibly turbid within 3–5 days and had to be freshly prepared.

RESULTS AND DISCUSSION

The relative effective mobilities of the components studied were measured by using the leading electrolytes given in Table I. The chloride anion was used as the leading ion in all instances. Its concentration was kept constant in order to suppress the effect of the variation of the ionic mobilities with concentration as much as possible. The pH range 3.2–7.4 was selected as it lies inside the so-called[20] "safe region" of isotachophoresis in aqueous media, *i.e.*, the pH range where the contributions of H^+ and OH^- ions to the total conductivity of zones can be neglected and, additionally, where the interference of carbonates is substantially suppressed. The terminating electrolyte was always selected at a given pH such that it was well separated from the other components of the sample and permitted the use of the highest possible driving current and thus also the attainment of the shortest possible separation time. The value of the driving current corresponds to the maximum voltage of the source used (16 kV) and to the conductivity of the terminator, or it is limited by the maximum of the stabilised current, *i.e.*, 400 μA.

TABLE I

CHARACTERISTICS OF THE OPERATING SYSTEMS

System	*Leading electrolyte*		*Terminating electrolyte*	*Driving current (μA)*
	pH	*Composition*		
Gl–Cl	3.2	0.011 *N* HCl + glycine	0.004 *M* acetic acid	220
Al–Cl	3.8	0.011 *N* HCl + β-alanine	0.004 *M* acetic acid	260
An–Cl	4.6	0.011 *N* HCl + aniline	0.005 *M* glutamic acid	400
Ur–Cl	5.1	0.011 *N* HCl + urotropine	0.005 *M* glutamic acid	400
Hi–Cl(1)	6.0	0.011 *N* HCl + histidine	0.005 *M* glutamic acid	400
Hi–Cl(2)	6.6	0.011 *N* HCl + histidine	0.005 *M* ascorbic acid	400
Im–Cl	7.4	0.011 *N* HCl + imidazole	0.005 *M* ascorbic acid	400

The measurement of the relative effective mobilities of the components investigated was carried out in such a way that the components were injected separately while the concentrations of the solutions were $3–8 \cdot 10^{-4}$ *M* and the amounts injected were approximately 4–8 μl. The relative effective mobilities were calculated from the measured step heights and their values are listed in Table II.

In order to select the most suitable pH for the separation of all of the components considered, it is advantageous to plot the dependences on a graph, as shown in Fig. 1. The components investigated, with the exception of oxalate, have very similar effective mobilities. The numerous intersections on the curves represent instances when different components have the same effective mobilities at a given pH, which results in the formation of undesirable stable mixed zones when the separation of the components is impossible.

The problem of interference by carbonates that are present in the sample and in the electrolytes used owing to the absorption of atmospheric carbon dioxide is an important aspect for the selection of a pH suitable for a given separation. The effective mobility of carbonate is also shown in Fig. 1. From the course of its curve, the mobility of the carbonate ion is obviously greater than the mobility of lactate at pH

TABLE II

RELATIVE EFFECTIVE MOBILITIES OF THE SPECIES IN VARIOUS OPERATING SYSTEMS

Chloride was used as the reference species; $u_{Cl} = 1.00$.

Ionic species	*System*						
	Gl–Cl	*Al–Cl*	*An–Cl*	*Ur–Cl*	*Hi–Cl (1)*	*Hi–Cl (2)*	*Im–Cl*
Chloride	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Oxalate	0.49	0.67	0.79	0.83	0.88	0.89	0.89
Oxaloacetate	0.36	0.52	0.54	0.55	0.77	0.78	0.78
Fumarate	0.30	0.45	0.63	0.74	0.74	0.74	0.74
α-Ketoglutarate	0.36	0.41	0.55	0.66	0.71	0.72	0.72
Citrate	0.25	0.37	0.53	0.65	0.69	0.69	0.69
Malate	0.23	0.36	0.54	0.64	0.70	0.71	0.72
Isocitrate	0.23	0.34	0.51	0.64	0.70	0.72	0.73
Succinate	0.12	0.27	0.44	0.56	0.67	0.70	0.72
Lactate	0.22	0.32	0.46	0.47	0.48	0.48	0.48
Terminator	0.08	0.20	0.35	0.37	0.40	0.42	0.42
	acetate			glutamate		ascorbate	

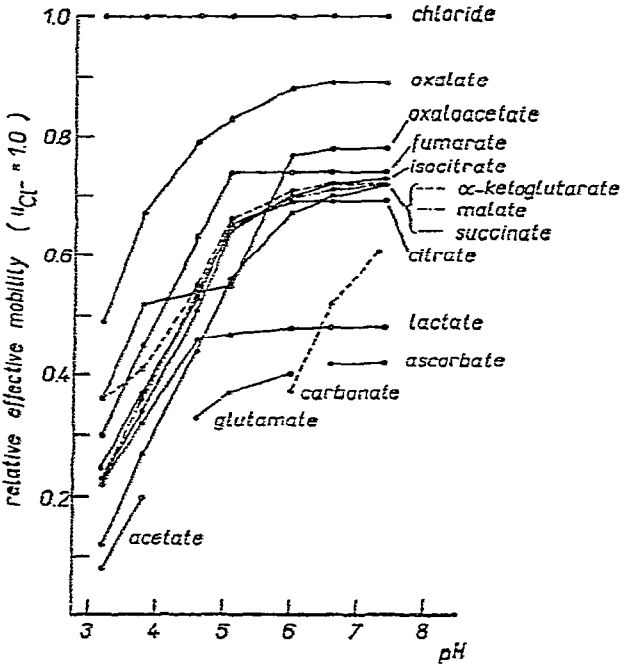

Fig. 1. Dependence of the relative effective mobilities of the species on the pH of the leading electrolyte. Chloride was used as the reference species, $u_{Cl} = 1.00$, independent of the pH.

above *ca.* 6.5 and the isotachophoretic migration of the lactate zone is obviously affected. At pH below 6.2, the mobility of the carbonate ion is very low (lower than the mobility of the terminator), which means that it does not create its own isotachophoretic zone in the given system and that its interference is virtually eliminated.

It is further necessary to consider the fact that the substances separate more easily, the greater is the relative difference in their mobilities[22]. It can be further seen from Fig. 1 that the mobilities decrease with decreasing pH, while their absolute differences (parallel course of the curves) were maintained in a number of instances. At lower pH, the relative differences in the mobilities increase. For instance, the mobilities of citrate and malate are 0.69 and 0.70 at pH 6.0, *i.e.*, their difference (0.01) is 1.4%. At pH 3.8, the mobilities are 0.37 and 0.36, *i.e.*, the same difference of 0.01 unit represents 2.7%.

If the conditions are known for such a separation, in which the composition of the leading electrolyte is the main factor, a suitable terminator must be selected, the basic criterion for which is that its mobility should be sufficiently lower than that of any component of the sample. Another requirement that should be fulfilled, which is particularly important in practice, originates from the fact that the conductivity of the terminating zone is a limiting factor for the increase in the driving current and thus also a limiting factor for the reduction of the analysis time. The maximum usable current is given by the maximum voltage of the power supply and by the conductivity of the terminating zone. From this viewpoint, a terminator should be selected that

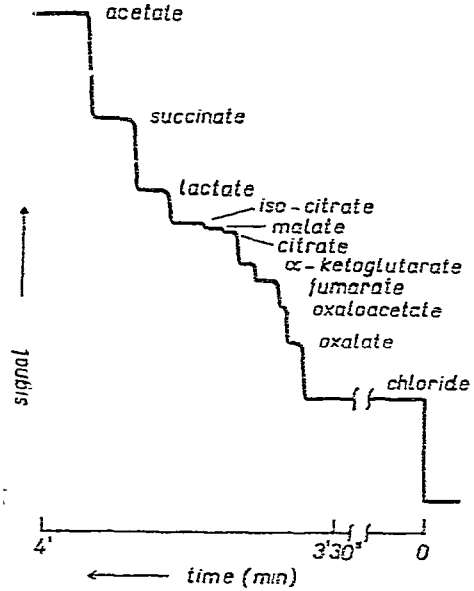

Fig. 2. Analysis of a model mixture of some acids of the Krebs cycle. The leading electrolyte consisted of 0.011 *M* HCl + β-alanine. The terminating electrolyte consisted of 0.004 *M* acetic acid. The composition of the model mixture was $4.4 \cdot 10^{-4}$ *M* oxalic acid, $5.0 \cdot 10^{-4}$ *M* oxaloacetic acid, $7.5 \cdot 10^{-4}$ *M* fumaric acid, $6.6 \cdot 10^{-4}$ *M* α-ketoglutaric acid, $7.1 \cdot 10^{-4}$ *M* citric acid, $5.1 \cdot 10^{-4}$ *M* malic acid, $1.1 \cdot 10^{-3}$ *M* isocitric acid, $9.7 \cdot 10^{-4}$ *M* lactic acid and $1.7 \cdot 10^{-3}$ *M* succinic acid. The volume injected was about 4 μl. The driving current used was 260 μA.

separates well from the other components of the sample and, at the same time, whose mobility is as high as possible.

Based on these considerations, a pH of 3.8 was selected as the most suitable for the separation of the components under study, *i.e.*, a leading electrolyte with the composition 0.011 *M* hydrochloric acid plus *β*-alanine; 0.004 *M* acetic acid was used as the terminator. Fig. 2 shows the record of the separation of the nine components typical of Krebs cycle acids and the operating conditions in greater detail. It is obvious that the selected pH of the leading electrolyte was suitable and that all of the components can be separated successfully. Also, it can be seen that a complete separation of the mixture can be achieved in less than 4 min, which proves the suitability of acetate as the terminator.

CONCLUSIONS

The selection of suitable operating conditions for carrying out the separation in question can be performed experimentally by measuring the dependences of the relative mobilities on the pH of the leading electrolyte. The gradient detector is advantageous for such measurements as the relative mobilities can be measured directly as the ratios of the step heights on the record.

This procedure was applied to a set of acids typical of the Krebs cycle. It was found that the relative effective mobilities of these acids are very dependent on pH and that they are very similar. A pH of 3.8 was selected from the region of pH values (3.2–7.4) measured as being suitable for the separation. The components studied were separated successfully at this pH by using 0.011 *M* hydrochloric acid + *β*-alanine as the leading electrolyte. *β*-Alanine also has the advantage of a good stability of its stock solutions (longer than 1 month).

The formation of stable mixed zones can be expected at pH values outside this range, and at pH above 6 interference with the separation can be expected from carbonates present in the solutions.

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