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Note

Analytical isotachophoresis in capillary tubes

Transformation of pyruvate to succinate by calf heart mitochondrial enzymes

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Since analytical isotachophoresis can be used for simultaneous detection of nanomole quantities of different ion species, this technique seems suitable for the analysis of citric acid cycle metabolites. The transformation of pyruvate to succinate by the use of mitochondrial enzymes was chosen to demonstrate the separation of these metabolites from all other essential ion species in the reaction mixture. One of the advantages of analytical isotachophoresis is that crude samples can be injected directly, which means that incubation mixtures containing mitochondria could be analysed without pretreatment of the sample.

A review of isotachophoresis, including the theory, has been published by Haglund[1].

EXPERIMENTAL

The capillary apparatus used was the LKB 2127 Tachophor (LKB-Bromma, Bromma, Sweden). The separations were carried out in a 62-cm long PTFE capillary with I.D. 0.5 mm, which was maintained at a constant temperature of 19°. The apparatus[2] was equipped with a thermal detector, which was also used in a differential manner, and with a UV detector set at 254 nm.

The leading electrolyte consisted of 0.01 *M* chloride as the leading ion and 0.032 *M* β-alanine as the counter ion giving a pH of 3.9. The terminating electrolyte was 0.01 *M* caproic acid. The experiments were run at a constant current of 70 μA and lasted for about 30 min. The potential increased from 3 kV at the beginning to 19 kV at the end of the experiments.

The mitochondria reacted in an incubation mixture of 7.7% sucrose, 60 m*M* Tris, 30 m*M* HCl, 9 m*M* $MgCl_2$, 0.3 m*M* ADP, 3 m*M* phosphate, 1.2 m*M* malate, 6.0 m*M* malonate and 1.2 m*M* pyruvate. The pH of the reaction mixture was 8.0 both before and after the addition of the mitochondria. The calf heart mitochondria used had a final concentration of 2 mg of protein per millilitre. The reaction time was a minimum of 20 min at 25°. A 5-μl sample was used for separation.

Sucrose, Tris, maleic acid, sodium pyruvate and β-alanine were purchased from BDH Chemicals Ltd.; HCl, KCl, $MgCl_2$, Na_2HPO_4 and malonic acid from Merck; ADP from Sigma Chemical Co.; and caproic acid from Fluka AG Chemische

Fabrik. The mitochondria preparation was kindly provided by Dr. A. Vestermark, Stockholm, Sweden.

RESULTS AND DISCUSSION

The separation of the anions in the incubation mixtures is shown in Fig. 1. The thermal detector signals (Fig. 1, intermediate position) give both qualitative and quantitative information about the separated ions. The step-height of the thermal signal is a measure of the net mobility, and is therefore characteristic of an ion in a particular electrolyte system. Each sample zone has a uniform concentration in isotachophoresis, and the zone lengths are therefore a measure of the amounts of the separated ions. On the differentiated thermal signal (Fig. 1, bottom), the zone lengths are measured as the distance between two adjacent peaks. As the UV detector has a very high resolution, in addition to the predicted sample zones several zones are found that are too narrow to be detected thermally. These zones are due to impurities in the sample or in the electrolytes.

In Fig. 1a, a sample containing 6 nmoles of pyruvate, 30 nmoles of malonate, 15 nmoles of phosphate, 6 nmoles of malate and 1.5 nmoles of ADP was separated.

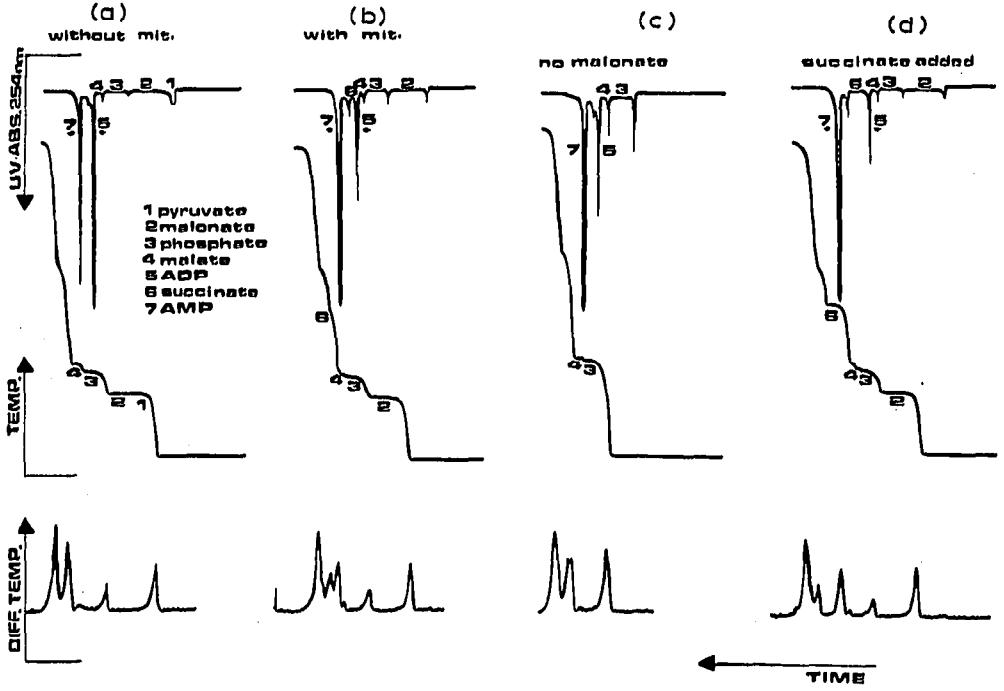

Fig. 1. The separation of an incubation mixture initially containing 6 nmoles of pyruvate, 30 nmoles of malonate, 15 nmoles of phosphate, 6 nmoles of malate and 1.5 nmoles of ADP, with (a) no mitochondria, (b) mitochondria, (c) mitochondria but no malonate and (d) mitochondria and an additional 10 nmoles of succinate. The leading electrolyte was 0.01 *M* HCl and 0.032 *M* β-alanine, pH 3.9, and the terminator was 0.01 *M* caproic acid. UV absorption (254 nm), thermal and differential thermal detectors were used.

The thermal detector will not resolve the pyruvate and malonate zones, and the ADP zone is too small to be detected thermally. On the UV detector signal, however, the pyruvate zone can be distinguished from the malonate zone. The ADP is also easily recognized as a UV-absorbing peak. The ADP used also contained small amounts of AMP (zone 7), seen by UV detection. Between all the non-UV-absorbing sample zones there are small amounts of UV-absorbing impurities that act as markers for the sample zones. Therefore, in this experiment, the UV detector identifies all the non-UV-absorbing sample components.

In Fig. 1b, the consumption of pyruvate is seen by the decreased zone length of the first thermal step and the absence of the first UV-absorbing zone that is present in Fig. 1a. The thermal step-height of the succinate zone is marked as 6, which corresponds to the zone between peaks Nos. 4 and 5 on the differentiated temperature signal. The UV detector shows the succinate zone to separate the ADP zone from a UV-absorbing impurity. When mitochondria are present in the reaction mixture, ADP is partly transformed into AMP, which is found by comparing zones 5 and 7 in Figs. 1a and 1b.

The consumption of pyruvate can also be seen when no malonate is present (Fig. 1c). Carbon dioxide and water were produced, as no inhibition occurred. An additional 10 nmoles of succinate was added to the incubation mixture (Fig. 1d) in order to verify the succinate production shown in Fig. 1b. The increase in zone 6 can be followed on all the detector signals in Fig. 1d. In all other respects, Figs. 1b and 1d are identical. Similarly, the AMP zone was identified by addition of synthetic AMP to the reaction mixture.

It can be seen that no ATP is produced. The ATP zone should be formed between the malonate and phosphate zones, easily recognized as being highly UV-absorbing. Obviously, the mitochondria used were uncoupled.

These experiments have shown that reaction mixtures containing mitochondria could be used directly for separation with analytical isotachophoresis. In these experiments, all the anions involved in the enzymatic transformation were separable, and could consequently be detected simultaneously.

The combination of the thermal and UV detectors permits the identification and quantitation of the sample ions with high resolution. Not only small amounts of UV-absorbing components can be detected, but also all the non-UV-absorbing ions were resolved by the UV detector, owing to the very small amounts of UV-absorbing impurities of diffierent mobilities that always seem to be present in the chemicals that are normally used.

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REFERENCES

1 H. Haglund, *Sci. Tools*, 17, No. 1 (1970) 2.
2 L. Arlinger, in H. Peeters (Editor), *Protides of the Biological Fluids*, 1971, p. 513.