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Analytical Isotachophoresis in Capillary Tubes Used for the Separation of Ions Involved in the Enzymatic Transformation of Glucose to 6-Phosphogluconate

ANN KOPWILLEM

LKB-Produkter AB, Fack, S-161 25 Bromma 1, Sweden

The ions involved in the enzymatic transformation of glucose to 6-phosphogluconate have been separated by means of analytical isotachophoresis. The reactants and the reaction products simultaneously analysed were ATP, ADP, $NADP^+$, NADPH, glucose-6-phosphate, and 6-phosphogluconate. Nanomol quantities of these ions were separated within 30 min. Detection of the sample ions was done with a thermal and a UV-detector.

A two-step enzymatic reaction, the transformation of glucose to 6-phosphogluconate, has been chosen as a model system to demonstrate the separability of some biochemically important ions by isotachophoresis in capillary tubes. The method gives qualitative as well as quantitative information about the ion species involved. A great number of reactants and reaction products can be followed simultaneously, *e. g.* ATP, ADP, $NADP^+$, NADPH, glucose-6-phosphate, and 6-phospho-gluconate. Nanomol quantities of these ions were analysed in 30 min. In addition to the compounds mentioned a number of impurities were also found. No pre-treatment, *i.e.* deproteinization or concentration of the sample, was necessary. Previously, isotachophoresis has also been used for the separation of nucleotides in capillary tubes [1] and for the separation of glucose metabolites on thin layer.[2–3]

In isotachophoresis, as in all electrophoretic separation methods, the ion species migrate in an electric field. In contrast to ordinary electrophoresis, the sample ions, when the steady state is reached, migrate with the same velocity and form zones according to their electrophoretic mobilities. In isotachophoresis, the electrolyte system consists of a leading electrolyte, containing a leading ion with a mobility higher than that of the sample ions and a terminating electrolyte, containing a terminating ion with a lower mobility than that of the sample ions. The leading electrolyte also contains a counter-ion, generally buffering, which migrates into the terminating electrolyte, and thus is common to both electrolytes. A constant current is applied to the system

creating a discontinuous electric field and the sample ions separate and form zones of constant concentrations. In the steady state, the electric field rises step-wise at each zone boundary. A high electric field in a sample zone is associated with a low mobility of the sample ion and *vice versa*. In isotachophoresis, the boundaries between the sample zones remain sharp due to the high and discontinuous electric field.

The concentration and pH in the sample zones are regulated by the concentration and mobility of the leading ion and the pH in the leading electrolyte. The separability of the sample ions is based on the differences in their net mobilities. The net mobility is defined as: ionic mobility × degree of dissociation. It is therefore pH-dependent. Consequently, the net mobility of the sample ion is dependent on the pH in the leading electrolyte.

A more detailed theoretical treatment of isotachophoresis is given by Haglund [4] in a review, which also contains different applications.

EXPERIMENTAL

The capillary apparatus used in this investigation was the LKB 2127 Tachophor (LKB-Produkter AB, Bromma 1, Sweden). The separations took place in a 62 cm long, teflon capillary with 0.5 mm I. D., which was kept at a constant temperature of 19°C. The apparatus [5] was equipped with a thermal detector, which was also used in a differential manner, and with a UV-detector set at 254 nm.

The ATP and glucose were obtained from British Drug Houses Ltd., Poole, Dorset, U.K. $NADP^+$ was purchased from Boehringer und Söhne, Mannheim, Germany, as well as the enzyme preparation containing both hexokinase and glucose-6-phosphate dehydrogenase activity. In all incubation mixtures the enzyme was present in a final dilution of 1:30.

Two series of experiments were carried out. First, a 1.2 mM glucose solution was transformed to glucose-6-phosphate in the presence of ATP (3 mM). In the second series, glucose (2 mM) was transformed to 6-phosphogluconate in the presence of ATP (3 mM) and $NADP^+$ (1 or 3 mM). All experiments were performed in triethanolammonium chloride buffer (24 – 36 mM) at pH 7.5. The incubation mixtures were kept at 25°C for at least 10 min to complete the reactions. The reaction products were then stoichiometrically formed. 5 µl samples were used for analytical isotachophoresis in all experiments. The quantities of the ion species are given in the figure texts.

The leading electrolyte consisted of 0.01 M chloride with different amounts of β-alanine as a counter-ion, giving varying pH-values in the leading electrolyte. The terminating electrolyte was 0.01 M caproic acid. The experiments were run at a constant current of 100 µA for about 30 min. The voltage increased from 4 kV at the beginning to 23 kV at the end of the experiment.

RESULTS AND DISCUSSION

Fig. 1 shows the thermal step-heights of the ions to be separated at different pH-values of the leading electrolyte. The thermal step-height is a measure of the net mobility [6–8] and thus also separability.

In the separation of the ions involved in the transformation of glucose to glucose-6-phosphate the pH in the leading electrolyte was 3.8, whereas in the separation of the ions present in the two-step enzymatic reaction, the leading electrolyte pH was 4.2 in order to ensure full separation.

Results of separations of different mixtures of ions are shown in Figs. 2 and 4. The curves with sharp peaks in Fig. 2 (bottom) are the differential

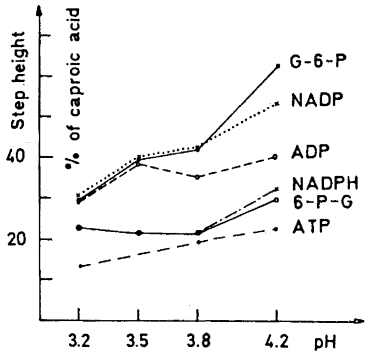

Fig. 1. Thermal step-heights of the ions involved in the enzymatic transformation of glucose to 6-phosphogluconate related to the step-height of the terminator, caproic acid, as a funtion of pH in the leading electrolyte.

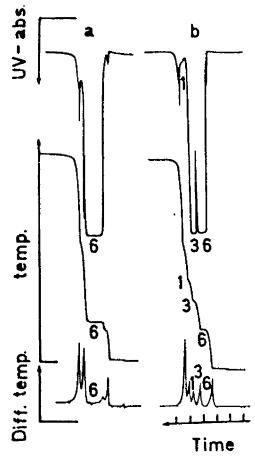

Fig. 2. Separation of ATP (zone 6), ADP (zone 3) and glucose-6-phosphate (zone 1). Leading electrolyte was 0.01 M HCl and 0.027 M β-alanine. (pH = 3.8). Terminator was 0.01 M caproic acid. UV-absorption (254 nm), thermal and differentiated thermal detectors were used.
Sample ions were: (a) ATP 15 nmol; (b) ATP 9 nmol, ADP 6 nmol, G-6-P 6 nmol.

temperature recordings. The distance between two peaks is easily measured from this type of curves. This distance is proportional to the zone length in the capillary tube. Since the concentration of an ion is uniform throughout its zone, quantitative determination of an ionic species can be made by simply measuring the length of the zones. The curves with steps in Fig. 2 (intermediate position) are the temperature recordings. The step-height is a function of the net mobility of the isotachophoretically moving ion in the corresponding zone. The UV-recordings in Fig. 2 (top) show, in addition to the nucleotide peaks of more or less rectangular shape a number of small peaks of different shapes. These small peaks represent UV-absorbing impurities either in the leading or terminating electrolyte or in the sample. For reference a sample containing no glucose is separated (Fig. 2a), where in addition to the ATP (6) zone of 15 nmol, the first low thermal step that occurs is derived from the sulphate ions in the enzyme solution. Another reproducible non-UV-absorbing zone comes from the impurities in the counter-ion, β-alanine. This zone can be seen following the ATP zone on all detector signals. The UV detector also resolves another three UV-absorbing impurities, which are separated by less UV-absorbing components.

The enzymatic consumption of ATP and glucose is seen in Fig. 2b by the occurrence of the glucose-6-phosphate (1) and ADP (3) zones and also from the decreased ATP (6) zone length. The zone lengths are directly related to the quantities of the ions involved in separation (Fig. 3). The UV detector signals

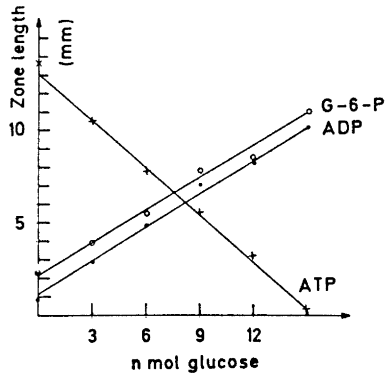

Fig. 3. The zone length (mm) of ATP, ADP, and glucose-6-phosphate as a function of initial glucose quantity in the reaction mixtures. Initial quantity of ATP was 15 nmol. Chart speed 10 mm/min.

were used to measure the zone lengths. Since the glucose-6-phosphate zone was measured together with the impurities in β-alanine up to the well-defined UV-peak (Fig. 2), the zone length function did not pass the origin. Similarly, the ADP zone length is enlarged due to the UV-absorbing zone following the ATP zone (Fig. 2a).

In the separation including $NADP^+$ used in the transformation of glucose-6-phosphate to 6-phosphogluconate, a large UV-absorbing impurity is present in front of $NADP^+$ (Fig. 4a). This impurity can be followed as the UV-absorbing peak following the 6-phosphogluconate (5) zone (Figs. 4b and 4c).

Figs. 4b and 4c show the separation of ions involved in the two-step enzymatic reaction, where glucose (10 nmol), ATP (15 nmol) and $NADP^+$ (5, 15 nmol) react with the enzymes. The reaction products, glucose-6-phos-

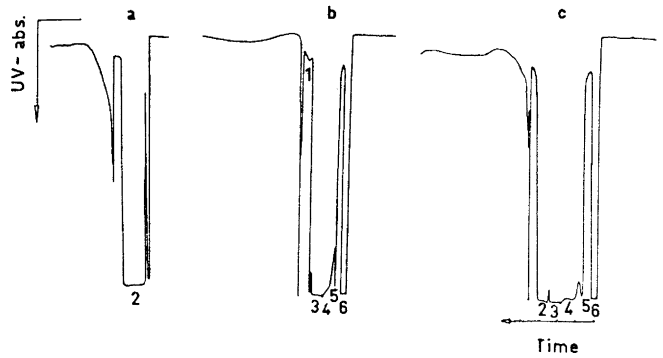

Fig. 4. Separation of glucose-6-phosphate (zone 1), $NADP^+$ (zone 2), ADP (zone 3), NADPH (zone 4), 6-phosphogluconate (zone 5), and ATP (zone 6). Leading electrolyte was 0.01 M HCl and 0.055 M β-alanine (pH = 4.2). Terminator was 0.01 M caproic acid. UV-absorption (254 nm) was measured.
Sample ions were: (a) $NADP^+$ 15 nmol; (b) G-6-P 5 nmol, ADP 10 nmol, NADPH 5 nmol, 6-P-G 5 nmol, ATP 5 nmol; (c) $NADP^+$ 5 nmol, ADP 10 nmol, NADPH 10 nmol, 6-P-G 10 nmol, ATP 5 nmol.

phate (1), ADP (3), NADPH (4), and 6-phosphogluconate (5), and the reactants ATP (6) and $NADP^+$ (2), are separable and recognizable as distinct zones. In addition to the compounds mentioned, a number of impurities are found, which act as markers of the zone boundaries. In fact, each predicted sample zone is surrounded by impurities. This can be most useful when two adjoining zones have the same UV-adsorption (*e.g.* zones 2 and 3 in Fig. 4c).

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