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Isotachophoresis used alone or in two-dimensional combination with zone electrophoresis for the small-scale isolation of labelled ribulose-1,5-diphosphate

By the electrophoretic method known as isotachophoresis, it is possible to arrange ionic species into consecutive zones. Each zone will, at equilibrium, contain only one ionic species and a corresponding amount of a counter ion. The ionic concentrations in the different zones will depend upon the concentration of the leading ion and the mobilities of a number of ions in the system[1]. The length of the zones will depend upon the concentration of the leading ion and the amounts of the ionic species present in the system. The zone length is independent of the concentration of the ionic species at the beginning of the experiment. An ionic species present in only a trace amount will therefore concentrate to give a very short zone (Fig. 1).

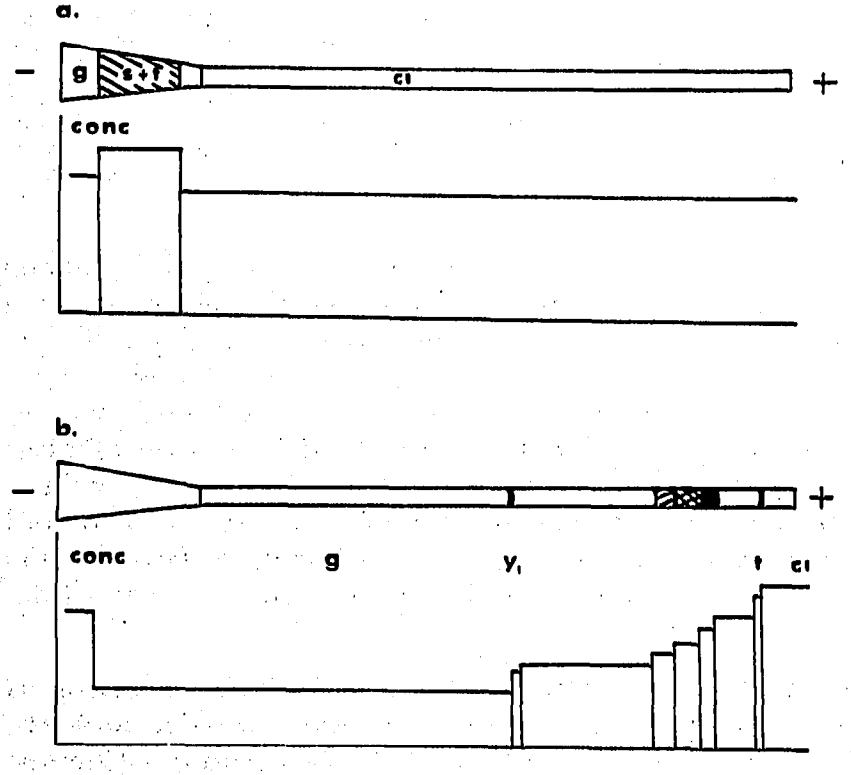

Fig. 1. (a) The upper part of the figure shows a cellulose acetate strip in contact with a wedge-shaped filter-paper (left) on which the sample(s) and the blue reference substance (t) are placed. The lower part of the figure shows the ionic concentrations along the strip and filter-paper. (b) All the ions in the sample have collected into consecutive zones between the yellow impurity (Y_1) from the strip and the reference substance (t).

Brief discussions of the basic theory of isotachophoresis are included in recent review papers[2,3].

In this paper we describe how isotachophoresis has been used for the small-scale isolation of labelled ribulose-1,5-diphosphate (RuDP), synthesized enzymatically. The two-dimensional combination of isotachophoresis with zone electrophoresis is also described.

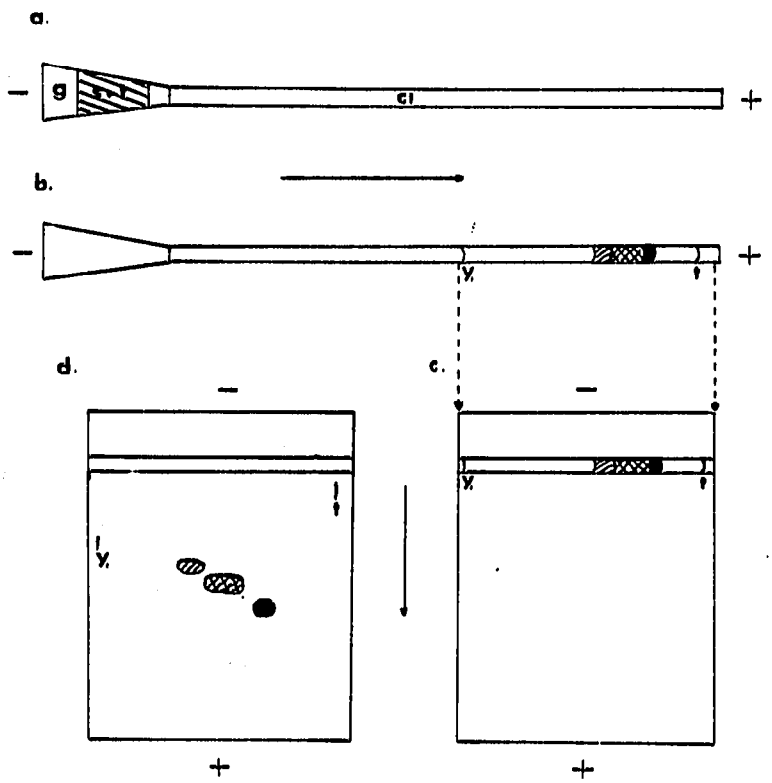

Fig. 2. Two-dimensional combination of isotachophoresis with zone electrophoresis: (a) the start and (b) the end of the isotachophoresis. In (c), the anodic part of the cellulose acetate strip used in the first direction has been transferred to a cellulose thin layer equilibrated with sodium-EDTA (0.2 *M*, pH 7.4). In (d), the zone electrophoresis has been completed.

Isotachophoresis and zone electrophoresis

One-dimensional isotachophoresis was carried out on 1 × 20 cm cellulose acetate strips (Macherey-Nagel & Co., Düren, G.F.R.). The strips were moistened with the leading electrolyte and placed on a siliconized glass plate. During the experiment, the plate was cooled with tap water[4]. The sample (s), together with a small amount of a blue reference substance, indigo tetrasulphonate (t), was applied on a wedge-shaped filter-paper and placed in contact with the terminating electrolyte (g) and one of the moistened cellulose acetate strips (Fig. 2). Samples of up to 0.2 ml were used. At 800–1000 V, the experiments lasted for about 1 h. The position of the separation zone was indicated by the blue indigo tetrasulphonate band. Some of the impurities in the cellulose acetate concentrated to sharp zones. Three of them, all yellow, were also used as mobility references. All sugar phosphates were found between one of the low-mobility yellow zones (Y_1) and the high-mobility indigo tetrasulphonate. When the electrophoresis was finished, the strips were transferred to a glass plate and dried in a warm air-stream (not above 40°). The drying was completed in about 1 min. The electrolyte systems used in the isotachophoretic and zone electrophoretic experiments are summarized in Table I.

Isotachophoresis for two-dimensional purposes was carried out on 0.4 × 20 cm cellulose acetate strips under the same conditions as the one-dimensional separations. When the separation on the 0.4-cm wide strip was finished, the anodic part of the strip containing the separation zone was rapidly transferred without drying to a

TABLE I

ELECTROLYTES USED IN ISOTACHOPHORESIS AND ZONE ELECTROPHORESIS

Experiment	*Buffer system*	*Concentration (M)*	*pH*	*Cathode vessel*	*Anode vessel*	*Strip or thin layer*	*Direction*
Isotachophoresis	Tris–HCl	0.4	7.5		+	+	1
	Tris–glycine	0.2	8.0	+			1
Zone electrophoresis	Sodium--EDTA	0.2	7.4	+	+	+	2

12 × 16 cm cellulose powder thin layer moistened with 0.2 *M* sodium-EDTA (pH 7.4) (Fig. 2). The thin layer was cooled with water during the electrophoresis, which lasted for about 1 h. The potential was adjusted in such a way that the heating of the thin layer was not more than 25 W.

Radioactive zones and spots were localized on the dried strips or plates radioautographically.

Preparation of labelled RuDP

[U-^{14}C]RuDP was prepared in three steps.

Step 1. 0.08 μmole (8.6 μCi) [U-^{14}C]glucose-6-phosphate ([U-^{14}C]G6P) was oxidized to [U-^{14}C]6-phosphogluconate ([U-^{14}C]6PG) in the presence of glucose-6-phosphate dehydrogenase (EC 1.1.1.49), NADP and Mg^{2+} (ref. 5). The total reaction mixture (0.2 ml) was subjected to isotachophoresis after 20 min of incubation at 30° (Fig. 3A). The resulting narrow zone containing [U-^{14}C]6PG was cut out from the cellulose acetate strip and used as "substrate" in the next step.

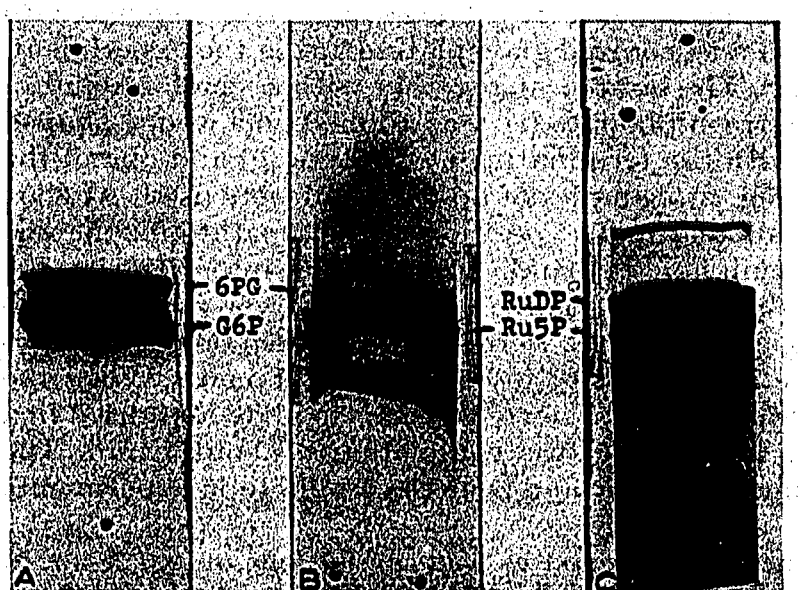

Fig. 3. Radioautographs of isotachophoretic separations of the three incubation mixtures used in the enzymatic synthesis of [U-^{14}C]RuDP from [U-^{14}C]G6P. (A) Transformation of G6P to 6PG; (B) 6PG to Ru5P; (C) Ru5P to RuDP.

Step 2. The piece of cellulose acetate strip containing the [U-^{14}C]6PG-zone from step 1 was incubated in the presence of 6-phosphogluconate dehydrogenase (EC 1.1.1.44), NADP and Mg^{2+} in optimal concentrations[6]. The volume of the solution was 0.2 ml. The [U-^{14}C]ribulose-5-phosphate ([U-^{14}C]Ru5P) was isolated isotachophoretically (Fig. 3B).

To check the efficiency of the one-dimensional isotachophoresis, a two-dimensional combination with zone electrophoresis was used. Also in these experiments the total reaction mixture (0.2 ml) was placed on the wedge-shaped filter-paper used for application purposes (Fig. 4).

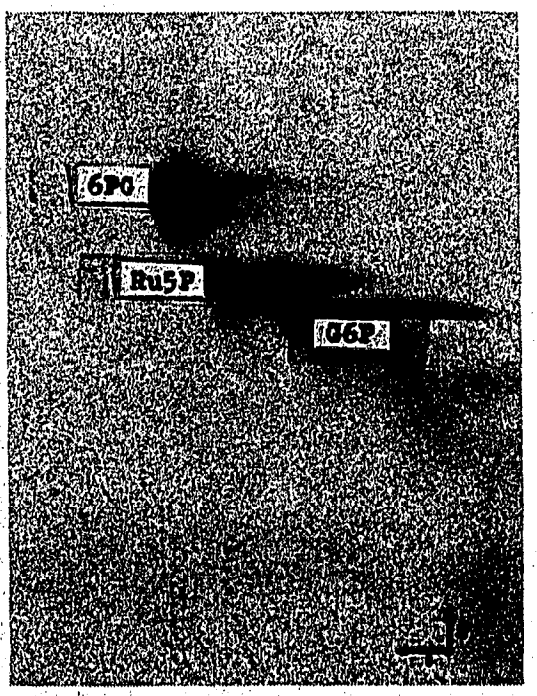

Fig. 4. Radioautograph of a two-dimensional separation of an enzymatic reaction mixture in which 6PG is transformed to Ru5P. The corresponding one-dimensional isotachophoresis is shown in Fig. 2b.

Step 3. [U-^{14}C]Ru5P was phosphorylated in the presence of phosphoribulokinase (PRK, EC 2.7.1.19), ATP and Mg^{2+} (ref. 6) to give [U-^{14}C]RuDP (Fig. 2c).

[1-^{32}P]RuDP was prepared in one step in a corresponding way. In this instance, [γ-^{32}P]ATP and Ru5P was the substrate for PRK[7].

The method presented here for the isolation of labelled substances was very convenient to use, as the electrophoretic system involved the use of the whole incubation mixture without pre-treatment, de-proteinization or concentration. During the isotachophoretic procedure, the labelled substances collected in zones of high concentration. Because of their high concentrations, the labelled substances could be localized radioautographically after only 5 h of exposure. As steps 1 and 2 could be completed in 1 day and step 3 the following day, the whole synthesis of [U-^{14}C]RDP from [U-^{14}C]G6P could be completed in two days.

As two continuous radioactive zones on a one-dimensional isotachophoretic separation strip would give only one black band on radioautography, it was necessary

to check the homogeneity of the radioactive zones by an additional method. For this purpose, the two-dimensional combination of isotachophoresis with zone electrophoresis was used. By this method, the degree of completeness of the isotachophoretic separations could also be checked.

Isotachophoresis either alone or in combination with zone electrophoresis seems to be a useful tool for the small-scale isolation of labelled substances synthesized by enzymatic reactions.

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