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Note

Liquid chromatographic analysis of α-D-glucose-1-phosphate

Determination of the activity of phosphorylase

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One of the intermediates in carbohydrate metabolism which may have a certain industrial value in the near future is α-D-glucose-1-phosphate (G-1-P). A possible application may be to the elongation of amylopectin chains[1], producing a starch solution of very high viscosity.

G-1-P can be prepared from starch[2] or sucrose[3] and inorganic phosphate (P_i) using phosphorylases, or chemically by reactions such as the phosphorylation of glucose pentaacetate with phosphoric acid in an anhydrous medium[4]. In any case it must be separated from a reaction mixture containing P_i and carbohydrate.

Recently a one-step chromatographic purification process using an anion-exchange resin with potassium acetate as single isocratic eluting buffer was described[5]. G-1-P is separated from both carbohydrate and P_i by a combination of ion-exchange and exclusion effects. Analytical scale chromatographic separations of (in)organic phosphates with anion-exchange resins or gels, *e.g.*, in enzyme assays, are well known, but these generally employ complex salt and pH gradients[6–9] which necessitate post-column colorimetric analysis.

We describe here a quantitative chromatographic analysis of G-1-P using an anion-exchange resin suitable for high-performance liquid chromatography (HPLC) with a single isocratic buffer and a differential refractometer as detector. The method is not sensitive enough for use in research on cell metabolism, but was not developed for that purpose. Our aim is to control preparative production of phosphorylases and G-1-P.

MATERIALS AND METHODS

Reagents

Disodium glucose-6-phosphate (G-6-P) and disodium β-glycerin phosphate were obtained from E. Merck (Darmstadt, F.R.G.). Dipotassium α-D-glucose-1-phosphate (G-1-P) was prepared in our laboratory[5].

Chromatography

Chromatographic separations were carried out with the resin Aminex A-27 (particle size 13.5 ± 1.5 μm), or with a size fraction of the resin AG1-X8 (−400

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mesh), both from Bio-Rad. A homogeneous particle-size fraction of the resin AG1-X8, >85% of particle size 40–60 μm, was prepared by a sequence of sedimentations in water. The particle-size distribution was determined by microphotography. The resins were used in the acetate form. Column dimensions for Aminex A-27 were 15 × 0.46 cm and for AG1-X8, 15 × 0.62 cm. Samples of 50 or 100 μl were analysed. Potassium acetate (0.3 *M*, pH 5) was used as single isocratic buffer with flow-rates up to 1.5 ml/min at elevated temperatures. Separations were optimized with a Hewlett-Packard liquid chromatograph, Model 1084 B, equipped with a differential refractometer, Model 1031. For routine analysis a Kipp Analytica liquid chromatography system, Model 9200, was combined with a Knauer differential refractometer and a Hewlett-Packard 3390 A integrator.

Phosphorylase assay

Phosphorylase from potatoes (E.C. 2.4.1.1) was partly purified as described by Lee[10]. The activity of phosphorylase preparations was determined colorimetrically, essentially according to Lee[10] and Chen *et al.*[11], or with our chromatographic method. A solution containing 2% G-1-P (0.05 *M*) and 1% soluble starch was incubated at pH 6.5 and 35°C with a suitable amount of enzyme. Samples were taken at different times, heated for 5 min at 100°C in a water-bath and chromatographically analysed for G-1-P content in comparison with a G-1-P standard solution. A unit of activity is defined as the transfer of 1 μmol G-1-P per minute under these conditions.

RESULTS AND DISCUSSION

Chromatographic analysis of G-1-P on Aminex resins

Comparable to the preparative chromatographic purification of G-1-P[5], good separations of G-1-P from P_i and carbohydrate were achieved on an analytical scale within 20 min using Aminex A-27 at 30°C. Various buffers may be used as an isocratic mobile phase, but best results were obtained with acetate (p*K* 4.75). The conditions given in Fig. 1 were used in further experiments. The two negative peaks are inherent to the chromatographic system used: the first is the solvent front; the position of the second peak depends on buffer composition, concentration and pH. Applying an acetate mobile phase, both G-1-P and P_i were eluted after the negative peaks and

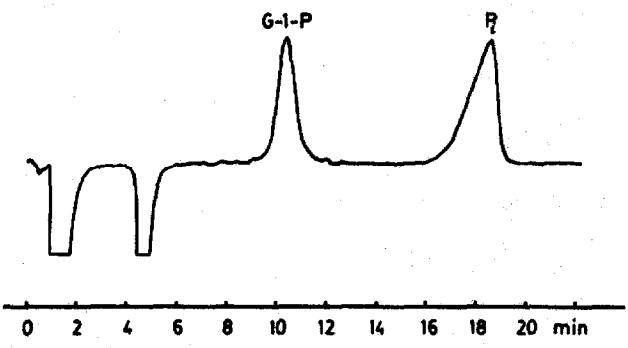

Fig. 1. Chromatographic analysis of G-1-P on an Aminex A-27 column (15 × 0.46 cm) with 0.3 *M* acetate buffer pH 5.0 as mobile phase. Flow-rate 1.0 ml/min; sample volume 50 μl; column temperature 30°C.

TABLE I

THE pK_a VALUES OF SOME (ORGANO)PHOSPHATES, MOLECULAR WEIGHTS OF THEIR MONOVALENT ANIONS AND RETENTION TIMES ON AMINEX A-27

Chromatographic conditions: 0.3 *M* acetate, pH = 5; 30°C; flow-rate 1 ml/min.

(Organo)phosphate	pK_1	pK_2	*Mol. weight monovalent anion*	*log M*	*Retention time (min)*
Glucose-1-phosphate	1.10	6.51	259	2.41	9.53 ± 0.03 (n = 5)
Glucose-6-phosphate	0.94	6.11	259	2.41	9.77 ± 0.02 (n = 5)
β-Glycerin phosphate	1.37	6.34	169	2.23	13.5
Orthophosphate	2.23	7.21	97	1.99	18.4 ± 0.5 (n = 5)

could be determined quantitatively by integration methods. Uncharged carbohydrates such as starch, sucrose or fructose were eluted from the column without any retardation, in or near the chromatographic front.

Anion size-exclusion effects must play an important rôle in the separation mechanism because G-1-P, being a stronger acid (Table I), is eluted before P_i. This is further demonstrated by the almost linear relationship between the retention times, t_R, of some (organo)phosphate anions and the logarithm of the molecular weight (Fig. 2).

Isomers such as G-1-P and G-6-P show only small but significant differences in retention times, related to their pK_a values. However they cannot be separated from each other with the described system due to the relatively broad peaks (0.8 min at half peak-height).

The lower limit of detection (signal-to-noise ratio = 3) with 50-μl G-1-P samples is 10^{-3} *M* [0.02% (w/v), calculated as $C_6H_{11}O_6PO_3H^-$]. The linear dynamic range of the detector response for G-1-P is more than two orders of magnitude when peak

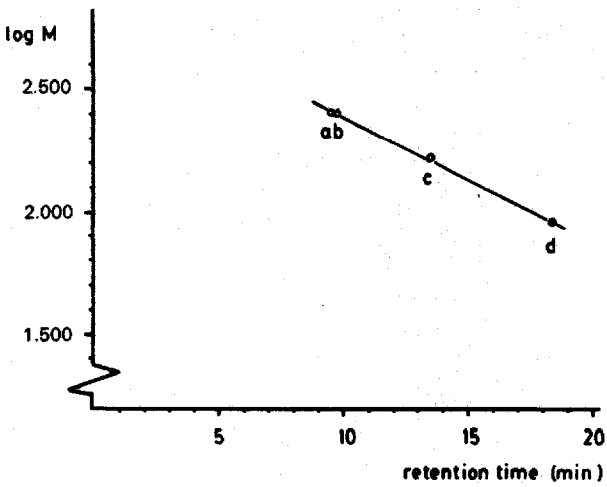

Fig. 2. Plot of the logarithm of the molecular weight, *M*, *versus* the retention time of some (in)organic phosphates. Chromatographic conditions as in Fig. 1. a = G-1-P; b = G-6-P; c = β-glycerin phosphate; d = P_i.

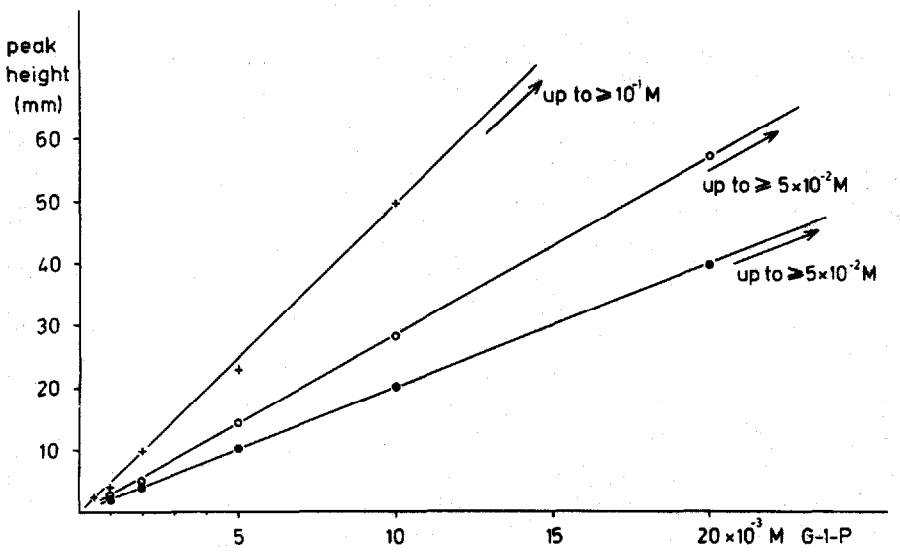

Fig. 3. The relationship between G-1-P concentration and refractive index detector response. +, Aminex A-27 column, conditions as in Fig. 1; ●, AG1-X8 column, mobile phase 0.3 *M* acetate pH 5.0, flow-rate 1.5 ml/min, sample volume 100 µl, column temperature 30°C; ○, AG1-X8 column, at 80°C, other conditions as before.

height is taken as a measure of concentration (Fig. 3). For P_i no linear relationship between concentration and detector response was found due to asymmetric peak shape. The lower limit of detection for P_i was 5×10^{-3} *M* [0.05% (w/v) $H_2PO_4^-$].

The resin AG1-X8 (−400 mesh) is chemically identical to Aminex A-27 but more than a 100 times cheaper. It is obvious that, because of the larger particle size and therefore increased dispersion, the peaks eluted from this column are broader than from the Aminex A-27 column. This results in a decrease in sensitivity. The column dimensions however allow the use of a larger sample volume without affecting significantly the peak width and thus the resolution. Furthermore, by increasing the diffusion coefficients by using elevated column temperatures (Wilke–Chang equation) the peak dispersion can be considerably decreased (Fig. 4).

With these adaptations, the use of an AG1-X8 fraction for chromatographic analysis of G-1-P may well be possible with a reasonable sensitivity as compared to Aminex A-27 (Fig. 3). We expect the resin A-27 to show comparable temperature effects to the AG1-X8 fraction, but for economic reasons we did not pursue this any further.

Determination of phosphorylase activity

α-Glucan phosphorylase (E.C. 2.4.1.1) catalyzes specifically phosphate-transfer reactions:

$$(\alpha\text{-1,4-glucosyl})_n + \text{G-1-P} \leftrightharpoons (\alpha\text{-1,4-glucosyl})_{n+1} + P_i$$

As shown in Fig. 5, using the substrate concentrations in Table II, the enzyme causes

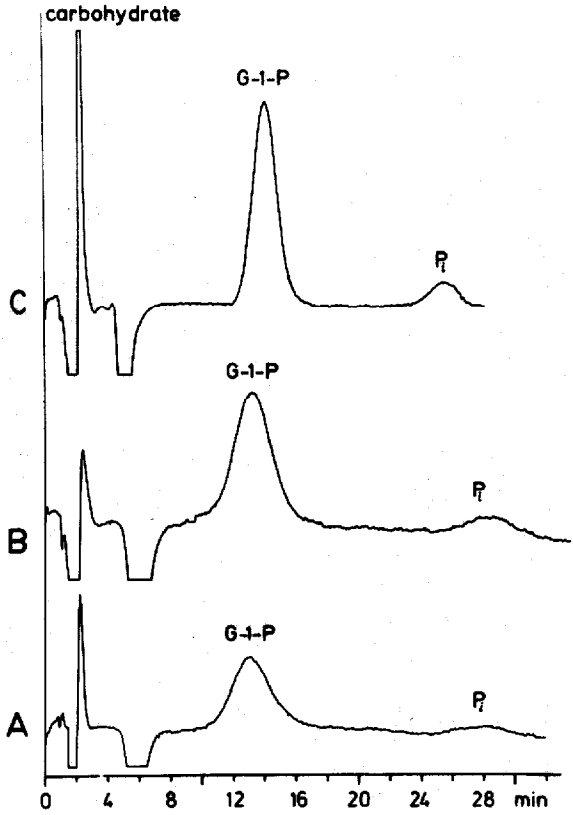

Fig. 4. Chromatographic analysis of 10^{-2} *M* test samples of carbohydrate, G-1-P and P_i on an AG1-X8 column. Mobile phase: 0.3 *M* acetate pH = 5.0, flow-rate 1.5 ml/min. A, Column temperature 30°C, sample volume 50 µl; B, column temperature 30°C, sample volume 100 µl; C, column temperature 80°C, sample volume 100 µl.

a linear decrease in G-1-P concentration from 5×10^{-2} to 3.3×10^{-2} *M*, a concentration which is still high with respect to the K_M value. When the reaction proceeds, the reverse reaction, formation of G-1-P from the produced phosphate ($\geqslant 1.7 \times 10^{-2}$ *M*) and starch, starts to interfere. Using a G-1-P standard solution, the phosphorylase activity can easily and accurately be calculated from the changes in G-1-P concentration in the linear range (Fig. 5), because the detection limit for G-1-P, 10^{-3} *M*, is low compared to these changes.

In the literature other methods for the determination of phosphorylase activity have been described, *i.e.*, colorimetric analysis of the liberated P_i[10,11], enzymatic analysis of G-1-P through linkage to a NADP-reducing system[12,13] or analysis based on pH changes[14].

For some phosphorylase preparations, values obtained for phosphorylase activity using the chromatographic method were compared with results from colorimetric tests[10,11]. Generally, there was a reasonable agreement (Fig. 6). However, an extensive comparison has not been carried out.

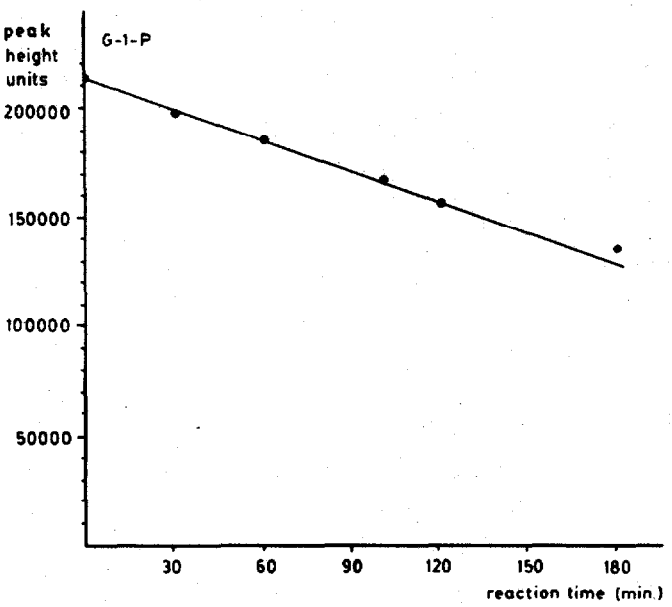

Fig. 5. Chromatographic analysis of G-1-P for determination of phosphorylase activity. Enzymatic transformation of G-1-P by potato phosphorylase as a function of reaction time. Original concentration of G-1-P: 5×10^{-2} *M*. Chromatographic conditions as in Fig. 1.

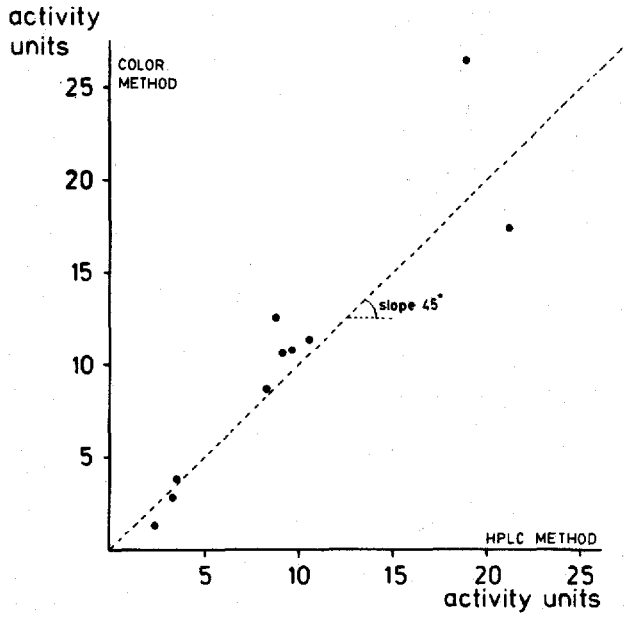

Fig. 6. Comparison between phosphorylase activity measurements by the HPLC method and the colorimetric method.

The chromatographic analysis described for starch phosphorylase may in principle be used for other phosphorylases or phosphatases. The reaction product, P_i or organophosphate, must differ sufficiently from the original substrate in molecular size or pK value. As shown in Table I, the difference between β-glycerin phosphate and P_i is already sufficient.

Isomerization reactions, such as the reaction catalyzed by phosphoglucomutase, cannot be analyzed in this way unless the reaction causes a considerable change in pK_a of the phosphate compound. However, it may be concluded that the described method with a single isocratic buffer as mobile phase and refractive detection offers a simple and reliable alternative to existing methods.

TABLE II

INITIAL SUBSTRATE CONCENTRATIONS IN THE CHROMATOGRAPHIC ANALYSIS OF PHOSPHORYLASE ACTIVITY AND K_M VALUES FOR POTATO PHOSPHORYLASE[15]

	K_M	*Initial substrate concentration (M)*
Glucose-1-phosphate	$3.5 \cdot 10^{-3}$	$5 \cdot 10^{-2}$
Orthophosphate	$7.5 \cdot 10^{-3}$	$5 \cdot 10^{-2}$
Amylopectin	$5 \cdot 10^{-5}$*	$2.5 \cdot 10^{-3}$*

* Determined as non-reducing chain-ends.

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