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ANION-EXCHANGE CHROMATOGRAPHY OF GLYCOLYSIS INTERMEDIATES

G. BEDETTI, G. D'AGNOLO AND F. POCCHIARI
Laboratorio di Chimica Biologica, Istituto Superiore di Sanità, Rome (Italy)

SUMMARY

A purification procedure for glycolysis intermediates is described. The separation of each compound was achieved by means of anion-exchange chromatography on a column of AG-1 X4 (Cl^-) and an eluting gradient of ammonium chloride, containing sufficient alkaline borate to ensure complexing of the sugar phosphates.

The eluting buffer was afterwards removed by evaporating to dryness with methanol and subsequent chromatography on an AG-50W X4 (H^+) column. Under such conditions the quantitative separation, identification and recovery of the components of the glycolytic pathway can be obtained.

INTRODUCTION

The chromatographic separation of the intermediates of glycolysis is of great interest as a tool for solving the problems of metabolic interrelationship[1], flux rates[2] and compartmental situations[3] within the EMBDEN-MEYERHOF pathway.

Several methods have been developed in which borate complexes of phosphate esters could be separated by ion-exchange chromatography[4-8]. Procedures using thin-layer chromatography[9,10], paper[11] and thin-layer electrophoresis[12] as well as gas chromatography[13] have been described. All these methods however, while yielding good results, allow the separation of only a few glycolysis intermediates.

The present paper describes a method in which sugar phosphates complexed with borate are eluted from an anion-exchange resin by means of a concave gradient of ammonium chloride. This method gives reproducible results and allows the separation, with good recoveries, of phosphorylated glycolysis intermediates.

EXPERIMENTAL

Materials

[U-^{14}C]Glucose; [U-^{14}C]glucose-6-phosphate; [U-^{14}C]glucose-1-phosphate; [U-^{14}C]lactate and [U-^{14}C]pyruvate were obtained from the Radiochemical Centre, Amersham, Bucks., Great Britain. [U-^{14}C]Fructose-1,6-diphosphate and [U-^{14}C]-phosphoenolpyruvate were purchased from Boehringer, Mannheim, G.F.R. [U-^{14}C]-

Fructose-6-phosphate was from Sorin, Saluggia, Italy, while [U-^{14}C]3-phosphoglycerate and [U-^{14}C]2,3-diphosphoglycerate were from Calbiochem, Los Angeles, Calif., U.S.A.

Labelled dihydroxyacetone phosphate and glyceraldehyde-3-phosphate were prepared from [U-^{14}C]fructose-1,6-diphosphate according to BEISENHERZ *et al.*[14]. Uniformly labelled 1,3-diphosphoglycerate was prepared from [U-^{14}C]3-phosphoglycerate by KRIMSKY's procedure[15] as modified by ALPERS[16].

Unlabelled compounds and enzymes were obtained from Boehringer. Ion-exchange resins were from Bio-Rad Laboratories, Richmond, Calif., U.S.A.

Analytical procedures

Phosphorus was assayed by the method of BARTLETT[17] as modified by MARINETTI[18]. Phosphoglyceric acids were determined with pyrogallol in sulphuric acid[19]. Anthrone was used as reducing agent[20]. Borate was determined spectrophotometrically with curcumin reagent[21].

After elution and removal of buffer, glycolysis intermediates were determined enzymatically according to BERGMEYER[22].

Column chromatography

The columns (1 × 50 cm) used were packed with AG-1 X4 (Cl^-) (200–400 mesh) until a 30-cm-high bed was obtained. After applying the mixtures of sugar phosphates, the column was washed with 100 ml of 0.001 *N* ammonium hydroxide to remove glucose and any other free sugar that may be present. Sugar phosphates were eluted, at room temperature, with a concave gradient made up of 0.0025 *M* NH_4OH + 0.25 *M* NH_4Cl in the reservoir (1.95 l in a 2.5-l Erlenmeyer flask) and of 0.0025 *M* NH_4OH + 0.005 *M* $K_2B_4O_7$ + 0.03 *M* NH_4Cl in the mixing chamber (2 l in a 4-l Mariotte bottle). The flow rate was maintained at 0.5 ml/min by means of an LKB 10200 peristaltic pump. Radioactivity in the effluent was monitored with a Nuclear Chicago 6770 Chroma/Cell; 10-ml fractions were collected. Individual column runs were carried out in order to establish the elution behaviour.

Fractions, under each peak, were pooled separately and evaporated to dryness in a Büchi evaporator (about 30° inside the flask).

Borate was easily removed by three to four evaporations with methanol. The residue, dissolved in 3–5 ml of water, was passed down a column (1 × 10 cm bed) of the resin AG-50W X4 (H^+) (200–400 mesh) to ensure the removal of the ammonium ion. The column was washed with water to neutrality and the effluent evaporated to dryness.

After this treatment the amount of borate was less than 0.01 μmoles per peak and did not interfere in the successive enzymatic analyses.

RESULTS AND DISCUSSION

The results obtained with a mixture of glycolysis intermediates are shown in Fig. 1. Similar patterns have been obtained by KHYM AND COHN[4] with a stepwise elution, utilizing a combination of borate complexing and pH.

The anion-exchange behaviour of the compounds examined is, as expected, the resultant effect of the p*K* values of the phosphate groups and the stability constants

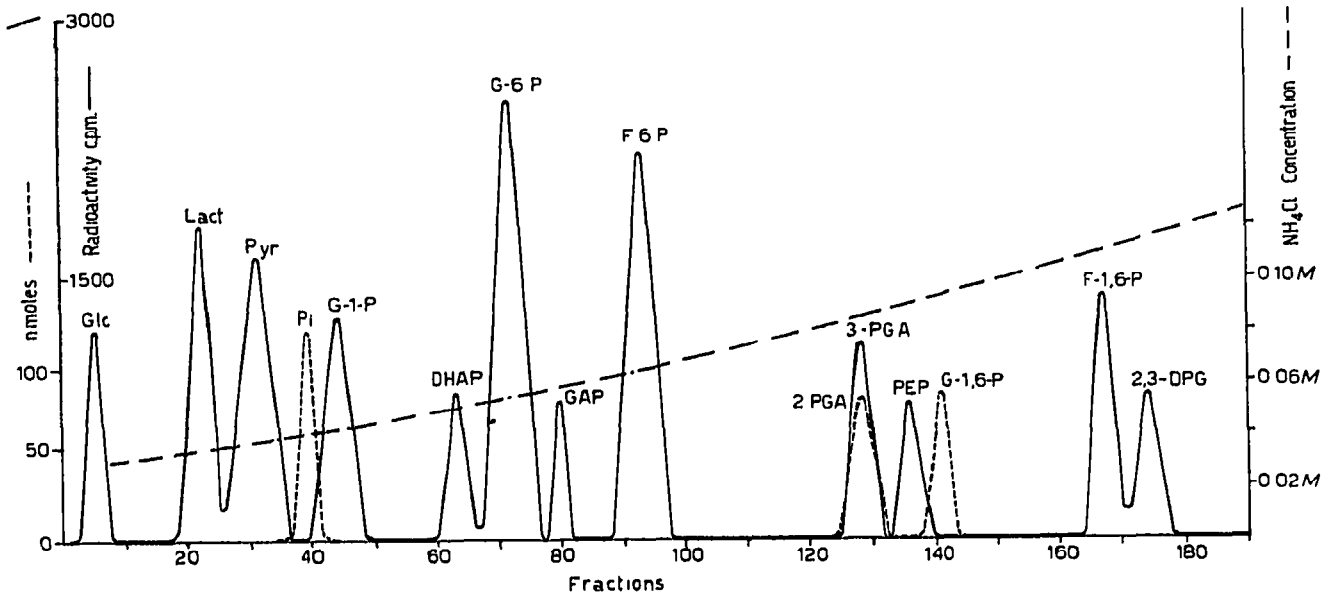

Fig. 1. Anion-exchange chromatography of glycolysis intermediates. The mixture applied to the column was: glucose (Glc); glucose-6-phosphate (G-6-P); glucose-1,6-diphosphate (G-1,6-P), glucose-1-phosphate (G-1-P); fructose-6-phosphate (F-6-P); fructose-1,6-diphosphate (F-1,6-P); dihydroxyacetone phosphate (DHAP); glyceraldehyde-3-phosphate (GAP); 3-phosphoglyceric acid (3-PGA); 2,3-diphosphoglyceric acid (2,3-DPG), 2-phosphoglyceric acid (2-PGA); phosphoenolpyruvate (PEP); pyruvate (Pyr), lactate (Lact). (————) c.p.m., (— — —) unlabelled compounds assayed for inorganic phosphate (Pi); (—·—·—) gradient from 0.03 *M* to 0.12 *M*.

of the borate complexes, so that large differences were found among members of the same series such as glucose-1-phosphate, glucose-6-phosphate and glucose-1,6-diphosphate. Steric hindrance of the phosphate group can, in fact, make the borate complex less stable, which is reflected as lower binding to the anion exchanger. The latter hypothesis, together with the participation of the hydroxyl group arising from the furanosic ring formation in reactions with boric acid, can also explain why aldoses are eluted ahead of ketoses.

TABLE I

AMOUNT OF GLYCOLYSIS INTERMEDIATES RECOVERED

Glycolysis intermediates	*nMoles added to column*	*nMoles recovered*	*Percent recovery*
Glucose	743	691	93
Glucose-6-phosphate	110	105	95
Glucose-1,6-diphosphate	100	88	88
Glucose-1-phosphate	180	152	84
Fructose-6-phosphate	160	144	90
Fructose-1,6-diphosphate	142	122	86
Dihydroxyacetone phosphate	100	78	78
Glyceraldehyde-3-phosphate	100	66	66
3-Phosphoglyceric acid	380	330	87
2,3-Diphosphoglyceric acid	75	68	90
2-Phosphoglyceric acid	95	83	87
Phosphoenolpyruvate	120	113	94
Pyruvate	170	155	91
Lactate	630	554	86

In different experiments 85–95% of the initial samples were recovered (Table I). Recoveries for dihydroxyacetone phosphate and glyceraldehyde-3-phosphate were, however, between 15–30%; but if the two peaks were lyophilised as fast as possible after elution and the deionising procedure was carried out in a cold room, recoveries rose to 70–80%.

In our system separation of 2-phosphoglyceric acid and 3-phosphoglyceric acid could not be achieved, and therefore their specific radioactivities must be measured as a whole after enzymatic conversion of the former in the latter[22].

Under the conditions employed 1,3-diphosphoglycerate was always recovered as 3-phosphoglycerate and inorganic phosphate because of spontaneous decomposition. However, the method can equally be applied to biological material; in the living cell the concentration of 1,3-diphosphoglycerate is so low that it can hardly be estimated.

As the same recoveries were found both by measuring the radioactivity of the sample and its amount, the method is suitable for obtaining valid specific radioactivities of most glycolytic intermediates in experiments with labelled substrates.

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