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High-performance liquid chromatographic system for separating sugar phosphates and other intermediary metabolites

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Abstract

A simple method for separating intermediates of carbohydrate metabolism, including the difficult-to-resolve sugar phosphates, using anion-exchange high-performance liquid chromatography is described. A gradient of decreasing borate concentration (10 to 0 m*M*) and increasing ionic strength (0 to 150 m*M* NH_4Cl) separates phosphorylated sugars based on the strength of the ester complex that they form with borate anion. Lyophillized samples are reconstituted in a low ionic strength aqueous medium (5 m*M* triethanolamine-HCl, pH 8.1) and chromatographed on a commercially available anion-exchange column (Hamilton PRP-X100). The process requires only 3 h and permits nanomole detection of the phosphorylated intermediates of the glycolytic and pentose shunt pathways.

1. Introduction

Cell physiological studies sometimes require the chromatographic separation of intermediary metabolites, e.g. to determine their concentrations under different metabolic conditions or to determine specific radioactivities in isotope tracer experiments. Sugar phosphates are a particularly challenging class of metabolites to separate due to the similarities in their charge and structure. The most successful chromatographic protocols for resolving the various sugar phosphates have relied on the formation of sugar–borate complexes (esters) that occurs under alkaline conditions [1]. The various sugar phosphates differ in the strength of their complexation with borate, and thus a separation based on charge, i.e. ion-exchange chromatography, can be utilized [2]. In one procedure [3] sugar phosphates were bound to a strong anion exchanger (Dowex) and then eluted with a sigmoidal gradient of salt in the presence of borate. Excellent resolution of phosphorylated intermediates of glycolysis was obtained, however elution of the columns required 2 to 4 days, and the elution peaks obtained were broad, causing a significant dilution of the intermediates.

Despite its widespread use in the separation of several classes of biologically important low-molecular-mass compounds (e.g., nucleotides, amino acids, organic acids), high-performance liquid chromatography (HPLC) has rarely been applied to the resolution of phosphorylated in-

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termediates, except to separate the inositol phosphates [4,5]. One HPLC system has been put forth to analyze the intermediates of photosynthesis [6], however in this scheme the hexose monophosphates all elute in one peak, and this peak barely resolves from the pentose monophosphates. Another HPLC protocol has been described that does separate sugar phosphates, however three independent HPLC runs (30 min each), using different elution conditions, must be performed to achieve complete separation of phosphorylated sugars [7]. Finally, an HPLC anion-exchange system has been described that affords excellent separation of sugar phosphates in 5 h (although the total gradient requires 15 h to complete), but it uses a homemade column of mixed resins that would, therefore, not be generally available to researchers [8].

In this paper is described an HPLC protocol for separating sugar phosphates and other intermediary metabolites on a commercially available, strong anion exchanger, using a borate-based elution gradient. The chromatography has been used to separate phosphorylated intermediates of the glycolytic and pentose shunt pathways in less than 3 h.

2. Experimental

2.1. Chemicals

Ammonium chloride and sodium borate were Baker Analytical Reagent Grade obtained from VWR (San Francisco, CA, USA). Tritiated water was from New England Nuclear (Boston, MA, USA), and ^{14}C-sodium bicarbonate was from ICN (Costa Mesa, CA, USA). All other chemicals were obtained from Sigma (St. Louis, MO, USA), including 1-^{14}C-glucose and 5-^{3}H-glucose used to synthesize radioactive standards for the column.

Radioactive glucose-6-phosphate was prepared by reacting 1-^{14}C-glucose with ATP and hexokinase. After chromatographic purification, this was then reacted with phosphohexose isomerase to generate 1-^{14}C-fructose-6-phosphate, with phosphoglucomutase to generate 1-^{14}C-glucose-1-phosphate, or with glucose-6-phosphate dehydrogenase plus NADP to generate 1-^{14}C-6-phosphogluconate. 4-^{3}H-ribulose-5-phosphate was generated by reacting 5-^{3}H-glucose sequentially with ATP plus hexokinase, then with glucose-6-phosphate dehydrogenase plus 6-phosphogluconate dehydrogenase plus NADP. The reaction conditions for all enzymatic conversions are as described in Bergmeyer [9]. All radioactive metabolites were purified chromatographically before use.

2.2. Biological samples

Eggs from the sea urchin, *Strongylocentrotus purpuratus*, were collected and handled as described previously [10]. Eggs were sedimented by low-speed hand centrifugation either before fertilization or at 2.5 min after fertilization. The sea water supernatant was aspirated and discarded; the resulting packed cell pellets were 0.1 ml in volume. An aliquot of 1 ml of trichloroacetic acid (10%, v/v, 0°C) was added to the cell pellets (at $T = 3$ min post-insemination for the fertilized sample), and extraction of the acid-soluble metabolites proceeded for 5 h on ice. These samples were then centrifuged (15 000 g; 5 min) to remove insoluble material, and the trichloroacetic acid in the supernatants was removed by extraction with diethyl ether (4 extractions; 5 ml each). The aqueous phase was then brought to neutrality by adding 0.01 ml of 1 M ammonium bicarbonate, and the samples were taken to dryness under vacuum. After reconstituting in 200 μl of 5 mM triethanolamine-HCl (pH 8.1) and centrifuging (2 min, 15 000 g), the entire sample was injected into the chromatographic system for analysis, and 1-min fractions were collected in test tubes containing 2 units of bovine intestinal alkaline phosphatase to hydrolyze phosphate esters after their separation. Each fraction was analyzed for phosphate content [11] after an overnight incubation at room temperature to ensure that the phosphatase reactions went to completion.

2.3. Chromatographic system

Analyses were carried out on an ISCO (Lincoln, NE, USA) Model 2350 HPLC system that

has a Model 2360 ternary gradient programmer. Injections were made manually through a 1-ml injection loop; typical injection volumes were 50–500 μl. Separations were performed with a Hamilton (Reno, NV, USA) PRP-X100 column (250 × 4.1 mm; 10 μm particle size), a poly(styrene–divinylbenzene) trimethylammonium exchanger. This column has been used successfully without replacement for one year. All steps were carried out at room temperature.

The elution buffers were as follows: buffer A = 5 m*M* triethanolamine-HCl (pH 8.1); buffer B = 10 m*M* sodium borate (pH 8.9, titrated with HCl); buffer C = 150 m*M* ammonium chloride (pH unadjusted, but typically 5.0). The ternary gradient programmer was used to generate the following gradient: 100% A (0 min to 5 min), 100% A to 100% B (5 min to 5.1 min), 100% B (5.1 min to 25.1 min), 100% B to 50% B and 50% C (25.1 min to 125.1 min), 50% B and 50% C to 100% C (125.1 min to 145.1 min), 100% C (145.1 min to 155.1 min), 100% C to 100% A (155.1 min to 165.1 min). The flow-rate was maintained at 1.5 ml/min, which results in a pressure of 1600–1800 p.s.i. (1 p.s.i. = $6.9 \cdot 10^3$ Pa) in this system.

After each chromatographic run, the column was washed with 15 ml of 0.2 *M* HCl. In cases where hydrophobic compounds were present the column was then washed with 30 ml of methanol to remove nonpolar materials from the polymer backbone of the exchanger. After these washes, the column was re-equilibrated with 5 m*M* triethanolamine-HCl (equilibration monitored by pH of the effluent) for the next application.

2.4. Detection methods

Fractions (1 min; 1.5 ml) were collected and assayed for the presence of metabolites spectrophotometrically. The amino acids alanine, aspartate, and glutamate were detected by the ninhydrin reaction [12]. Glucose was determined with the anthrone reagent [13]. Pyruvate and lactate were determined with lactate dehydrogenase [9]. Octopine was determined with octopine dehydrogenase [14]. Acetate was determined with acetase kinase coupled to pyruvate kinase and lactate dehydrogenase [15]. Succinate was determined in a coupled enzyme system containing succinyl thiokinase, nucleoside diphosphate kinase, pyruvate kinase and lactate dehydrogenase [16]. All phosphorylated intermediates were determined by analyzing for inorganic phosphate after ashing each fraction with magnesium nitrate, as described by Ames and Dubin [17]. Radioactive metabolites were assayed by liquid scintillation counting.

3. Results and discussion

The elution strategy described here basically follows that outlined by Khym and Cohn [2], i.e., a gradual reduction in borate concentration coupled with increases in the ionic strength and acidity of the elution medium. However in the scheme proposed here the gradient is continuous, whereas in the original method the elution proceeds by a step elution protocol. The salt/borate/pH gradient is divided into two phases of different steepness (Fig. 1). The first part of the gradient is shallow in order to maximize the resolution of the sugar phosphates that are difficult to separate (e.g., glucose-6-phosphate and ribulose-5-phosphate), then the steepness of the gradient is increased to facilitate rapid elution of more easily resolvable compounds (e.g.,

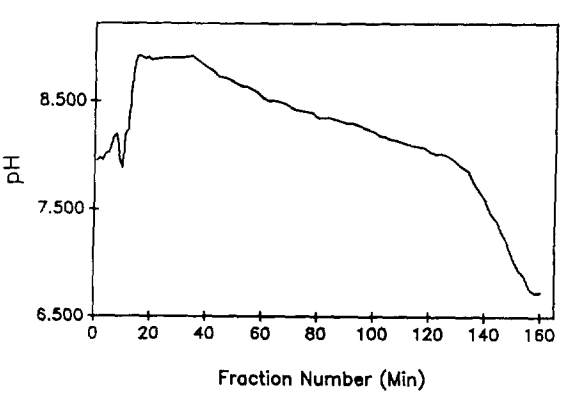

Fig. 1. pH profile of gradient. Fractions (1 min, 1.5 ml) were collected and their pH was determined with a glass pH electrode. The gradient is as described in section 2 and the pH of the buffers were: buffer A pH 8.1, buffer B pH 8.9, buffer C pH 5.0. Note that in this system there is approximately a 7-min lag between formation of the gradient and collection of fractions.

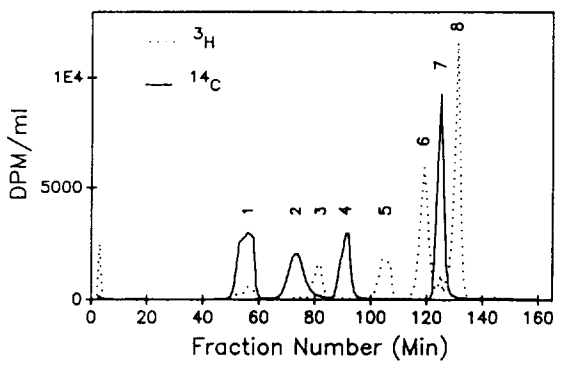

Fig. 2. Separation of representative sugar phosphates. Synthetic radiolabeled sugar phosphates were injected into the HPLC system, and eluted as described in section 2. Fractions (1 min, 1.5 ml) were collected and assayed for radioactivity by liquid scintillation counting. The compounds are: 1 = 1-^{14}C-glucose-1-phosphate; 2 = 1-^{14}C-glucose-6-phosphate; 3 = 4-^{3}H-ribulose-5-phosphate; 5, 6 = decomposition products of unknown structure derived from evaporating and re-dissolving 4-^{3}H-ribulose-5-phosphate; 7 = 1-^{14}C-6-phosphogluconate; 8 = 5-^{3}H-glucose-1,6-bisphosphate.

6-phosphogluconate and glucose-1,6-bisphosphate).

Fig. 2 shows the elution profile for the chromatographic separation of six sugar phosphates by HPLC, while Table 1 provides a list of the retention times for the thirty one metabolites thus far characterized. Note that peaks 5 and 6 in Fig. 2 are of unknown chemical structure; they are decomposition products arising from evaporation and redissolving of chromatographically pure ^{3}H-ribulose-5-phosphate (peak 3). In addition to the intermediates of the glycolytic and pentose shunt pathways, the retention times for some of the commonly found end products of anaerobic energy metabolism are characterized. These include lactate (for mammals), as well as alanine, acetate, octopine, and succinate (for invertebrates).

The overall elution order of the metabolites in Table 1 follows that previously seen with low pressure anion-exchange systems carried out in the presence of borate ion. Uncharged (glucose, alanine, water) or positively charged (octopine) metabolites do not bind to the exchanger, and thus elute with the void volume (3 min in our setup). Negatively charged metabolites carrying one charge (lactate, acetate, bicarbonate, glutamate, aspartate, and pyruvate) elute during the borate wash step, whereas succinate (net charge = −2 at pH 8.9) elutes only after the ammonium chloride gradient has begun.

Table 1
Retention times of common metabolic intermediates

Compound	Retention time (min)
Alanine	3
Glucose	3
Tritiated water	3
Octopine	3
Lactate	9
Arginine phosphate	12
Acetate	15
HCO_3	18
Glutamate	18
Inorganic phosphate	18
Aspartate	19
Pyruvate	23
Succinate	46
α-Glycerol phosphate	46
1,2-Propanediol phosphate (mixed isomers)	49, 56
Glucose-1-phosphate	54
Erythrose-4-phosphate	64
Xylulose-5-phosphate	66
Glyceraldehyde-3-phosphate	70
Sedoheptulose-7-phosphate	70
Adenosine-5′-monophosphate	72
Glucose-6-phosphate	75
Ribulose-5-phosphate	81
Fructose-6-phosphate	92
3-Phosphoglyceric acid	89
Phosphoenolpyruvate	90
UDP-glucose	101
6-Phosphogluconate	124
Glucose-1,6-bisphosphate	131
Fructose-1,6-bisphosphate	136
Adenosone 5′-diphosphate	154

Among the phosphorylated intermediates, glucose-1-phosphate has a very low affinity for borate [2], and thus elutes at a lower salt concentration than does glucose-6-phosphate. Furanose sugars (ribose phosphates and fructose phosphates) bind more tightly to borate [2] than do pyranose sugars, and are thus eluted from the column only at higher concentrations of salt/lower concentrations of borate. Finally, compounds carrying more than one charged group

(UDP-glucose, 6-phosphogluconate, glucose-1,6-bisphosphate, fructose-1,6-bisphosphate, and adenosine-5′-diphosphate) all require higher ionic strengths for their elution.

Fig. 3 gives a biological example for this method. Here the phosphorylated intermediates in sea urchin eggs before and after they have been fertilized were chromatographed to follow changes in metabolic pools attending fertilization. As has been reported [18,19], there is a large increase in arginine phosphate levels upon fertilization, and the major organic phosphate in both developmental states (other than ATP which does not elute under these conditions) is likely to be 1,2 propanediol phosphate, a metabolite of unknown function. In contrast to the findings of earlier workers [19] using ^{31}P-NMR, inorganic phosphate is a minor component of the

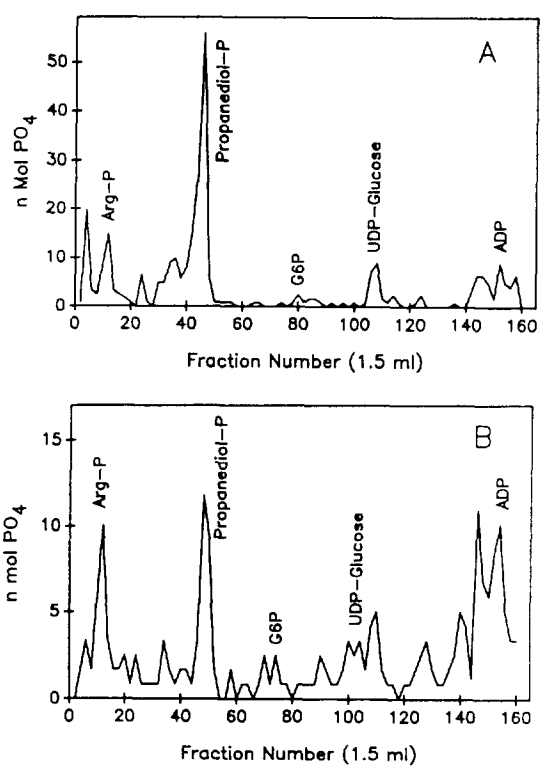

Fig. 3. Separation of acid-soluble phosphorylated metabolites from unfertilized sea urchin eggs (A), or from fertilized eggs 3 min post-insemination (B). Fractions (1.5 ml) were collected in tubes containing 2 units of alkaline phosphatase and analyzed for phosphate content after overnight incubation at room temperature.

acid-soluble compounds in sea urchin eggs. An increase in ADP upon fertilization is also apparent from the chromatograms in Fig. 3.

The experimental protocol presented here is a compromise to achieve adequate separation of the intermediary metabolites of glycolysis and the pentose shunt in a minimal amount of time. This basic protocol could be easily modified to suit other experimental needs. For example, the change from 5 m*M* triethanolamine-HCl to 10 m*M* sodium borate occurs in six s, but this could be extended to several min if greater resolution of singly charged organic acids or amino acids is desired. At the maximal concentration of salt (150 m*M* ammonium chloride) the ionic strength is insufficient to elute highly charged metabolites such as ATP and inositol polyphosphates (these compounds only elute in the 0.2 *M* HCl wash of the column). By increasing the final concentration of ammonium chloride, and modifying timing of the gradients to maintain the same slopes, thus preserving the resolution of the phosphorylated sugars, it should be possible to design a gradient system capable of eluting more highly charged compounds, albeit with longer chromatographic runs. In preliminary experiments it was found that decreasing the time of the 10 m*M* borate wash (20 min) resulted in poor separation of glucose-1-phosphate and glucose-6-phosphate, and thus shortening this step in the interest of saving time is not advised.

In summary, a scheme for the chromatographic separation of phosphorylated and nonphosphorylated metabolites is presented. This approach combines the exquisite resolving capability of HPLC with the use of anion-exchange in the presence of borate to discriminate various sugar phosphates on the basis of the strength of complexation with borate anion. The chromatography requires only three hours to effect the separation of metabolites that are closely related in chemical structure.

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