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HPLC Separation and Indirect Ultraviolet Detection of Phosphorylated Sugars[1]

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ABSTRACT

An HPLC method for the separation and analysis of phosphorylated sugars is presented. Ion-exchange chromatography coupled to indirect ultraviolet detection has produced good resolution and sensitivity. Fructose 6-P, glucose 6-P, ribose 5-P, 3-phosphoglyceric acid, ribulose 1,5-P_2, fructose 1,6-P_2, and sedoheptulose 1,7-P_2 can be separated at a sensitivity down to 10 nanomoles. The system resolves 2-carboxy-D-arabinitol 1,5-P_2 from 2-carboxy-D-ribitol 1,5-P_2. The natural inhibitor of ribulose bisphosphate carboxylase, 2-carboxy-D-arabinitol 1-P, has been separated from its 5-P isomer and most other phosphorylated compounds. This method is applied to identification of the products obtained upon ion-exchange purification of synthetic 2-carboxyarabinitol 1-P.

A technique for the HPLC separation and UV quantitation of some physiologically important monophosphate and bisphosphate sugar esters has been developed. This same method also separates the C-2 epimers of a transition state analog of the carboxylation reaction of RuBP[2] carboxylase/oxygenase, CABP (7). Also discussed will be the separation from other phosphorylated compounds of a natural inhibitor of RuBP carboxylase, CA1P (5, 8).

Most modern liquid chromatography methods for separating phosphate esters have relied on ion-exchange techniques using gradient elution in high salt (1, 3, 4). Due to the low UV absorption of most sugar phosphates, detection requires either hydrolysis of the phosphate ester bond after elution followed by quantitation of the released Pi by colorimetric methods (2, 3, 6) or radiometric detection of labeled compounds (6, 8). The method presented here utilizes conventional anion-exchange chromatography coupled to indirect UV detection (9). For indirect or vacancy detection a compound that absorbs light in the UV region is used as the eluting anion. When a negatively charged sample ion elutes, there is a compensating decrease in the amount of UV absorbing eluent. It is this decrease or vacancy in the amount of UV absorbing eluent that is used to monitor the UV transparent sample ion eluting from the column. A low capacity ion-exchange column is best since separation can proceed at low concentrations of eluting anion whereby small changes, corresponding to elution of the sample, can easily be observed.

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[2] Abbreviations: RuBP, ribulose 1,5-bisphosphate; CABP, 2-carboxy-D-arabinitol 1,5-bisphosphate; CaIP, 2-carboxy-D-arabinitol 1-phosphate; G6P, glucose 6-phosphate; R5P, ribose 5-phosphate; F6P, fructose 6-phosphate; PGA, 3-phosphoglyceric acid; FBP, fructose 1,6-bisphosphate; SBP, sedoheptulose 1,7-bisphosphate; CRBP, 2-carboxy-D-ribitol, 1,5-bisphosphate; CA5P, 2-carboxy-D-arabinitol 5-phosphate; DHAP, dihydroxyacetone phosphate; Ru5P, ribulose 5-phosphate.

MATERIALS AND METHODS

HPLC System. Polymer based anion-exchange columns (PRP-X100) were obtained from Hamilton Co. The pump and solvent delivery system was a Spectra Physics SP8700. Detection is accomplished with a Kratos 783 absorbance monitor set at 260, 280, or 300 nm. Peaks were integrated with Varian 4270 integrator.

Solvent Systems. All HPLC mobile phases were made with reagent grade chemicals and distilled, deionized water. Different solvent compositions were used for the analysis of different classes of compounds. The four classes of compounds studied were: (a) monophosphorylated compounds including G6P, R5P, F6P, and PGA; (b) monophosphorylated compounds containing carboxylate moieties such as PGA and CA1P; (c) bisphosphates including RuBP, FBP, and SBP; (d) bisphosphorylated carboxylate compounds including CABP and CRBP.

For monophosphates the eluent was 0.3 mM trimesic acid (1, 3, 5-benzenetricarboxylic acid, Aldrich Chemical Co.), 10 mM borate (pH 8.7) with LiOH. For monophosphorylated carboxylates the eluent was 0.5 mM trimesic acid and 10 mM borate (pH 9.7) with LiOH. For the bisphosphates the eluent was 1.5 mM trimesic acid and 10 mM borate (pH 8.7) with LiOH. The bisphosphorylated carboxylates were eluted with 3 mM trimesic acid and 10 mM borate (pH 9.7) with LiOH. Flow rates were 1.0 ml/min.

Sample Preparation. The monophosphates and bisphosphates were detected best when applied in low ionic strength solutions to the HPLC column. Leaf extracts or mixtures of pure sugar phosphates were initially separated on a silica ion-exchange precolumn (Sepralyte SAX from Analytichem International, Harbor City, CA). Monophosphates were selectively eluted with 4 ml of 0.05 N HCl, bisphosphates were eluted with 4 ml of 0.15 N HCl, and bisphosphorylated carboxylates were eluted with 5 ml of 1 N HCl. Samples were evaporated to near dryness under vacuum in a Savant SpeedVac concentrator and resuspended in a small volume of water, adjusted to pH 7 with NaOH, and applied to the column. Carboxypentitol bisphosphates were prepared according to (7).

CA1P and CA5P were prepared by limited alkaline phosphatase digestion of CABP. [2-^{14}C]CABP was incubated with alkaline phosphatase in 50 mM glycine buffer at pH 9.3 containing 1 mM $ZnSO_4$, and 10 mM $MgCl_2$ for 15 min. The monophosphates were separated from unreacted CABP and enzyme by fractionation on a small silica anion-exchange precolumn as described above. The sample was evaporated to dryness, dissolved in 1 ml H_2O and adjusted to pH 9.0 by addition of 25 μl of 1 M glycine buffer (pH 9.0) and small amounts of base. After 12 h of incubation at this pH no lactones were detected in the solution. The

sample was loaded on a 1.5 × 55 cm Dowex AG-50 (H^+) column and eluted with a 1 L linear gradient from 0 to 0.2 M LiCl in 3 mM HCl. Fifteen ml fractions were collected and assayed for radioactivity. The peak fractions were pooled, the total volume was reduced to approximately 10 ml with a rotating evaporator, the pH was adjusted to 8.2, and the sugar phosphates were precipitated by addition of 1 M barium acetate. Samples were redissolved using Bio-Rad AG 50 (H^+) and analyzed by HPLC. All other phosphorylated samples were obtained from Sigma Chemical Corporation.

RESULTS

The separation of the sugar monophosphates is shown in Figure 1. These conditions were optimized for the resolution of G6P, F6P, R5P, and PGA. G6P, Pi, and DHAP co-elute at the beginning of the elution profile and cannot be determined. Quantitation of peaks within the first 3 to 5 min can be difficult because of interference due to the positive and negative system peaks that occur at this position under these conditions and are induced by salt in the sample.

Optimal quantitation of PGA and CA1P occurred by increasing the eluent strength and the pH. This separation is shown in Figure 2. These same conditions can be used to resolve CA1P and CA5P, the two isomers that are produced from limited alkaline phosphatase digestion of CABP. This separation is shown in Figure 3. The identity of the CA1P was verified using inhibitor purified from dark-adapted bean leaves (8). The identity of the CA5P peak was verified using CA5P synthesized by reacting Ru5P with KCN at pH 8.5.

The profile of radioactivity that elutes from the preparative ion-exchange separation of the products of limited hydrolysis of [^{14}C]CABP is shown in Figure 4. Analysis by HPLC indicated that peak 1 was CA5P and peak 2 was CA1P. The presence of $ZnSO_4$ in the reaction mixture during the slow hydrolysis by alkaline phosphatase favored the formation of CA1P over CA5P.

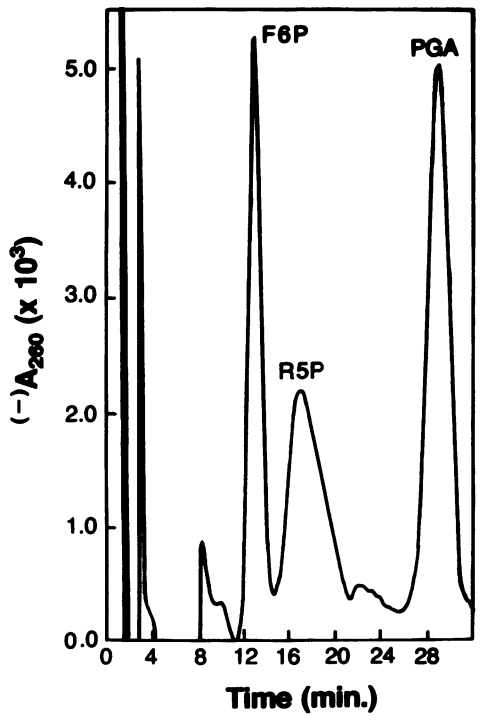

FIG. 1. Separation of monophosphorylated sugars. Fifty nmol of each sample was injected. The mobile phase contained 0.3 mM trimesate and 10 mM boric acid (pH 8.7) with detection at 260 nm.

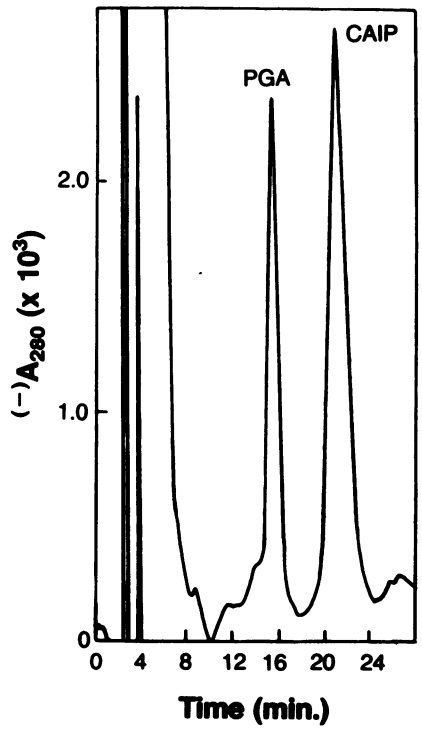

FIG. 2. Separation of monophosphorylated carboxylates. Conditions were specifically adjusted to resolve CA1P from PGA. Fifty nmol of each component was injected. The mobile phase contained 0.5 mM trimesate and 10 mM borate (pH 9.7) with detection at 280 nm.

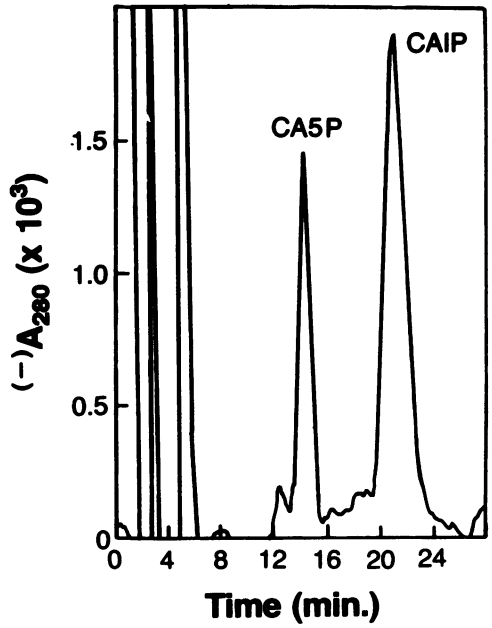

FIG. 3. Resolution of the 1- and 5-P isomers of 2-carboxy-D-arabinitol. Samples were incubated at pH 9.0 prior to injection to form the free acid. The mobile phase contained 0.5 mM trimesate and 10 mM borate (pH 9.7) with detection at 280 nm. This analysis was used to develop the procedure described in Figure 4.

The HPLC resolution of the sugar bisphosphates (RuBP, FBP, and SBP) is shown in Figure 5. Resolution of the arabinitol and ribitol isomers of 2-carboxypentitol 1,5-P_2 required increasing the trimesate concentration and pH. These compounds were best resolved as the lactones (Fig. 6A), while they were inseparable

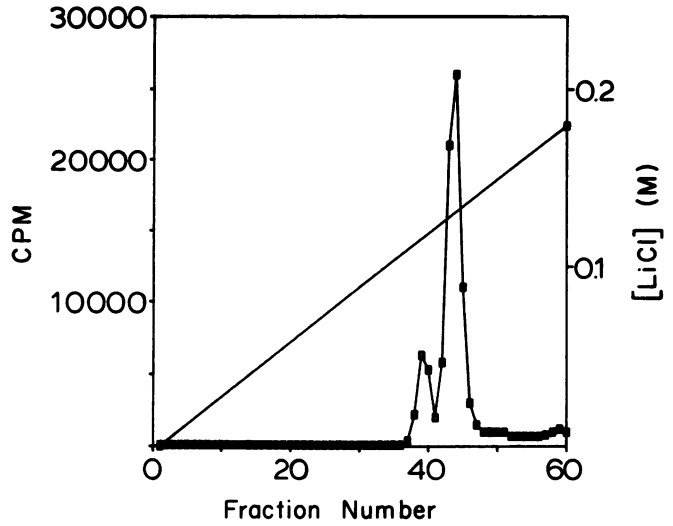

FIG. 4. Separation of CA1P from CA5P on Dowex AG-50 (H^+). The mixture was loaded on a 1.5 × 55 cm Dowex AG-50 anion exchange column and eluted with a linear gradient of 0 to 0.2 M LiCl in 3 mM HCl. Compounds were detected by assaying for radioactivity and identified by HPLC analysis. The presence of $ZnSO_4$ in the hydrolysis step led to the prevalence of CAIP over CA5P.

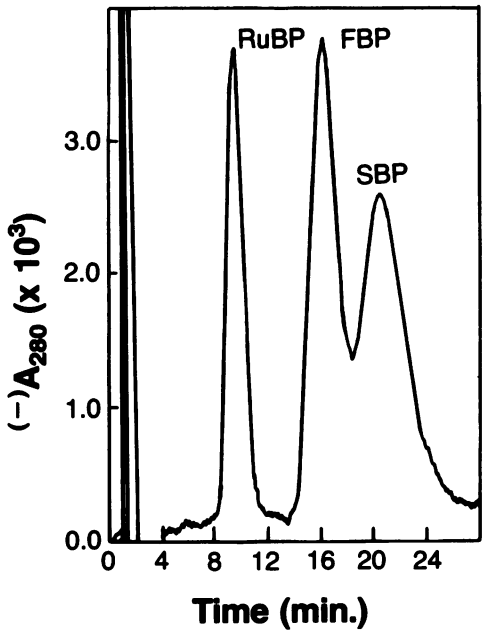

FIG. 5. Separation of bisphosphorylated sugars. Fifty nmol of each compound was injected. The mobile phase contained 1.5 mM trimesate and 10 mM borate (pH 8.7) with detection at 280 nm.

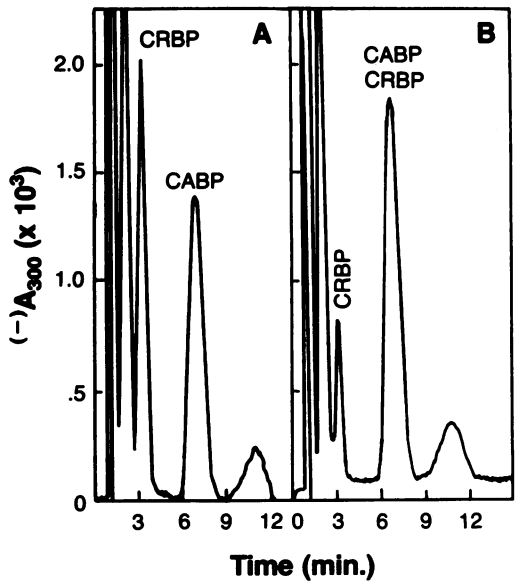

FIG. 6. Separation of CABP and CRBP. The mobile phase contained 3 mM trimesate and 10 mM boric acid (pH 9.7) with detection at 300 nm. A), Separation of the lactones of CRBP and CABP. To form the lactones, the sample was evaporated to dryness at pH 2, desiccated overnight under vacuum, and resuspended in 200 μl H_2O. Fifty μl was injected. B), Separation of a mixture of free acids and lactones of CABP and CRBP. The mixture from (A) was partially hydrolyzed at pH 9 for 1 h. As lactones, CABP and CRBP separated into individual peaks; however, as free acids no resolution was possible.

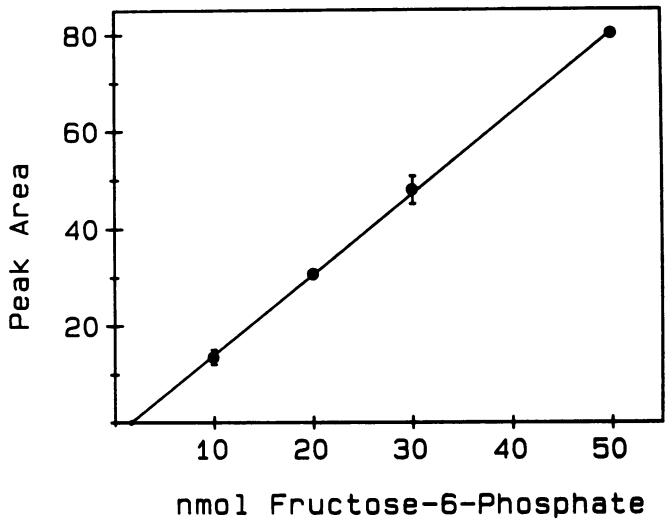

FIG. 7. Indirect UV detection following HPLC column separation of F6P is linear over the range shown. As little as 10 nmol of sugar phosphate can be detected.

as the free acids (Fig. 6B). When either compound alone was eluted as the lactone there was no indication of free acid formation. As the free acids, the two compounds co-eluted. Figure 6B shows the same sample as in Figure 6A after partial hydrolysis at pH 9. After an overnight incubation at pH 9.0 the CABP and CRBP lactones were completely converted to the free acid form.

Samples with as little as 10 nmol of F6P were detectable by this system and the response was linear with increasing amount (Fig. 7). The sensitivity of other sugar phosphates was similar. Smaller amounts of sugar phosphates less than 10 nmol can be detected depending on the resolution of the signal to noise.

DISCUSSION

In spite of some limitations, this HPLC method is useful because significant resolution and detection is achieved without requiring any highly specialized equipment or sample derivitization. A conventional HPLC system with a low capacity ion-exchange column and a UV detector is sufficient. The separation system is highly versatile in that the conditions can be adjusted to meet the specific requirements of a given analysis.

The key parameters that can be manipulated are the nature and concentration of the eluting anion, the concentration of boric

acid, and the pH. Increasing concentrations of boric acid and eluting anion tend to have counterbalancing effects. Increased boric acid tends to cause compounds containing cis-diols to be retained. Increasing the eluent concentration decreases the retention of the phosphorylated sugars. In order to maintain optimum optical sensitivity the eluent concentration is best kept at a minimum (9).

A disadvantage of this technique is that anions, other than sugar phosphates, such as Cl^-, ClO_4^-, $HCOO^-$, and SO_4^{2-} can interfere with detection. Monophosphate separation is most sensitive to interference. About 1 μequivalent of contaminating anion causes the system peak to overlap the first sample peak. For this reason considerable pretreatment and fractionation of the sample mixture may be required. For example, G6P, Pi, and DHAP co-elute in the beginning system peak region. These compounds can best be separated if a weaker eluting anion such as *O*-phthalate is used. Leaf tissue samples gave similar separations, but only if the content of interfering anions was low.

We have shown that a number of difficult separations can be achieved with this system. Of particular interest to plant biochemists is the detection of CAlP which has been shown to be a potent inhibitor of photosynthesis (5, 8). This technique offers a rapid analytical method for its determination.

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