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Note

High-performance liquid chromatographic determination of L-ascorbate-2-phosphate in phosphorylation reactions*

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Several of us recently reported[1] the synthesis of L-ascorbic acid 2-phosphate (A-2-P, Fig. 1). In that work we used a rapid, high-performance chromatographic method to assay the products of reaction between 5,6-O-isopropylidene-L-ascorbic acid (IAA) and phosphorus oxychloride. A number of reactions were done under a variety of conditions to maximize the yield of A-2-P. Previous workers[2] used gravity column chromatography to separate the phosphorylation products. In this paper we present the details of the high-performance liquid chromatographic (HPLC) procedure used in the synthesis work.

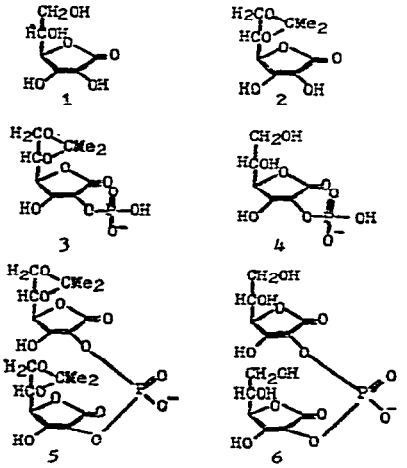

Fig. 1. Compounds involved in this work: 1 = AA; 2 = IAA; 3 = IA-2-P; 4 = A-2-P; 5 = BIA-2-P; 6 = BA-2-P.

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EXPERIMENTAL

Reagents

The following compounds were used in analytically pure form: IAA[3], m.p. 217–223°; tricyclohexylammonium A-2-P[1], m.p. 178–182° (decomposition); barium bis(L-ascorbate)-2,2′-phosphate (BA-2-P)[1], m.p. 250° (decomposition), dipotassium L-ascorbate-2-sulfate[4] (A-2-S), m.p. 96–97°; and L-ascorbic acid (AA), m.p. 192° (Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A.).

Standard solutions (0.05–1.0 m*M*) of the water-soluble salts were prepared in water. In the case of the sparingly soluble phosphodiester, barium BA-2-P (50 mg, 0.081 mmole) was added to water (100 ml) containing sodium sulfate (20 mg, 0.141 mmole). After barium sulfate had been removed by gravity filtration, standard solutions were prepared.

Phosphate buffers (0.1–0.5 *M*) of pH 3.5–5.0 were prepared by mixing aqueous potassium hydrogen phosphate (0.1–0.5 *M*) with aqueous phosphoric acid (0.1–0.5 *M*). The buffers were degassed by boiling momentarily.

Apparatus

Separations were carried out on a Waters Model ALC/GPC 201 high-performance liquid chromatograph fitted with a septum injector and a UV detector (Waters Assoc., Milford, Mass., U.S.A.). The detector operated at 254 nm with a cylindrical flow cell of 1 mm diameter and 10 mm length (8 μl cell volume). The detector, which was equipped with a binary attenuator (2× to 64×), produced a linear output over $A = 0.02$–0.64. The stainless-steel column (1.22 m × 3.17 mm) was packed with a pellicular, strongly basic anion-exchange resin (Bondapak AX/Corasil, 37–50 μm, Waters Assoc.). The chromatography was done under ambient temperature conditions. Samples (10 μl) were injected (Series "B-110" syringe, Precision Sampling, Baton Rouge, La., U.S.A.) and the components were eluted with potassium dihydrogen phosphate buffer (0.1 *M*) at a flow-rate of 0.5 ml/min. Peaks were recorded on a 0.1 mV recorder at a chart speed of 0.5 cm/min. The retention times are given with reference to the mobility of AA (Table I). When retention times changed gradually with sustained use of the column, spiking was done to verify peak assignments.

Analysis of phosphorylation reactions

To a mixture of IAA (6.15 g, 28.5 mmoles) in water (50 ml) with or without pyridine (10 ml, 124 mmoles; or 12 ml, 155 mmoles), was quickly added 10 *M* aqueous potassium hydroxide to a final pH of 12 or 13. Each reaction mixture was kept at about 0–5° under an atmosphere of nitrogen while phosphorus oxychloride (3.65 ml, 39.9 mmoles) was added dropwise and the pH of the reaction mixture was maintained constant by periodic addition of 10 *M* aqueous potassium hydroxide. The reaction mixture was allowed to warm to 25° and was made to volume (250.0 ml). Each reaction mixture was examined by HPLC before hydrolytic removal of the isopropylidene group. For that purpose, an aliquot (1.0 ml) of the diluted reaction mixture was added to dipotassium A-2-S (0.1 mmole), and the resulting mixture was diluted again (250 ml). For quantitative analysis, the isopropylidene group was hydrolyzed and the pyridine removed by treatment with a cation-exchange resin at 25°. An aliquot (1.0 ml)

TABLE I

RELATIVE MOBILITIES (R_A) OF DERIVATIVES OF AA

Relative mobilities (R_A) = (distance migrated by derivative)/(distance migrated by L-ascorbic acid). HPLC; stationary phase, pellicular anion-exchange resin; mobile phase 0.1 *M* KH_2PO_4, pH 4.4, at 0.5 ml/min and 25°. The retention time of AA was 4.5–6.0 min depending on the age of the column. The figures in the table were observed using fresh resin. Paper chromatography (PC): development solvent, *n*-propanol–water–trichloroacetic acid (15:4:1, v/v/w).

Compound	R_A	
	HPLC	*PC*
AA	1.0	1.0
IAA	1.3	1.6
A-2-S	1.8	0.5
A-2-P	2.8	0.6
BA-2-P	2.3	0.4
IA-2-P*	3.4	—
BIA-2-P*	6.0	—
Inorganic phosphate	—	1.2

* Compounds not isolated; structures assigned based on products identified after mild acid hydrolysis.

of the once diluted reaction mixture was passed through Amberlite IR-120 (H^+) (15 ml) which had been previously washed free of UV-absorbing material. The reaction mixture became strongly acidic (pH 0.5–1.0) after passing through the exchange resin, because of the exchange of sodium ions. The column was washed generously with water (200 ml), the effluent was made to volume (250 ml), and an aliquot (10 μl) was injected in triplicate into the liquid chromatograph. Standard curves were prepared from analytically pure samples of known tricyclohexylammonium A-2-P. The precision of the assay was $\pm 3\%$.

Paper chromatography was also used to examine the reaction mixtures. The products of a reaction mixture were converted to sodium salts by a cation-exchange resin, concentrated to a small volume, and spotted on Whatman No. 1. The papers were developed in a descending manner at 25° using *n*-propanol–water–trichloroacetic acid (15:4:1, v/v/w). Components were visualized with three spray reagents. Strongly reducing compounds such as IAA reacted with silver nitrate spray[5] (1 ml of saturated aqueous silver nitrate in 100 ml of acetone). Ferric chloride spray[6,7] (1%, w/w, $FeCl_3$ in ethanol–water, 95:5) gave a permanent intense red color on a yellow background for the 2-substituted esters of AA and permanent white spots for strongly reducing ascorbic acids. Phosphate containing spots were detected as blue spots using an acid–molybdate spray[8] followed by treatment of the paper with hydrogen sulfide.

RESULTS AND DISCUSSION

Fig. 2 shows the HPLC separation of the products when IAA was reacted at a constant pH of 12 with 1.4 equivalents of phosphorus oxychloride in alkali. The peak with the shortest retention time is the internal standard A-2-S. The other two prominent peaks represent the principal products of the phosphorylation reaction, namely 5,6-O-isopropylidene-L-ascorbate 2-phosphate (IA-2-P) (51%) and bis(5,6-O-

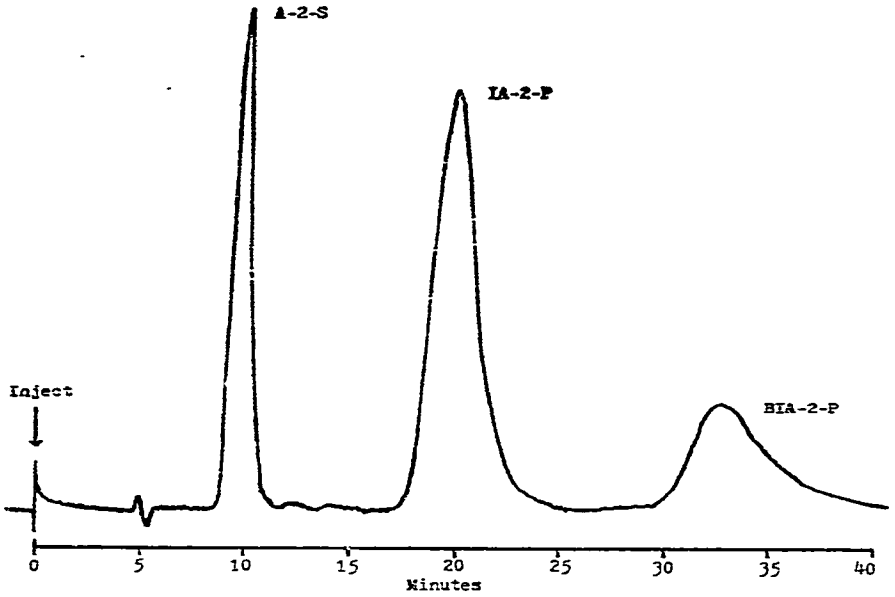

Fig. 2. HPLC separation of reaction products between IAA and phosphorus oxychloride at pH 12 and 2°. An internal reference standard was added, A-2-S. Other abbreviations identified in Fig. 1.

isopropylidene-L-ascorbate)-2,2′-phosphate (BIA-2-P) (22%). Those two products were not isolated, but upon hydrolytic removal of the acetal group two new peaks appeared with a ratio of intensities equal to that of the acetonated products, and with mobilities identical to those of analytically pure samples of A-2-P and BA-2-P. The chromatographic mobilities of various AA compounds are given in Table I. The mobilities of the compounds varied somewhat as the use of the column increased. However, the relative positions of the peaks did not change.

Fig. 3 shows the effect of adding pyridine (initially 1.9 *M*) to the phosphorylation reactions mixture at pH 12. The chromatogram shows that the quantity of phospho-diester (retention time, $t_R = 37$ min) is greatly reduced compared to that seen in Fig. 2. In Fig. 3 the first peak to elute is pyridine followed by the internal standard A-2-S ($t_R = 14.5$ min).

The optimum reaction medium for the conversion of IAA to IA-2-P was found to be a mixture of potassium hydroxide at pH 13 and pyridine at an initial concentration of 2.3–2.8 *M*. Under those conditions, as seen in Fig. 4, IA-2-P ($t_R =$ 24.5 min) was formed in 97% yield and only a trace (<1%) of BIA-2-P was observed. When the optimum reaction mixture was treated at 25° with a strongly acidic cation-exchange resin in the H^+ form, pyridine was removed and the 5,6-acetal was quantitatively cleaved (Fig. 5). The response area of the major peak ($t_R = 14.5$ min) compared to that of the pure standard tricyclohexylammonium A-2-P showed a 97% conversion of IAA to the monophosphate ester. Prior studies[9] on the hydrolysis of A-2-P have indicated that the phosphate ester would probably be stable in acid at pH 1.0 and 25°. Fig. 5 shows a slight shoulder on the left side of the major peak due to traces of BA-2-P ($t_R \approx 12$ min) in the reaction mixture. It should be noted that

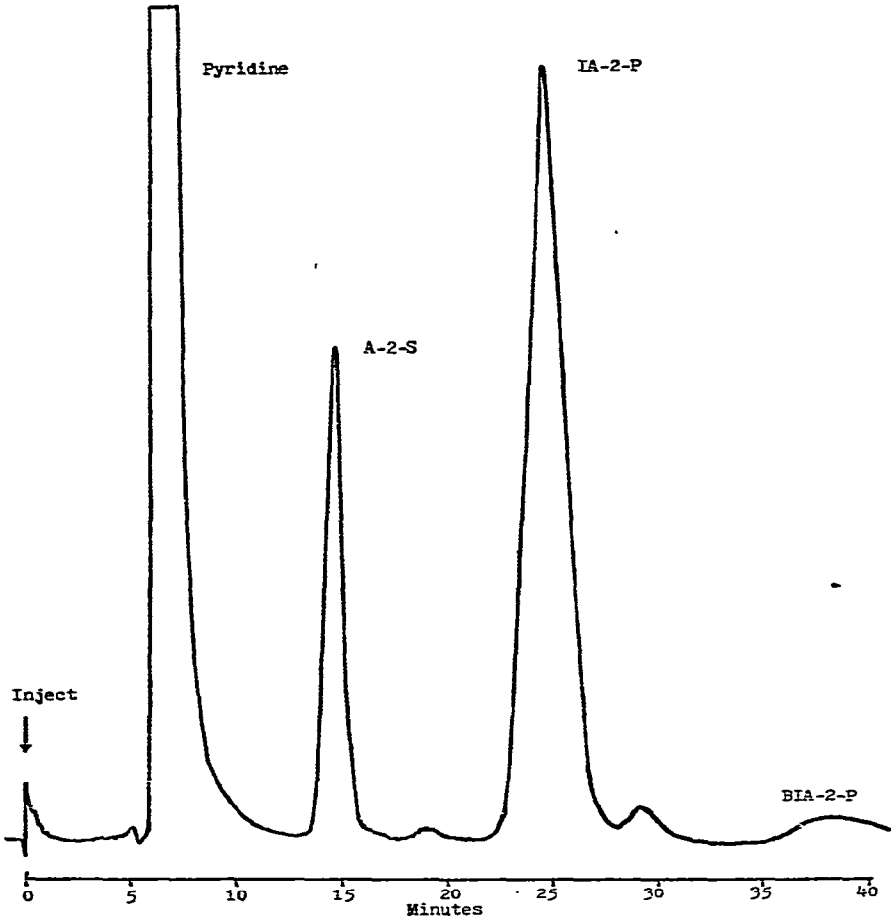

Fig. 3. HPLC separation of reaction products between IAA and phosphorus oxychloride at pH 12 and 2° in presence of pyridine (1.9 *M*). See Figs. 1 and 2 to identify abbreviations.

BIA-2-P has a slower mobility on the analytical column than IA-2-P, but the reverse is true of BA-2-P and A-2-P.

In another phase of this work, several phosphorylation reactions were done directly on AA in strong alkali (pH 13–13.5) without pyridine. In one example, we repeated the conditions of a reaction described in a German patent[10]. AA (8.3 g) was dissolved in a mixture of water (30 ml) and calcium hydroxide (11.5 g), and phosphorus oxychloride (7.5 g) was added at 0°. After 90 min the reaction mixture was diluted and examined by HPLC (Fig. 6). At pH 13–13.5 with no pyridine, the reaction mixture contained mainly BA-2-P (37%) along with A-2-P (24%) and unreacted AA (24%). The barium salt of BA-2-P, which crystallizes rather easily from water, was readily isolated[1] in pure form from that reaction mixture.

The HPLC separation of A-2-P and BA-2-P depended on the ionic strength and pH of the eluting buffer (Table II). The best resolution was found with a mobile

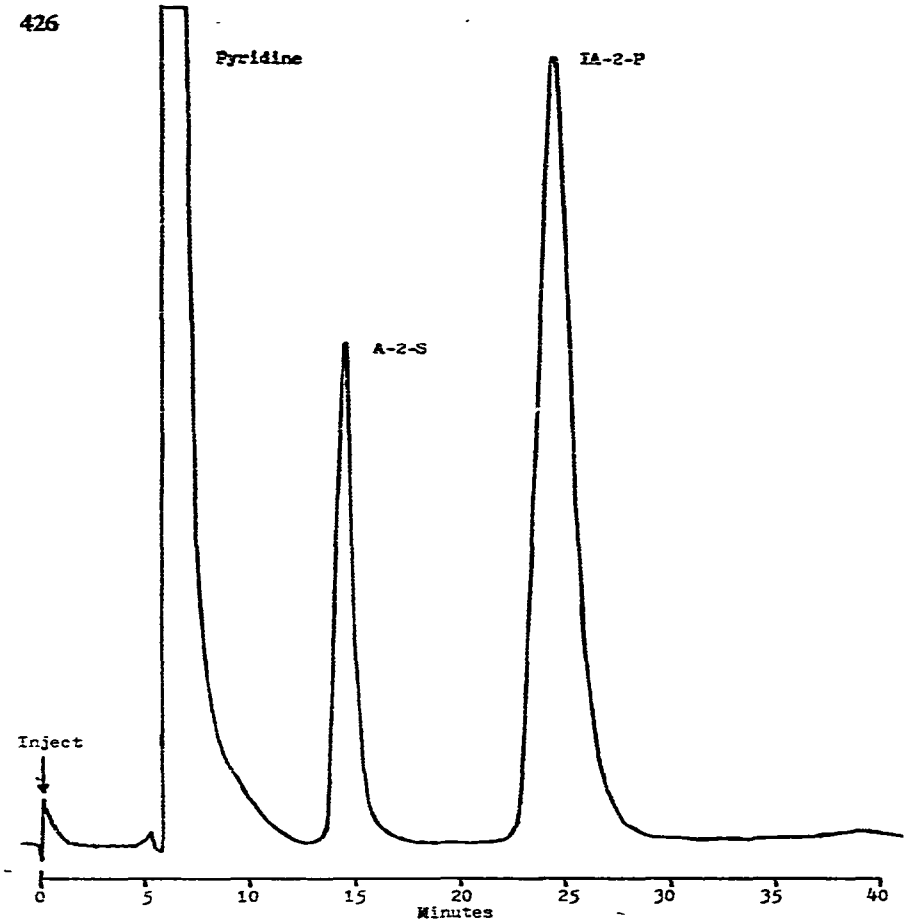

Fig. 4. HPLC separation of reaction products between IAA and phosphorus oxychloride at pH 13 and 2° in the presence of pyridine (2.3 *M*). See Figs. 1 and 2 to identify abbreviations.

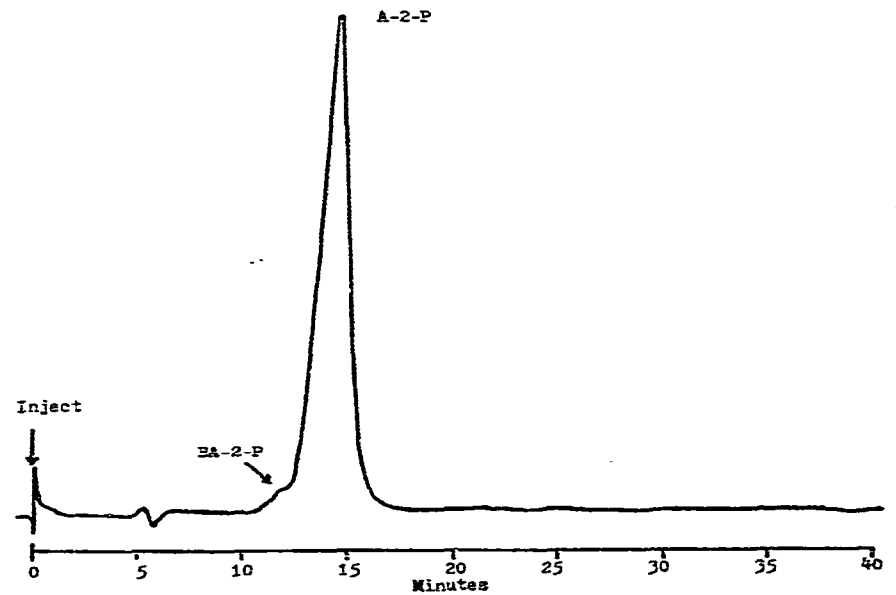

Fig. 5. HPLC separation of de-acetonated reaction products obtained at optimal conditions (pH 13, 2°, and pyridine at 2.3 *M*) for formation of A-2-P.

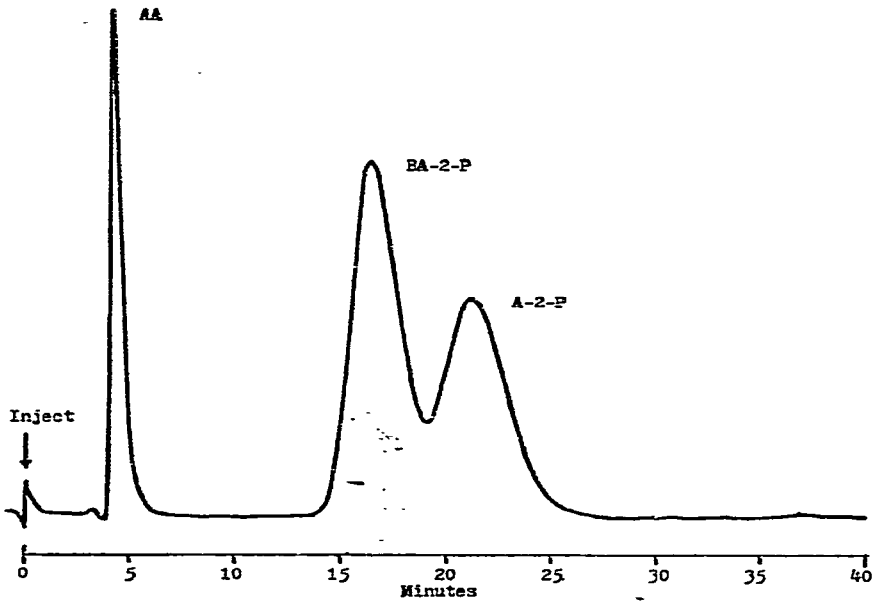

Fig. 6. HPLC separation of reaction products formed when phosphorus oxychloride is added to AA in aqueous calcium hydroxide (pH 13.1) at 2°.

TABLE II

MOBILE PHASE FOR SEPARATION OF PHOSPHO-MONOESTER AND PHOSPHO-DIESTER OF AA

Eluent flow-rate, 0.5 ml/min.

Potassium dihydrogen phosphate (M)	*pH*	*Retention time (min)*	
		Phospho-diester	*Phospho-monoester*
0.5	4.4	5.8	6.2
0.3	4.4	6.6	7.2
0.1	4.4	12.8	15.4
0.1	5.0	12.4	13.6
0.1	4.7	12.6	14.8
0.1	4.0	14.0	16.4
0.1	3.5	17.2	17.2

phase of 0.1 *M* potassium dihydrogen phosphate at pH 4.4. That same eluent gave baseline resolution of the 5,6-acetal derivatives of those two phosphate esters (Fig. 2).

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