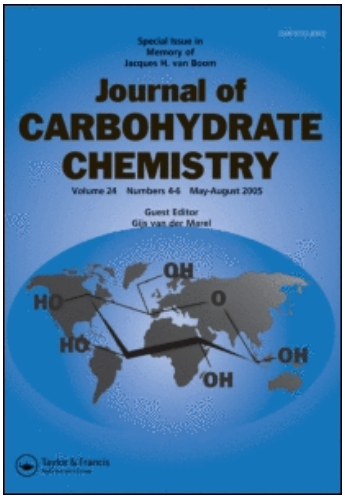

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An Improved Synthesis of L-Ascorbate 2-Polyphosphate

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AN IMPROVED SYNTHESIS OF L-ASCORBATE 2-POLYPHOSPHATE

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ABSTRACT

L-Ascorbate (**0**) reacted rapidly with sodium trimetaphosphate (STMP) at 20-25 °C and constant pH 9.5-10.0 in the presence of calcium ion. Barium and strontium ions also catalyzed the phosphorylation reaction, but not magnesium ion. The optimum reaction mixture was initially 1.4 M L-ascorbate, 1.8 M STMP, 1.7 M calcium hydroxide, and 0.4 M calcium chloride. Over a 20-min reaction period, pH was maintained by adding approximately 0.1 g calcium hydroxide in a 20% aqueous slurry. The initial and final Ca/P ratios were ~0.36 and 0.42, respectively. The reaction products were 98.8% 2-phosphorylated L-ascorbate, 1.2% unreacted **0**, and a trace of 4,5-ene, probably formed through 5,6-phosphorylated L-ascorbate 2-polyphosphate. High-performance liquid chromatography with UV detection and preparative anion-exchange chromatography showed the presence of eight 2-phosphorylated derivatives of **0** and two of the 4,5-ene skeleton. Rapid 2-phosphorylation prevented accumulation of the 4,5-elimination reaction, and three 5,6-cyclophosphoesters of **0** were found in the reaction products. A Ca/Na (3/1) salt of L-ascorbate 2-polyphosphate was isolated in 88% yield as a white, odorless powder containing 33% of L-ascorbate equivalents. Crystalline cyclohexylammonium salts of L-ascorbate 2-mono, di, and triphosphates were prepared.

INTRODUCTION

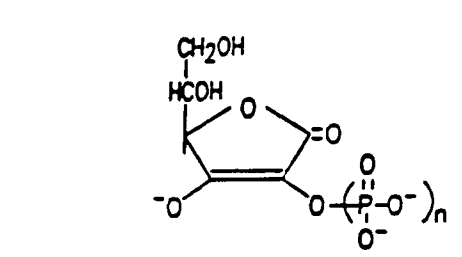
L-Ascorbate 2-polyphosphate was prepared previously[1,2] by reaction of L-ascorbate (**0**) with 1.3 equivalents of sodium trimetaphosphate at 35 °C and constant pH ~10.5 using potassium or sodium hydroxide. After 24 h, the reaction mixture contained 90% of L-ascorbate 2-mono (**1**), 2-di (**2**), 2-triphosphate (**3**); 4% of unreacted **0**; and 3% of a 4,5-unsaturated, phosphorylated by-product (**5**). 2-Phosphorylated derivatives of **0** have been shown[2-4] to have vitamin C activity equivalent to that of **0**. In a model aqueous system at 28-45 °C, pure **3** was one, two, and three orders of magnitude more stable towards O_2-oxidation than **0** at pH 3, 6, and 8, respectively.[2]

Metal-catalyzed phosphorylation of various acceptors with adenosine triphosphate (ATP) was reported by Lowenstein.[5-8] Orthophosphate, adenosine monophosphate (AMP), and a fatty acid were used as acceptors to give pyrophosphate, adenosine diphosphate (ADP), and acylphosphate, respectively. The bivalent metal ions, Ca, Sr, and Ba, were effective catalysts, Mn and Cd were moderately effective, but Fe, Co, Ni, Sn, and Cu were ineffective. The metal ion-catalyzed phosphorylation reaction depended on pH and on the ratio of ATP to metal ion. In the case of the reaction of ATP with orthophosphate, the optimum reaction was at pH 9 with a ratio of metal ion to ATP of 1.0-1.3.

The objective of this work was to reexamine the phosphorylation of L-ascorbate (**0**) using sodium trimetaphosphate (STMP) in the presence of bivalent metal ions. Because calcium ion was found to be a potent catalyst for the phosphorylation of **0** with STMP, a second objective was to isolate from a calcium-catalyzed phosphorylation, without chromatography, a 2-phosphorylated L-ascorbate product.

RESULTS AND DISCUSSION

Reaction of L-Ascorbate with STMP in the Presence of Calcium Ion. Calcium ion-catalyzed phosphorylation of **0** with



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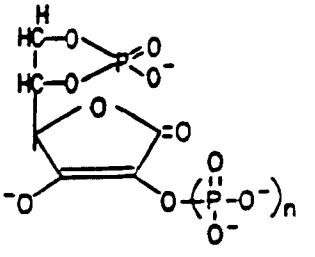
3. n=3
7. n=4



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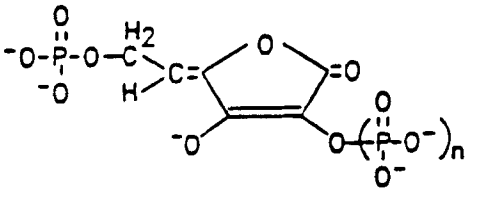

5. n=3
6. n=1

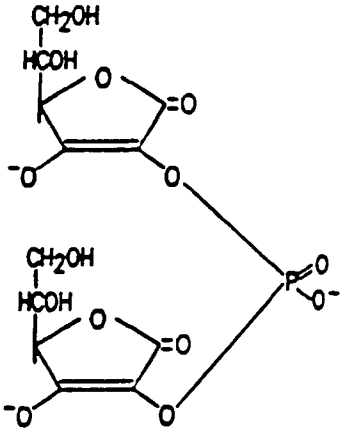

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Figure 1. Phosphate derivatives of L-ascorbate isolated from the reaction mixture of Run 7, Table 1.

STMP was carried out at pH 9.5-10.0, but calcium hydroxide alone was not sufficiently soluble to give initially the desired ratios of Ca/P 0.36 or Ca/STMP 1.08. Therefore, a small amount (0.2 g/g **0**) of calcium chloride was added at the beginning of the reaction. With periodic addition of calcium hydroxide to maintain pH, the optimum reaction gave >98% 2-phosphorylation, 1% unreacted **0**, and less than 0.3% of 4,5-ene by-product (Run 7, Table 1). The Ca/P ratio in the reaction

Table 1. Reaction of L-Ascorbic Acid (**0**) or 5,6-*O*-Isopropylidene-L-Ascorbic Acid (IAA) with 1.3 Equivalents of Sodium Trimetaphosphate (STMP) at an Initial Metal Ion to Phosphorus Ratio of 0.36

Run No.	AsA M	Ionic Catalyst	Temp. °C	pH	Time, Min	Unreact[a] AsA,%	2-Phosphorylation, % UV Spec[b]	2-Phosphorylation, % HPLC-UV[c]
1	1.42	Ca	20	10.5-11.2	20	4.1	97.9	92.9
2	1.42	Ca	20	9.5-10.0	25	1.8	101.7	99.3
3	1.42	Ca	20	7.5- 8.0	45	11.2	92.3	90.1
4	1.42	Ca	20	10.5-11.2	27	3.0	100.5	94.3
5	1.14	Ca	20	10.5-11.2	20	6.9	95.1	91.8
6	1.42	Ca	25	10.5-11.2	15	6.7	94.7	90.5
7	1.42	Ca	25	9.5-10.0	20	1.9	103.9	99.6
8	1.42	Ca	25	9.5-10.0	25	1.3	101.7	97.3
9	1.42[d]	Ca	25	9.5-10.0	20	12.2	89.9	-
10	1.42	Ba	25	9.5-10.0	25	40.0	55.0	65.7
11	1.42[e]	Ba	25	10.5-11.2	25	11.1	89.3	90.0
12	1.42[e]	Mg	25	9.5-10.0	22h	7.5	92.4	91.0
13	1.42[f]	Sr	25	9.5-10.0	25	0.4	103.9	101.2
14	1.62[g]	-	35	10.5-10.7	24h	1.6	97.1	-
15	1.42[h]	Ca	25	9.5-10.0	25	1.6	101.0	103.6
16	1.42[i]	Ca	25	9.5-10.0	25	2.2	101.1	84.8

a. It was determined by iodine titration.
b. UV assay at 259 nm and pH 10 using ε_{mM}16.0 $Lmmol^{-1}cm^{-1}$.
c. HPLC-UV assay using standard curves of reference standards (**1**, **2**, & **3**) with UV monitoring at 250 nm. The standard curve of compound **3** was used to estimate yields of **4** and **7-10** except that for **4**, molar extinction was taken as twice the value of **3**.
d. Initial metal ion to phosphorus ratio was 0.20.
e. Sodium hydroxide was used to maintain pH.
f. Strotium chloride was added to **0**, the mixture adjusted to pH 9.5-10.0 by adding 10 M sodium hydroxide, STMP added, and the pH maintained by adding 10 M sodium hydroxide.
g. Sodium hydroxide was used to maintain pH. No catalyst was added. Data from Ref.2. The 2-phosphorylated reaction mixture contained 3% of 4,5-ene by-product.
h. D-Erythorbic acid (D-isoascorbic acid) was the starting material.
i. 5,6-*O*-Isopropylidene-L-ascorbic acid was the starting material.

gradually increased from 0.36 to a final ratio of 0.42. HPLC-UV analysis showed that the reaction mixture contained, on a molar basis, 14.7% of **1**, 15.9% **2**, 61.1% **3**, ~0.5% **4**, and 6.7% higher phosphorylated derivatives of **0** (Fig. 2), which together accounted for 98.9% of starting material (**0**). The structures of the compounds detected in the chromatogram (Fig. 2) were established as discussed below.

The phosphorylation reaction was accelerated greatly by calcium ion. Under optimum synthesis conditions, L-ascorbate (~1.43 M) reacted completely with 1.3 equivalent of STMP in 20 min at 25 °C in the presence of initially ~1.62 M calcium ion, or a Ca/P ratio of 0.36 (Ca/STMP 1.08, Run 7, Table 1). When the amount of calcium ion in the phosphorylation reaction was reduced from an initial Ca/P molar ratio of 0.36 to 0.20 (Ca/STMP 1.08 to 0.60, Run 9, Table 1), the extent of 2-phosphorylation of **0** decreased from 98% to 90% at 25 °C and 20 min. Strontium ion (Run 13, Table 1) at the same molar ratio of metal ion to phosphorus also accelerated the phosphorylation reaction, and barium ion (Runs 10 & 11, Table 1) was somewhat less effective. Magnesium ion (Run 12, Table 1) gave no catalysis.

Apparently, metal ion catalysis of the phosphorylation of **0** with STMP follows the same trend observed in phosphorylation of a variety of acceptors using adenosine triphosphate.[5-8] That is, the alkaline earth ions, Ca, Sr, and Ba were active catalysts, whereas Mg, Co, Zn, Cu, and Fe ions were poor catalysts.

The speed of 2-phosphorylation of L-ascorbate by STMP in the presence of calcium ion reduced the yield of 4,5-ene by-product to 0.3%, in contrast to 3.4% in the reaction catalyzed by sodium or potassium hydroxide.[2] The 4,5-ene by-product is thought to form slowly by an elimination reaction on a 5,6-cyclic phosphate of **1**, **2**, and **3**, which forms through the intermediate 2,6-*bis*-(triphosphoryl)-L-ascorbate. A diminishing low proportion of 4,5-ene by-product in the solid sodium/calcium salts of L-ascorbate 2-polyphosphate would explain their white color and bland odor.

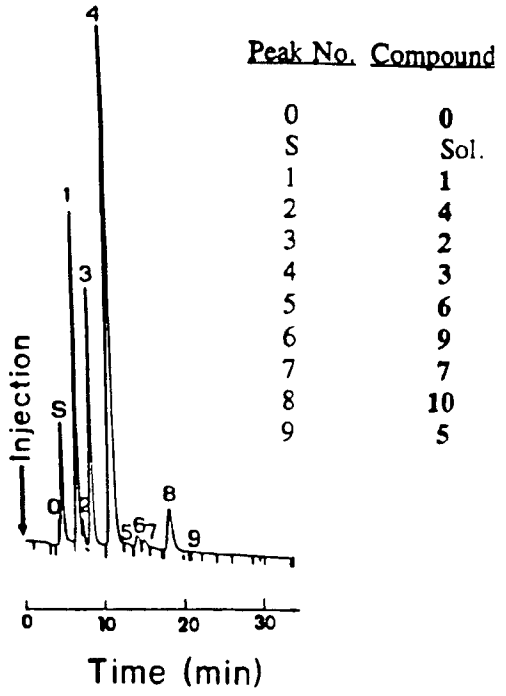

Figure 2. HPLC-UV chromatogram of reaction mixture from Run 7, Table 1. Compounds: **0**, L-ascorbic acid; **1**, L-ascorbate 2-monophosphate; **2**, L-ascorbate 2-diphosphate; **3**, L-ascorbate 2-triphosphate; **4**, 2,2'-*bis*-(L-ascorbyl)-phosphate; **5**, 4,5-ene-2-triphosphate; **6**, 4,5-ene-2-monophosphate; **7**, L-ascorbate 2-tetraphosphate; **9**, L-ascorbate 5,6-cyclophosphate 2-diphosphate; **10**, L-ascorbate 5,6-cyclophosphate 2-triphosphate

Components in L-Ascorbate 2-Polyphosphate Prepared by Reaction of L-Ascorbate with Trimetaphosphate in the Presence of Calcium Hydroxide. The HPLC-UV chromatogram in Fig. 2 shows the presence of six previously known phosphorylated derivatives of L-ascorbate plus three unknown peaks (peak nos. 6, 7, and 8).

To identify the unknown peaks, the reaction mixture was subjected to preparative anion exchange column chromatography. The elution profile showed no peak of L-ascorbate prior to elution of **1**, even though iodometric titration indicated that the reaction mixture contained 1.2% unreacted **0**. Apparently,

the low level of **0** in the reaction mixture was destroyed during the time required to develop the column.

The preparative column separation of the reaction mixture gave six pure fractions: I, II, III, VI, VIII, and IX, which contained compounds **1, 2, 3, 7, 10,** and **4**, respectively, and three impure fractions, IV, V, and VII. Rechromatography of the three impure fractions on anion exchange columns gave pure **6** from Fraction IV, pure **8** from Fraction V, and pure **5** and **9** from Fraction VII. Structural analysis of the compounds is discussed below. The unknown peak nos. 6, 7, and 8 in the HPLC-UV chromatogram of Fig. 2 coeluted with compounds **9**, **7**, and **10**, respectively.

Table 2 gives the yields of the major components isolated from reaction of **0** with STMP in the presence of calcium hydroxide compared to sodium hydroxide. Catalysis with calcium hydroxide gave decreased levels of unreacted **0** and 4,5-ene compounds (**5** and **6**) and increased yields of 5,6-cyclophosphates (**8-10**) and total phosphorylated L-ascorbate. The yield of pure **3** was higher in the sodium than in the calcium hydroxide reaction. The total yield of 4,5-ene derivatives (Compounds **5** and **6**) isolated by ion-exchange chromatography of the calcium hydroxide reaction mixture was 1.1%. That yield was higher than found (0.3%) by UV absorbance[2] at $\lambda_{313\ nm}$ before column separation. The increase may have been due to the conversion of the 5,6-cyclic phosphate compounds (**8**, **9**, and **10**) to the 4,5-ene derivatives (**5** and **6**) during column separation.

Structures of Minor Compounds Isolated from the Reaction Mixture. The structures of Compounds **6-10** isolated were determined by UV, NMR, and mass spectroscopy. Compound **6** exhibited UV properties similar to those of the previously[2] identified 4,5-ene 6-phosphate 2-triphosphate (**5**). Compound **6** was assigned the structure of 4,5-ene 6-phosphate 2-monophosphate (Fig. 1). The UV spectrum of **6** at pH 2 showed a major absorption band at 250 nm with a shoulder at 278 nm (Table 3, and Fig. 3, left side). After ionization of the 3-OH at pH 10, the two absorption peaks were resolved; one at 249 nm and the other at 319 nm. The absorption at λ_{max2} (319 nm, ε_{mM} 8.5

Table 2. Major Components (%) Isolated by Anion-Exchange Chromatography of Reaction Mixtures Using Sodium and Calcium Hydroxide to Control pH.

Compound	NaOH[a]	$Ca(OH)_2$
0	(4)[b]	(1.2)[b]
1	1.9	14.7
2	3.4	15.9
3	86.0	61.1
4	trace	trace
5	3.4	trace
Higher Esters	trace	7.2
Total	98.7	100.4

a. Data from Ref. 2.
b. Determined by iodometric titration.

$Lmmol^{-1}cm^{-1}$ at pH 10) was the one used throughout this investigation to measure the combined yields of compounds **5** and **6** in a reaction mixture. The absorbance of 2-phosphorylated derivatives of **0** was negligible[2] at 319 nm and pH 10, as illustrated by compound **10** in Fig. 3 (right side).

^{31}P NMR of **6** at pH 6.5 with 85% phosphoric acid as the external standard showed a singlet at 4.3 ppm, which was attributed to a 6-phosphate. Another singlet at 2.4 ppm was assigned to the 2-monophosphate group (Table 4).

The ^{13}C NMR spectrum agreed with the assigned structure of the 4,5-ene 2-monophosphate for compound **6**. Table 5 shows that the ^{13}C signals of **6** were similar to those of the 4,5-ene 2-triphosphate (**5**). The C-4 and C-5 signals of **6** at 147.9 and 104.8 ppm, respectively, were typical of vinyl carbons and also agreed with those (150.0 ppm and 106.3 ppm) reported[2] for **5**. The FAB-MS spectrum of **6** gave a $(M-1)^-$ ion equal to 317, which confirmed the structure.

Compound **7** showed UV properties (Table 3) and ^{13}C NMR chemical shifts (Table 5) similar to those[2] of **3**. The ^{31}P NMR spectrum (Table 4) at pH 6.5 gave four phosphorus signals typical of a tetraphosphoryl ester, and FAB-MS gave molecular ion $(M-1)^-$ at 495.

Table 3. UV Spectral Properties of Components Isolated from Reaction Mixture (ε_{mM} $Lmmol^{-1}cm^{-1}$)

Components		Acid (pH 2.0) λ_{max}, nm	Acid (pH 2.0) ε_{mM}	Neutral (pH 7.0) λ_{max}, nm	Neutral (pH 7.0) ε_{mM}	Base (pH 10.0) λ_{max}, nm	Base (pH 10.0) ε_{mM}
L-Ascorbic acid	(**0**)[a]	243	10.0	265	16.5	-	-
L-Ascorbate 2-monophosphate	(**1**)[a]	238	9.0	258	11.5	264	16.0
2-diphosphate	(**2**)[b]	235	10.3	255	13.2	259	15.9
2-triphosphate	(**3**)[b]	235	10.3	258	15.4	258	16.0
2,2′-*bis*-L-ascorbyl phosphate	(**4**)[a]	236	17.3	258	21.6	259	30.2
4,5-ene 2-triphosphate	(**5**)[b]	251[c]	13.7	246[d]	15.4	246[d]	15.6
				312[e]	8.2	313[e]	8.5
monophosphate	(**6**)	250[c]	-	248[d]	-	249[d]	-
				307[e]	-	319[e]	-
L-Ascorbate 2-tetraphosphate	(**7**)[f]	236	10.1	258	15.3	259	15.7
5,6-cyclophosphate 2-monophosphate	(**8**)	238	10.3	260	14.3	264	16.8
2-diphosphate	(**9**)	236	10.3	257	14.3	260	16.3
2-triphosphate	(**10**)	235	10.4	259	16.0	259	17.1

a. Data from Ref. 15.
b. Data from Ref. 2.
c. Single peak with shoulder at 278 nm.
d. Major peak.
e. Minor peak.
f. Liao and Seib[2] reported λ_{max} 258 nm at pH 7.0 and pH 10.0, and ε_{mM} 16.0 $Lmmol^{-1}cm^{-1}$ at pH 10.

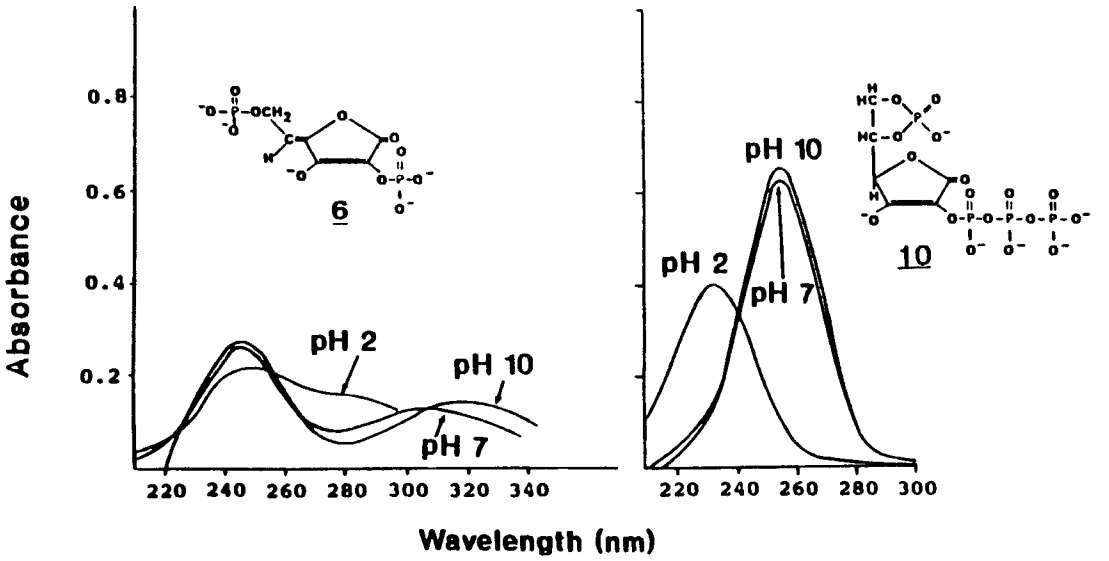

Figure 3. UV-Spectra of 4,5-ene 2-triphosphate (6) and L-ascorbate 5,6-cyclophosphate 2-triphosphate (10) isolated from the reaction mixture of Run 7, Table 1.

Compounds **8**, **9**, and **10** were assigned the structures of the 5,6-cyclophosphates, respectively, of 2-mono-, 2-di-, and 2-triphosphate of L-ascorbate (Fig. 1). The ^{31}P NMR spectra (Table 4, Fig. 4) of those compounds showed a singlet at 18.4 ppm, which is characteristic of a cyclic phosphate ester.[9,10] The ^{13}C NMR signals of C-5 and C-6 in **8-10** occurred at ~75.2 and 66.3 ppm, respectively, and were different from the signals of C-5 (70.6 ppm) and C-6 (63.6 ppm) in **0** (Table 4). Phosphorylation of the primary hydroxyl of **0** to give L-ascorbate 6-phosphate[11] deshielded C-6 by ~4 ppm. The 2-mono, di, and triphosphate groups of compounds **8**, **9**, and **10**, respectively, were deduced from ^{31}P and ^{13}C NMR data (Tables 4 and 5) and from UV data (Table 3, Fig. 3). Their molecular ions observed in FAB-MS spectra agreed with the assigned structures (Table 4).

Isolation and Purification of Calcium L-Ascorbate 2-Polyphosphate (AsPP). The AsPP formed by reaction of **0** with sodium trimetaphosphate at pH 10.5-11.0 in the presence of calcium hydroxide was purified and isolated easily without

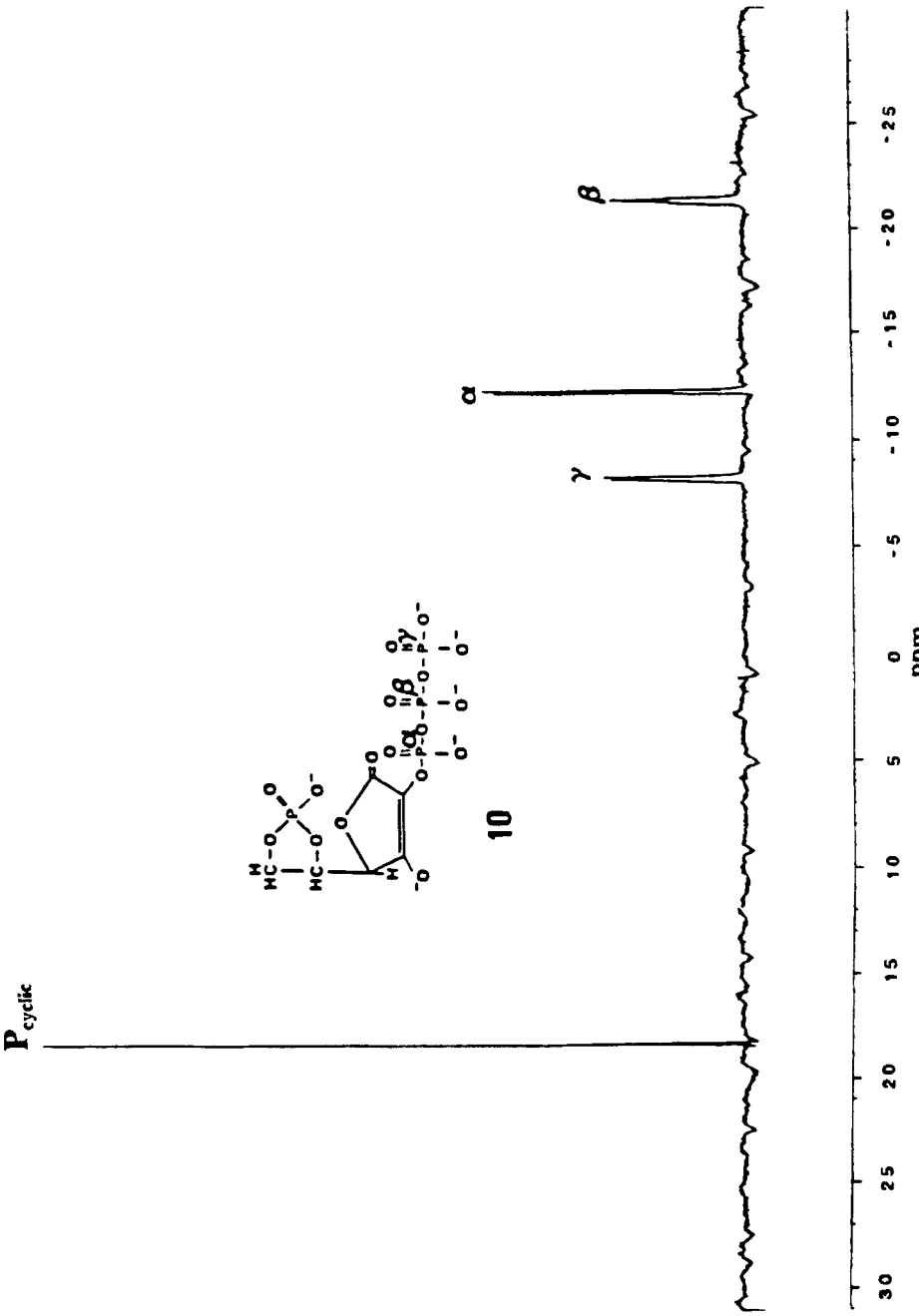

Figure 4. ^{31}P NMR spectrum of L-ascorbate 5,6-cyclophosphate 2-triphosphate (10) isolated from the reaction mixture of Run 7, Table 1.

Table 4. ^{31}P NMR Chemical Shifts[a](ppm) of 2-Phosphorylated Derivatives of L-Ascorbate in Deuterium Oxide.

Derivatives		pH	Chemical Shift of Phosphorus 2-α	2-β	2-γ	2-δ	5,6-cyclo	6
L-Ascorbate 2-monophosphate	(**1**)[b]	10.0	3.6d	--	--	--	--	--
2-diphosphate	(**2**)[c]	6.5	-9.8d	-3.6d	--	--	--	--
2-triphosphate	(**3**)[c]	8.2	-11.7d	-20.5t	-5.2d	--	--	--
2-tetraphosphate	(**7**)[d]	6.5	-12.4d	-21.6t	-21.2t	-5.6d	--	--
4,5-ene 2-triphosphate	(**5**)[c]	8.5	-15.2d	-23.6t	-8.4d	--	--	1.4s
monophosphate	(**6**)	6.5	4.3s	--	--	--	--	2.4s
L-Ascorbate 5,6-cyclophosphate 2-monophosphate	(**8**)	6.5	2.4s	--	--	--	18.4s	--
2-diphosphate	(**9**)	6.5	-11.7d	-5.9d	--	--	18.3s	--
2-triphosphate	(**10**)	6.5	-12.3d	-21.1t	-8.2d	--	18.3s	--
2,2′-*bis*-(L-ascorbyl) phosphate	(**4**)[e]	6.5	2.9s	--	--	--	--	--

a. All derivatives were ammonium salts. Chemical shifts in ppm referenced to external 85% H_3PO_4. Upfield shifts are given a negative sign. Singlet (s), doublet (d), triplet (t).
b. Data from Ref. 15.
c. Data from Ref. 2. Reference to external 50% H_3PO_4.
d. Liao and Seib[2] reported α-P, -10.7(d); β-P, -19.9(t); γ-P, -19.4(t); and δ-P, -3.8(d) at pH 6.5 using reference external 50% aqueous H_3PO_4.
e. Yang and Seib[16] reported -4.24 (s) for bis(D-erythorbyl) 2,2′-phosphate at pH 6.5.

Table 5. ^{13}C NMR Chemical Shifts[a](ppm) of L-Ascorbate and Its 2-Phosphorylated Derivatives in D_2O at pH 6.5-7.0.

Derivatives		Chemical Shift of Carbone					
		C-1	C-2	C-3	C-4	C-5	C-6
L-Ascorbate	(**0**)[b]	178.0	114.1	176.2	79.2	70.6	63.3
L-Ascorbate 2-monophosphate	(**1**)[c]	177.4	113.2	177.0	78.7	70.1	62.9
2-diphosphate	(**2**)[d]	176.4	111.8	175.3	78.2	69.3	62.3
2-triphosphate	(**3**)[d]	177.9	111.2	176.0	78.8	69.7	62.8
2-tetraphosphate	(**7**)[d]	177.4	110.8	175.5	78.3	69.3	62.2
4,5-ENE 2-triphosphate	(**5**)[d]	172.7	115.5	168.6	150.0	106.3	61.1
monophosphate	(**6**)	171.9	115.6	165.3	147.9	104.8	58.7
L-Ascorbate 5,6-cyclophosphate 2-monophosphate	(**8**)	176.9	113.5	174.9	78.0	75.3	66.3
2-diphosphate	(**9**)	176.8	112.1	176.5	78.2	75.2	66.3
2-triphosphate	(**10**)	176.7	112.0	176.1	78.3	75.1	66.3
L-Ascorbate 6-phosphate[e]		179.9	115.6	178.3	80.9	71.4	67.7

a. All derivatives were ammonium salts. Chemical shifts in ppm downfield from 1,4-dioxane (67.4 ppm).
b. Data from Ref. 17.
c. Data from Ref. 18.
d. Data from Ref. 2. Downfield from Me_4Si.
e. Data from Ref. 11.

chromatography. After the phosphorylation reaction, calcium chloride was added to give a final Ca/P ratio of 1/1. After removal of precipitated inorganic phosphates, the supernatant was concentrated to ~0.2 M in 2-phosphorylated L-ascorbate, which was then precipitated by addition of 1 volume of ethanol. The inorganic chloride salts occluded with the precipitated Ca/Na L-ascorbate 2-polyphosphate were extracted from the solids using 50% ethanol at 25 °C. The final dried product had a Ca/Na molar ratio of 3/1 and was obtained in 88% yield based on the original amount of **0**. The white, free-flowing powder had no caramel odor and contained ~33% by weight of active vitamin C (L-ascorbic acid) and ~5% moisture. The low concentration of 4,5-ene by-product explained the whiteness and absence of caramel odor.

Preparation and Isolation of L-Ascorbate 2-Monophosphate by Reaction of L-Ascorbate with Sodium Trimetaphosphate in the Presence of Calcium Ion. Calcium ion not only accelerated the reaction of **0** with sodium trimetaphosphate at alkaline pH, but also catalyzed hydrolysis of linear polyphosphate chains as previously reported.[12,13] That property of calcium ion was used to prepare the 2-monophosphate (**1**) with sodium trimetaphosphate as the phosphorylating agent. After phosphorylation of L-ascorbate with STMP according to Run 7, Table I, the Ca/P ratio was 0.42. Then more calcium chloride was added to give Ca/P = 1/1, and a slurry of calcium hydroxide was added to maintain pH 10.5-11.0. The hydrolysis of the polyphosphate chains was allowed to proceed for 2 h at 25 °C, at which point the Ca/P was 1.2/1. HPLC-UV analysis of the reaction mixture showed ~77% of L-ascorbate converted to compounds (**1**) and (**2**) in a ratio of 2.3/1.0.

A calcium/sodium salt of the product was isolated from the supernatant after centrifuging and removing insoluble calcium phosphates. A dry powdery product, with a Ca/Na ratio of 12/1, was obtained in 78% yield from **0** as determined by UV analysis. HPLC-UV assay showed that the solid was comprised mostly of **1** and **2** in a molar ratio of 2.7 to 1.0.

EXPERIMENTAL

General. All chemicals were reagent grade unless otherwise stated. L-Ascorbic acid (**0**) was from Fisher Scientific Co. (99.6%, reagent grade); D-erythorbic acid and 5,6-*O*-isopropylidene L-ascorbate, both in >98% purity, were from Aldrich Chemical Company, Inc. (Milwaukee, WI). Magnesium L-ascorbate 2-monophosphate (AsMP) was a gift from Showa Denko K. K. (Tokyo, Japan). Sodium hexametaphosphate (SHMP) was obtained from Alfa Chemical Company, Inc. (Ward Hill, MS), and sodium trimetaphosphate (STMP, 95-97% pure) and 3′:5′-cyclic nucleotide (AMP) phosphodiesterase from bovine heart were from Sigma Chemical Company (St. Louis, MO). Analytical grade anion exchange resin (AG-1X8, 200-400 mesh) was from Bio-Rad (Richmond, CA), and cation resin (Amberlite IR-120, medium porosity) was from Rohm & Haas Co. (Philadelphia, PA). Charcoal was in the form of activated carbon pellets (Norit RO 0.8) from Aldrich.

Solvent evaporations were done under diminished pressure below 40 °C. Moisture content was determined by weight loss upon oven-drying 1 h at 130 °C. NMR spectra were recorded on a Bruker WM-400 instrument (USA Bruker Instruments, Inc., Mountain View, CA), and the pH of deuterium oxide solutions of samples was adjusted prior to measurements using a pH meter. The values of pH reported are those read on the meter. NMR signals were reported in δ-values (ppm) using reference standards of internal 1,4-dioxane (^{13}C) and an external 85% phosphoric acid (^{31}P) in D_2O. UV spectroscopy was done using a U-3210 Hitachi Spectrophotometer.

High-performance liquid chromatography (HPLC) with ultraviolet (UV) or electrochemical (EC) detection was done using the method of Liao and Seib[2] and Wang et al.[14] Elemental assay was done by Galbraith Laboratories, Inc. (Knoxville, Tenn).

Fast-atom bombardment mass spectroscopy (FAB-MS) and FAB-Collision Induced Dissociation (CID)-MS were run on a ZAB HS mass spectrometer (VG Analytical Ltd, Manchester, UK) equipped with a 11/250 data system. FAB-MS experiments were performed

using a xenon gun operated at 8 Kev energy and 0.8 mA emissions. CID spectra were generated using the technique of linked-scan at constant B/E with precursor ions attenuated 60% with helium in the first field-free region gas cell. CID spectra were recorded in the multichannel analyzer mode (MCA) under control of the data system. The scan range was 800-10 amu; three scans were acquired at 20 s/decade. Aqueous solutions of the ammonium salts of L-ascorbate phosphates were added to glycerol, which served as the matrix on the sample probe. The ammonium salts of L-ascorbate phosphates were generated using a cation exchange resin (H^+) followed by neutralization of a column effluent with ammonium hydroxide.

Reference Standards for HPLC-UV. Pentacyclohexylammonium L-Ascorbate 2-Triphosphate (3). Compound **3** was prepared by reaction of L-ascorbate (30 g) with sodium trimetaphosphate (71.5 g) while pH 10.5-10.7 and temperature 35 °C were maintained over a 24-h period.[2] The reaction mixture was made to volume (250 mL) with water, so that the diluted mixture contained ~0.013 M inorganic phosphate in orthophosphate equivalents, 0.038 M **3**, and a trace of other phosphate esters of L-ascorbate. An aliquot (25 mL) of the diluted mixture, which contained ~15.3 mmole **3**, was applied to a column (45x5 cm) of anion exchange resin in the bicarbonate form. The column was developed stepwise with 0.40 M (5 L) ammonium bicarbonate followed by 0.42 M bicarbonate (8 L). The flow rate was 1.0 mL/min, and fractions (15 mL each) were collected and monitored by UV at 258 nm. Fraction Nos. 300 to 530, which contained pure **3**, identified by HPLC-UV, were combined and concentrated. Water (500 mL) was added several times, and the solution reconcentrated to remove ammonium bicarbonate. The last traces of ammonium bicarbonate were removed by addition of strongly acidic cation exchange resin in the H^+-form to reach pH 2. After filtration, the filtrate was concentrated to a volume of ~ 50 mL and adjusted to pH 9.0 with freshly distilled cyclohexylamine, and absolute ethanol (50 mL) was added. The mixture was concentrated to 20 mL, more (20 mL) ethanol added, and the mixture concentrated again to a small volume (~15 mL).

The concentrate was kept overnight at 4 °C, which induced crystallization of pentacyclohexylammonium L-ascorbate 2-triphosphate (**3**). Recrystallization from 95% ethanol produced needles (6.6 g, 52.3% yield) with mp 146-148 °C.

Anal. Calcd for $C_{36}H_{76}N_5O_{15}P_3$: C, 47.42; H, 8.34; N, 7.68; P, 10.21. Found: C, 47.16; H, 8.25; N, 7.72; P, 10.28.

Tetracyclohexylammonium L-Ascorbate 2-Diphosphate (2). A second aliquot (25 mL) of the diluted reaction mixture was adjusted to pH 2-3 using 4 M hydrochloric acid (10 mL). The solution was allowed to enter the bed of a charcoal column (40x2.5 cm), which had been washed previously with 4 M hydrochloric acid (600 mL), and the loaded bed allowed to stand for 2.5-3 h at 25 °C. The 2-phosphorylated L-ascorbate derivatives then were eluted using 0.2 M sodium hydroxide at a rate of 0.8 mL/min. The column effluent was monitored for UV absorbance at 250 nm, and the active fractions were examined by HPLC-UV. The first material (Fraction A) eluted was pure **3**; the second (Fraction B), a mixture of **3**, **2**, and **1**; and the third (Fraction C), pure **1**.

During elution of Fraction B, the effluent was adjusted continuously to pH 6-7 by addition of magnesium oxide. Then, the slurry (~5000 mL) was concentrated to 50 mL, and insoluble magnesium phosphate removed by filtration. An aliquot of the filtrate was assayed by HPLC-UV and found to contain **3/2/1** in a molar ratio of approximately 1/1.3/1.

The filtrate was loaded onto a strongly basic anion exchange column (45x5 cm, bicarbonate form), the column developed with aqueous ammonium bicarbonate solution (0.40 and 0.42 M), and fractions (10 mL) were collected and monitored as described above. These fractions containing **2**, which had been eluted with two bed volumes of 0.42 M ammonium bicarbonate, were combined and concentrated to approximately 100 mL. Ammonium bicarbonate in the concentrate was removed as described above; the final concentrate was dissolved in water and the solution passed through a strongly acidic, cation exchange resin (40x2 cm, H^+ form). The column effluent was adjusted to pH 9 with cyclohexylamine, and the mixture

concentrated to a small volume (5 mL). After adding ethanol (10 mL) and evaporating to a syrup (~2 mL), absolute ethanol (21 mL) was added, and the mixture kept overnight at 4 °C. The crystals of tetracyclohexylammonium L-ascorbate 2-diphosphate (**2**) were collected and recrystallized from 95% ethanol to give needles (~1.5 g, 20% yield based on **0**) with mp 148-150 °C.

Anal. Calcd for $C_{30}H_{62}N_4O_{12}P_2 \cdot \frac{1}{2}H_2O$: C, 48.58;H, 8.50; N, 7.56; P, 8.37. Found: C, 48.50; H, 8.34; N, 7.53; P, 8.47.

Tricyclohexylammonium L-Ascorbate 2-Monophosphate (1). The magnesium salt of **1** (3 g) was dissolved in water (50 mL), and the solution passed through a strongly acidic cation exchange column (45x5 cm, H^+ form). The column effluent was adjusted to pH 9.5 by addition of cyclohexylamine, and the mixture concentrated to a syrup. Addition of absolute ethanol (10 mL) followed by holding overnight at 4 °C gave crystalline tricyclohexylammonium L-ascorbate 2-monophosphate (**1**). The material was recrystallized from 95% ethanol to give needles (3 g, 73% yield) with mp 174-176 °C. Lit[15] mp 182 °C (dec).

Anal. Calcd for $C_{24}H_{48}N_3O_9P$: C, 52.08; H, 8.68; N, 7.59; P, 5.61. Found: C, 51.97; H, 8.50; N, 7.56; P, 5.63.

Barium 2,2'-Bis-(L-ascorbyl) Phosphate (4). The barium salt of **4** was made by the method of Lee et al:[15] mp 250 °C(dec); Lit., mp 250 °C (dec).

Anal. Calcd for $C_{12}H_{12}O_{14}PBa_{1.5}$: C, 23.35; H, 1.96; P, 5.02. Found: C, 23.45; H, 2.04; P, 4.70.

(2Z,4Z)-2,3,4,6-Tetrahydroxy-2,4-hexadienoate-1,4-lactone 6-Phosphate 2-Triphosphate (5). The ammonium salt of **5** was isolated in amorphous form as described by Liao and Seib.[2]

Response curves were established for the HPLC-UV assay using standard solutions of the purified cyclohexylammonium salts of **1**, **2**, and **3**. The response curves, based on peak areas, were linear over the range of 0.02-0.20 mM with injection of 20 μL. The relative slopes of the response curves were 0.70/0.96/1.0 for **3**/**2**/**1**.

Reaction of L-Ascorbate with Sodium Trimetaphosphate (STMP) at Alkaline pH in the Presence of Calcium Hydroxide and Chloride. A 100 mL beaker was fitted with a magnetic stirring bar and a pH electrode. To the beaker, which was placed in a

water bath at 25 °C, was added water (12 mL), **0** (3 g, 17.0 mmole, initially 1.42 M), calcium chloride dihydrate (0.6 g, 4.1 mmole), and a suspension of calcium hydroxide (1.5 g, 20.3 mmole) in water (3 mL). The starting mixture had pH ~9.5. STMP (7.2 g, 22.4 mmole) was added, and the pH maintained at 9.5-10.0 by periodic addition of 20% (w/v) calcium hydroxide in water. The reaction was stopped after 20 min, at which time the amount of calcium hydroxide added was 0.3 g (4.1 mmole). The reaction mixture (~30 mL), which contained insoluble phosphate salts, was transferred quantitatively to a volumetric flask, and made to volume (100 mL) with distilled water. While the mixture was stirred vigorously, an aliquot (1.0 mL) was removed and made to volume (250 mL) with 0.1 N hydrochloric acid to give a clear solution. An aliquot (1.0 mL) of the clear solution then was made to volume (25 mL) with 0.1 M carbonate buffer (pH 10), and its absorbance was determined at 259 nm. The absorbance was 0.432, which indicated[2] 99% 2-phosphorylation using a molar extinction of 16 $Lmmol^{-1}cm^{-1}$ for 2-phosphate esters of **0** at pH 10. The UV absorbance at 313 nm was 0.01, indicating that only a trace of the 4,5-ene by-product was present. HPLC-UV assay of the clear solution using the response curves of the reference standards showed that the reaction mixture contained 14.7% of **1**, 15.9% **2**, 61.1% **3**, ~0.5% **4**, and 6.7% of other phosphorylated derivatives of **0**, assuming the phosphorylated derivatives of **0** had the same UV response as **3**. Only a trace of **5** was observed in the chromatogram. An aliquot (20 mL) of the first diluted reaction mixture reduced 1.65 mL of 0.05 N iodine, indicating[15] 1.2% of unreacted **0** in the reaction mixture.

Phosphorylation of 5,6-*O*-isopropylidene-L-ascorbate (IsAsA) and D-erythorbic acid (EA) with STMP were done using the method described above, except that L-ascorbate was replaced by an equal molar amount (17.0 mmole) of IsAsA (3.69 g) or of EA (3.0 g).

Reaction of L-Ascorbate with Sodium Hexametaphosphate (SHMP) at Alkaline pH in the Presence of Calcium Ion. The

phosphorylation of L-ascorbate was done at alkaline pH in the presence of calcium ion using the method described above, except that SHMP replaced STMP at one-half the molar level of STMP.

Reaction of L-Ascorbate with Sodium Trimetaphosphate (STMP) at Alkaline pH in the Presence of Strontium or Barium Ion. The phosphorylation of L-ascorbate (3 g) was done with STMP at alkaline pH using the method described above, except that sodium hydroxide (10 N) was used to maintain pH 9.5-10.0, and strontium or barium chloride (1.50 g $SrCl_2$ or 1.96 g $BaCl_2$) replaced calcium ion. The molar ratio of Sr or Ba ion to P was 0.42. The reaction mixture (~30 mL) was analyzed for unreacted **0** and for its 2-phosphorylation yield using the methods described above.

Ion-Exchange Chromatography of the Reaction Mixture from the Calcium-Catalyzed Phosphorylation of L-Ascorbate with STMP. L-Ascorbate (3 g) was reacted as previously described with 1.3 equivalents of STMP in the presence of calcium ion at 25 °C and pH 9.5-10.0 for 20 min. The reaction mixture (~30 mL) was diluted to 100 mL with distilled water, and the mixture centrifuged for 10 min at 1700xg to remove insoluble calcium phosphate salts. The insoluble salts were washed with water (50 mL), and the supernatants combined. Ammonium bicarbonate (5 g) was added, and the mixture was adjusted to pH 9.5 with 1 M sodium hydroxide. After being stirred for 5 min at 25 °C, the mixture was centrifuged to remove insoluble calcium carbonate. The sediment was washed with water (4x50 mL) to recover occluded organophosphates, and the washings and supernatants were combined and evaporated to ~50 mL. Water (300 mL) was added to the concentrate, the mixture concentrated to 50 mL, and the procedure repeated until the concentrate reached pH ~8. The concentrate then was applied to a column (45x5 cm) of anion exchange resin in the bicarbonate form. The column was developed with 0.4-0.8 M ammonium bicarbonate at a flow rate of 1.0 mL/min, and fractions (15 mL each) were monitored for UV absorbance at 258 nm. The UV-active fractions were examined by HPLC-UV to monitor the L-ascorbate derivatives.

Inorganic phosphate salts were eluted from the column with 0.40 M ammonium bicarbonate (~2.5 L), followed by UV-active Fractions I, II, and III with 0.42 M ammonium bicarbonate[2] (~17 L). Fractions I, II, and III were found by HPLC-UV to be pure **1**, **2**, and **3**, respectively. Higher phosphate esters of **0** were present in Fractions IV, V, VI, VII, and VIII, which were eluted, respectively, with approximately 1.5 L each of 0.45 M, 0.50 M, 0.55 M, 0.65 M, and 0.80 M ammonium bicarbonate. The last fraction, IX, which was pure *bis*-phosphate ester (**4**), was eluted with 0.80 M ammonium bicarbonate. The UV activities of Fractions I, II, III, and IX showed that **1**, **2**, **3**, and **4** accounted for 14.1%, 16.9%, 57.3%, and 0.6%, respectively, of the reacted L-ascorbate.

Compounds in Fractions IV through VIII were purified and isolated. After their structures were established by NMR and FAB-MS, the levels of the compounds in the fractions were determined in L-ascorbic acid equivalents using HPLC-EC after phosphatase digestion.[14] Fractions IV, V, and VII from the anion-exchange column were found to be impure as indicated by HPLC-UV. Fraction IV was subjected to a second ion-exchange, chromatographic purification using a column (29x4 cm) of the strongly basic resin in the bicarbonate form. The column was developed with a linear gradient of 0.42 to 0.50 M ammonium bicarbonate (500 mL each), followed by 0.50 M ammonium bicarbonate (350 mL), and finally by 0.55 M ammonium bicarbonate (650 mL); 7 mL fractions were collected. The fractions containing pure compound **6** were combined, and the solution (~100 mL) treated as described previously to remove ammonium bicarbonate. The final concentrate (~5 mL) had pH 6. Insignificant amounts of other compounds were found in the other fractions obtained by chromatographing Fraction IV.

Fractions V and VII were subjected to anion exchange chromatography (column 29x4 cm) using the procedure described for Fraction IV, except the developing solvents were linear gradients of 0.75 to 0.80 M ammonium bicarbonate (500 mL each) for Fraction V and of 0.45 to 0.55 M ammonium bicarbonate (500 mL each) followed by 0.55 M ammonium bicarbonate (1000 mL) for

Fraction VII. Solutions (~5 mL each) of pure compounds **6** and **8** were obtained from Fraction V and of compounds **5** and **9**, from Fraction VII.

Fractions VI and VIII obtained from the anion-exchange column contained pure compounds **7** and **10**, respectively, as indicated by HPLC-UV. Fractions VI and VIII were concentrated, water was added, and they were reconcentrated to neutral pH. The solutions (~5 mL) of compounds **7** and **10** were kept at 4 °C until structure analysis and quantitation.

Reaction of STMP with L-Ascorbate in the Presence of Calcium Hydroxide and Isolation of Calcium/Sodium L-Ascorbate 2-Polyphosphate. Phosphorylation of **0** (3 g) with STMP was done in the presence of calcium hydroxide as described above, except no calcium chloride was added and the pH of the reaction was maintained at 10.5-11.0. After the 20-min reaction, the total amount of calcium hydroxide used in the reaction amounted to a Ca/P ratio of 0.42. The reaction mixture (about 30 mL) was diluted to about 200 mL, and calcium chloride dihydrate (6.6 g) was added. After the pH was adjusted to 4.5 using 8 M hydrochloric acid, the mixture was stirred 5 minutes at 25 °C and centrifuged at 1700xg to remove insoluble calcium phosphate. Water (200 mL) was added to the sedimented calcium phosphate, and the pH adjusted to 4.5 using 8 M hydrochloric acid. After being stirred for 5 min at 25 °C, the mixture was centrifuged, the supernatant saved, and the washing procedure repeated 4 times. The supernatant solutions were combined and concentrated to a volume of ~90 mL, the concentrate was filtered through Whatman filter paper No. 5, and the filtrate adjusted to pH 7 with 1 M sodium hydroxide. Ethanol (150 mL) was added with vigorous stirring, and the precipitated product collected by centrifugation. The sediment was washed with 50% ethanol (usually 4x120 mL) until the washings were free of chloride ion. Finally, the sediment was suspended in absolute ethanol (150 mL), collected by filtration, and dried in a vacuum desiccator over phosphorus pentoxide. The product contained about 5% moisuture as determined by weight loss and 1.5% sodium and 16.9% calcium on a dry-weight basis as determined by atomic absorption.

Phosphatase digestion[14] of the powdery Ca/Na L-ascorbate 2-polyphosphate followed by HPLC-EC showed that the product contained 33% by weight of L-ascorbic acid, which accounted for 88% of the original **0** used in its preparation. HPLC-UV of the Ca/Na L-ascorbate 2-polyphosphate using standard curves derived from reference standards showed the following yields of individual components; **1** (17.8% mole %, based on starting **0**), **2** (18.5%), and **3** (41.9%). The product also contained the *bis* Phosphate **4** plus higher phosphate esters of 2-phosphorylated L-ascorbate (8.3%), which were quantitated by the difference between total 2-phosphorylation determined from UV spectroscopy and the levels of **1**, **2**, and **3** determined by HPLC-UV. Unreacted **0** was not detected in the solid Ca/Na L-ascorbate 2-phosphate esters.

L-Ascorbate 2-Monophosphate (1) Prepared by Reaction of 0 with Alkaline Sodium Trimetaphosphate in the Presence of Calcium Ion. L-Ascorbic acid (1 g) was phosphorylated using STMP (1.3 equivalents) in the presence of calcium hydroxide and chloride at pH 9.5-10.0 as described above. The reaction mixture (~15 mL) was diluted with water to 80 mL, and 33% aqueous calcium chloride (3 mL total, 1 g solids) was added dropwise over 30 min. The mixture was stirred at 25 °C for 2 h, while pH was maintained at 10.5-11.0 by addition of a suspension of calcium hydroxide in water (0.3 g/1 mL). After 2 h, the milky-appearing reaction mixture (Mixture A) was measured for total volume (about 112 mL) using a graduated cylinder, and an aliquot (1 mL) of Mixture A was dissolved in 0.1 M HCl (100 mL). After an additional 10-fold dilution with 0.1 M potassium carbonate buffer at pH 10, the mixture had UV absorbance of 0.803 at λ_{max} 262 nm, indicating 99% of phosphorylation of **0** using ε_{mM} 16 $Lmmol^{-1}cm^{-1}$. A second aliquot (0.1 mL) of Mixture A was diluted with water (50 mL) and assayed by HPLC-UV. The chromatogram showed that **1** and **2** were the principal components in the mixture, which also contained traces of **0** and higher phosphate esters. The yield of **1** plus **2** was 77 %, and the molar ratio of **1/2** was 2.3/1.0.

To isolate the calcium/sodium salt of mainly **1** plus **2**, Mixture A (about 110 mL) was diluted with water to 200 mL and

adjusted to pH 4.5 with 8 M HCl. Calcium chloride (0.75 g) was added to the mixture so that the overall addition of calcium amounted to a P/Ca molar ratio at 1:1. The mixture was centrifuged for 15 min at 1700x*g*, and the supernatant was collected. The sediment was water-washed (4x100 mL) and centrifuged at 1700x*g*; the supernatants were combined and evaporated to ~30 mL. The concentrate was filtered through Whatman No. 4 filter paper, and the filtrate adjusted to pH 7 using 1 M NaOH. The calcium/sodium salts of mixture **1** plus **2** were precipitated, washed free of NaCl, and dried under vacuum as described previously.

The yield was 2.2 g of powdery solid containing 5.0% moisture. A small portion (43 mg) of the dry powder was dissolved in 0.1 M HCl, and an aliquot (0.5 mL) of the solution diluted 20-fold with 0.1 M potassium carbonate buffer at pH 10. UV absorbance of the solution at 262 nm was 0.69, indicating that the combined yield of calcium/sodium **1** plus **2** (HPLC-UV showed a 2.74:1 molar ratio of **1**:**2**) plus traces of higher phosphate esters of L-ascorbate was 78% based on starting **0**. Phosphatase digestion of the product followed by HPLC-EC assay for total **0** showed 80% yield of phosphorylated derivatives based on starting **0**. UV spectroscopy absorbance and the molar ratio of **1** and **2** by HPLC-UV showed that ~67% of the weight of the dry solid was accounted for by **1** plus **2** plus other higher phosphate esters of L-ascorbate, of which ~62% was accounted for by **1** plus **2**. The molar ratio of Ca/Na in the aqueous solid was about 12:1. The calcium phosphate removed from the reaction mixture was dissolved in 0.1 M HCl, and the solution combined with the aqueous alcoholic washings of the calcium salts of **1** and **2**. Assay by UV spectroscopy showed that losses during those two steps totaled 12-15% of starting **0**.

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REFERENCES

1. P. A. Seib and M. L. Liao, U.S. Patent 4 647 672, 1987.
2. M. L. Liao and P. A. Seib, *J. Agric. Food Chem.*, **38**, 355 (1990).
3. B. F. Grant, P. A. Seib, and M. L. Liao, *J. World Aquac. Soc.*, **20**, 143 (1989).
4. L. J. Machlin, F. Garcia, W. Kuenzig, and M. Brin, *Am. J. Clin. Nutr.*, **32**, 325 (1979).
5. J. M. Lowenstein, *Biochem. J.*, **65**, 40P (1957).
6. J. M. Lowenstein, *Biochim. Biophys. Acta*, **28**, 206 (1958).
7. J. M. Lowenstein, *Biochem. J.*, **70**, 222 (1958).
8. J. M. Lowenstein, *Nature*, **187**, 570 (1960).
9. D. G. Gorenstein, *J. Am. Chem. Soc.*, **97**, 898 (1975).
10. D. G. Gorenstein, in *Phosphorous-31 NMR, Principles and Applications*, D. G. Gorenstein, Ed; Academic Press: New York, 1984, p 7.
11. M. L. Liao, X. Y. Wang, C. Chung, Y. T. Liang, and P. A. Seib, *Carbohydr. Res.*, **176**, 73 (1988).
12. M. Tetas and J. M. Lowenstein, *Biochem.*, **2**, 350 (1963).
13. J. Green, *Ind. Eng. Chem.*, **42**, 1542 (1950).
14. X. Y. Wang, M. L. Liao, T. H. Hung, and P. A. Seib, *J. Assoc. Off. Anal. Chem.*, **71**, 1158 (1988).
15. C. H. Lee, P. A. Seib, Y. T. Liang, R. C. Hoseney, and C. W. Deyoe, *Carbohydr. Res.*, **67**, 127 (1978).
16. H. Yang and P. A. Seib, *J. Carbohydr. Chem.*, **11**, 485 (1992).
17. J. V. Paukstelis, D. D. Mueller, P. A. Seib, and D. W. Lillard, Jr., in *Ascorbic Acid: Chemistry, Metabolism, and Uses*, P. A. Seib and B. M. Tolbert, Eds; American Chemical Society: Washington, D. C., 1982, p 125.
18. C. H. Lee, Ph.D. Dissertation, Kansas State University, 1976.