[Chem. Pharm. Bull. 17(2) 387—393 (1969)]

UDC 615.356.011.5 : 577.164.2

Studies on L-Ascorbic Acid Derivatives. III.[1] Bis(L-ascorbic acid-3,3′)phosphate and L-Ascorbic Acid 2-Phosphate[2]

Hiroaki Nomura, Toshihiro Ishiguro and Shirō Morimoto

Research and Development Division, Takeda Chemical Industries, Ltd.[3]

(Received September 2, 1968)

Phosphorylation of 5,6-isopropylidene-L-ascorbic acid (I) in acetone with phosphoryl chloride gave, in addition to L-ascorbic acid 3-phosphate (II) and 3-pyrophosphate (III), the following two derivatives, *i.e.*, bis(L-ascorbic acid-3,3′)phosphate (IV) and L-ascorbic acid 2-phosphate (V). The structure of these phosphates was determined on the basis of elemental analysis, potentiometric titration, NMR-analysis, supplemented by studies of the hydrolysis.

IV was hydrolyzed by acid and alkali most readily among the four isomers. By being passed through a column of Dowex-1-chloride with 1N hydrochloric acid, this compound quantitatively afforded equimolar amount of II and L-ascorbic acid. On the other hand, during the hydrolysis of V in 0.5N hydrochloric acid at 100°, the phosphoryl migration to II was ob served. On the alkali treatment, V was shown to be stable and no migration was detected.

In the preceding paper,[1] the preparation of L-ascorbic acid 3-phosphate (II) and 3-pyrophosphate (III) was reported. This report describes the synthesis and some chemical properties of bis(L-ascorbic acid-3,3′)phosphate (IV) and L-ascorbic acid 2-phosphate (V), which were obtained besides II and III by the phosphorylation of 5,6-isopropylidene-L-ascorbic acid (I) with phosphorus oxychloride in acetone.

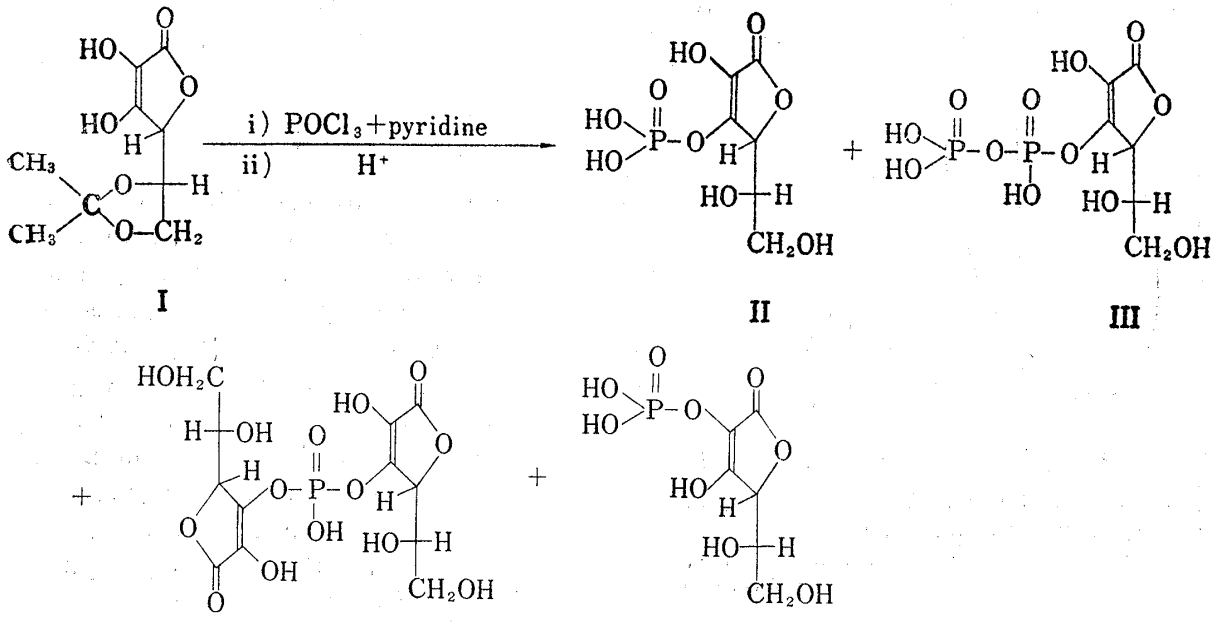


Chart 1

1) Part II: H. Nomura, T. Ishiguro and S. Morimoto, *Chem. Pharm. Bull.* (Tokyo), **17**, 381 (1969).
2) Presented at the 88th Annual Meeting of the Pharmaceutical Society of Japan, Tokyo, April 1968.
3) Location: *Juso, Higashiyodogawa-ku, Osaka.*

A mixture of the phosphorylation products was chromatographed on Dowex-1-bicarbonate using a solution of 0.7M sodium bicarbonate. The elution diagram is shown in Fig. 1. It is necessary to reduce the time for the elution of these phosphates, because the compound in peak III[4] is not stable and prone to decomposition during the chromatographic separation. When the elution was carried out over a period of three to four days the peak III has disappeared almost completely. The phosphate, isolated from the fractions making up peak III, was indicated to be a bis(L-ascorbic acid-3,3′)phosphate (IV) by the elemental analysis and the potentiometric titration.

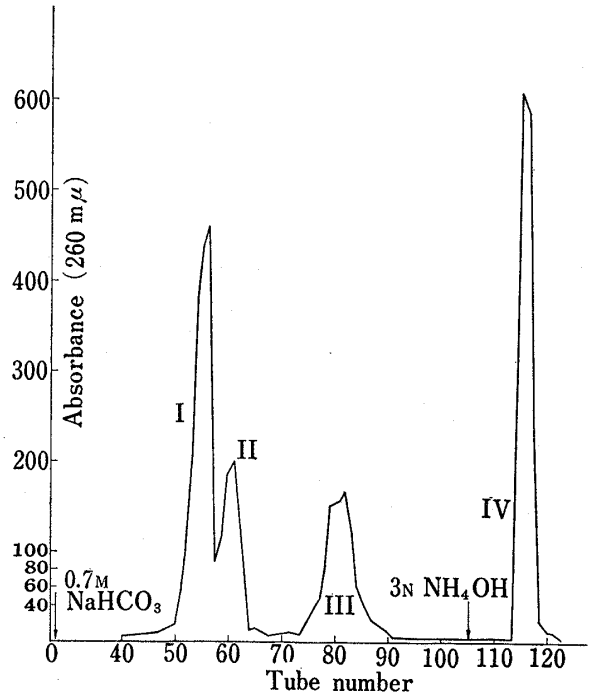


Fig. 1. Ion-exchange Column Chromatogram of the Phosphorylated Product of L-Ascorbic Acid

exchanger: Dowex-1×8-chloride (200—400 mesh, 4×20 cm)
elution buffer: I, 0.7M sodium bicarbonate (initial); II, 3N ammonia (beginning with tube no. 105)
Sorbed material, 10 g of the mixed phosphate.

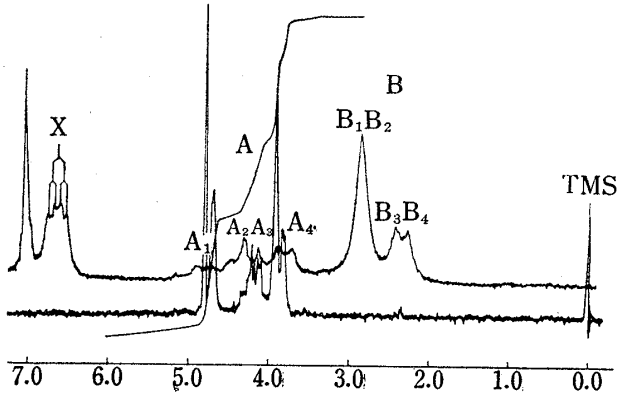
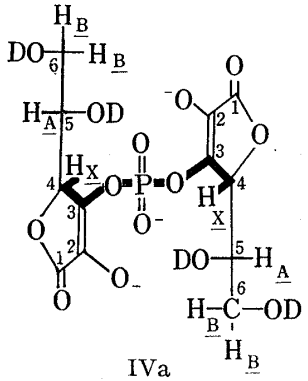


Fig. 2. Nuclear Magnetic Resonance Spectrum of bis(L-ascorbic acid-3,3′)phosphate

The NMR spectra were recorded at a frequency of 60M Hz on Varian A-60 spectrometer with external reference (TMS) using in D_2O solution.

In the NMR spectrum, as shown in Fig. 2, the four protons of the phosphate (IVa) (TMS as external reference) constitute an AB_2X system. Careful inspection and calculation[5] of the pattern reveal that the chemical shifts of the protons and the coupling constants between them are as follows: δ_A=4.24 ppm (1H), δ_B=3.92 ppm (2H); J_{AB}=6.25 cps,



IVa

Chart 2

δ_X=4.83 ppm; J_{AX}=1.25 cps. Resonance signals for the proton attached to C_4 was further complicated by a long range coupling to phosphorus and appeared as a quartet ($^4J_{XP}$=0.95 cps). This suggests that the phosphoryl group is attached to the C_3 hydroxyl group of ascorbic acid, because similar examples of four bond long range coupling of phosphorus to proton have been noted with other organophosphorus compounds.[6]

4) A relatively large band area of the peak III was observed in Fig. 1 as compared with that of the corresponding peak as described in the preceding paper. This may be due to the difference of the elution conditions as well as the elution period.
5) H.J. Bernstein, J.A. Pople and W.G. Schneider, *Can. J. Chem.*, 35, 65 (1957).
6) J. Herweh, *J. Org. Chem.*, 31, 2422 (1966); C.F. Griffin, R.B. Davison and M. Gordon, *Tetrahedron*, 22, 561 (1966).

When I was treated with phosphorus oxychloride in a molar ratio of 2:1, IV was obtained in relatively high yield.

It appears that the presence of neighboring groups considerably affect the stability of the phosphate. Thus, hydrolysis experiments in this paper (Table II) and Table I in the preceding paper indicate that the both acid and alkaline hydrolyses of IV proceed much faster as compared to the other three isomers. This was in accord with the general rule known for the hydrolysis of phosphodiesters which possess a neighboring hydroxyl group,[7] a typical example being the hydrolysis of glycerol 1-(2-hydroxycyclohexyl phosphate). It must be considered that the hydrolysis of IV in acid or alkali may proceed *via* the intermediate cyclic phosphate (VI, Chart 3), in which the lactone ring is extremely sensitive to hydrolysis.[8] Another possibility may be that Dowex-1-chloride exerts a definite catalytic effect on the hydrolysis (route *a*). As a matter of fact, when the ester was passed through the column with 1 N hydrochloric acid, stoichiometric amount of II and L-ascorbic acid were liberated.

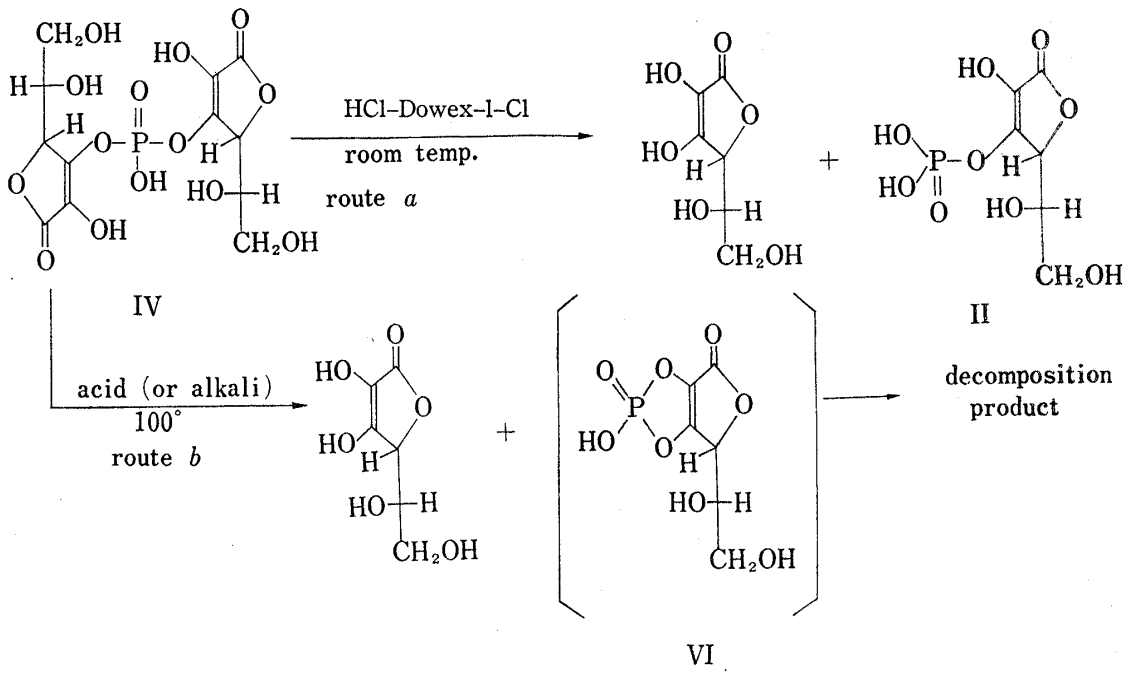


Chart 3

Finally, when the column was eluted with 3 N ammonia the substance corresponding to peak IV was obtained. The compound was isolated as the magnesium salt and did not consume an iodine solution. The elemental analysis as well as the potentiometric titration (Fig. 3) indicated that the compound was L-ascorbic acid monophosphate (V). The reaction of the compound with ferric chloride afforded an intense red color. As described in Table I, the colorations of II, III and IV showed the absorption maximum at 480 mμ, whereas that of V was observed at 460 mμ.

7) D.M. Brown, G.E. Hall and H.M. Higson, *J. Chem. Soc.*, **1958**, 1360; B. Maruo and A.A. Benson, *J. Am. Chem. Soc.*, **79**, 4564 (1957); T. Ukita and K. Nagasawa, *Chem. Pharm. Bull.* (Tokyo), **9**, **544** (1961).

8) The lacton ring of the 2,3-di-substituted ascorbic acid is very sensitive to the nucleophilic attack, for exampfe, 2,3-dimethyl ascorbic acid takes up one equivalent of sodium hydroxide on treatment with alkali in the cold.; W.N. Harworth, E.L. Hirst and F. Smith, *J. Chem. Soc.*, **1934**, 1556.

Table I. Absorption Maxima of the Coloration with Ferric Ion

Compound	Assignment	Maximum absorption[a] (mμ)	$(\varepsilon) \times 10^{-3}$
II	L-ascorbic acid-3-phosphate	480	1.14
III	L-ascorbic acid-3-pyrophosphate	480	1.07
IV	bis(L-ascorbic acid-3,3′)phosphate	480	1.78
V	L-ascorbic acid-2-phosphate	460	0.81

a) procedure: Twenty milligram of the sample, weighed to the nearest 0.1 mg, and 50 mg of ferric chloride ($FeCl_3 \cdot 6H_2O$) were transferred to a 100 ml volumetric flask. The mixture was diluted to the mark with 0.1 N hydrochloric acid. The absorbance of the solution at its maximum absorption was immediately determined.

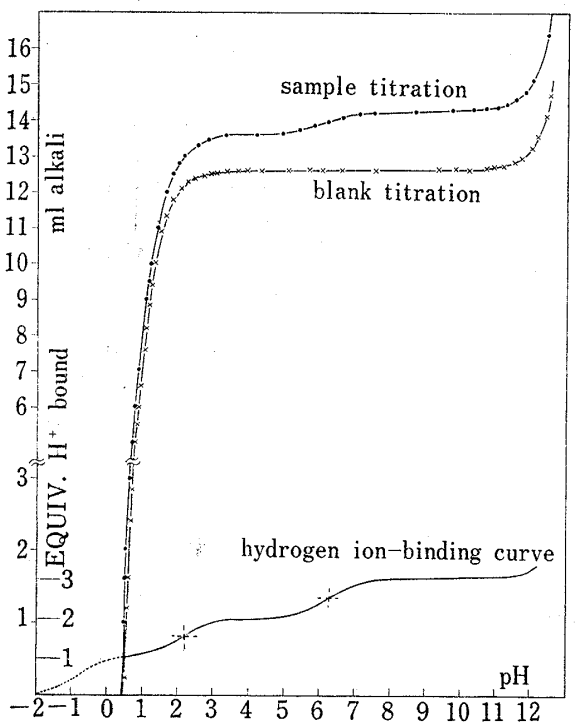


Fig. 3. Titration Curves for L-Ascorbic Acid 2-Phosphate

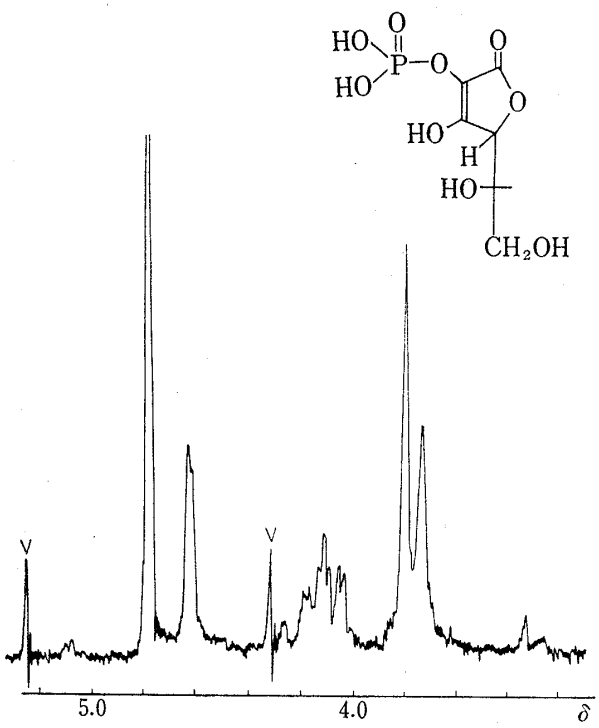


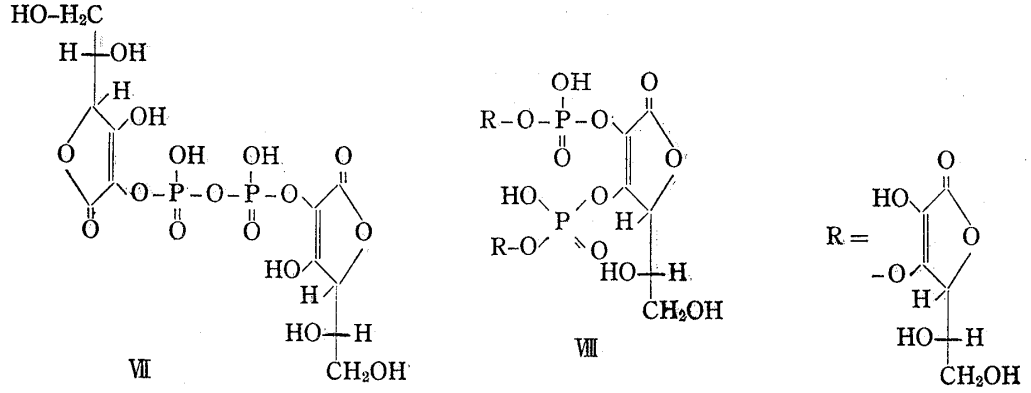
Fig. 4. NMR Spectrum of L-Ascorbic Acid 2-Phosphate in D_2O (60 Mc)

In the NMR spectrum of V (Fig. 4), the resonance signal due to the C_4 proton appeared as a diffused doublet (δ=4.63, J=1.8 cps) which was in contrast with the corresponding signals of II[1] and IV.

Treatment of V with diazomethane, followed by amidation and ozonization, led to oxamic acid and no methyl oxamate was detected on Paper Partition Chromatography. These experiments strongly suggested that V would likely be L-ascorbic acid 2-phosphate.

The hydrolysis studies were then undertaken to obtain further proof to determine the position of the phosphate. As compared with IV, V was relatively stable to acid and alkali as shown in Table II. Upon alkaline treatment, the compound showed a large stability,

which ruled out the possibility of the pyrophosphate structure (VII)[9] as well as the polymeric structure (VIII).[8,10]



While, on acid treatment, V was hydrolyzed to ascorbic acid or isomerized to II as shown in Chart 4. Visual estimation of V on the paper chromatogram after varying periods of the

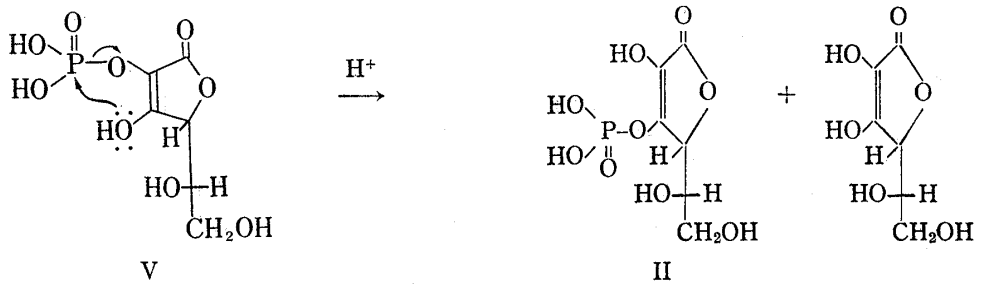


Chart 4

hydrolysis indicated that the phosphoryl group migration[11] to II had occurred within 10 min in 0.5 N hydrochloric acid at 100° (Fig. 6). Additional evidence for the characterization of the isomerized product was obtained by the comparison of the rate of hydrolysis with that of authentic II. The specific rate constant calculated from the values between 10 min and 60 min hydrolysis (Column 2 in Table II) showed 5.91×10^{-4} sec^{-1}, which was in good agreement with that of II.

These facts provided further support for the proposed structure for V.

Experimental

Paper Chromatography (PPC)——Ascending chromatography on Toyoroshi No. 51 paper (2×40) was carried out with solvent systems (A) propanol–water–trichloroacetic acid (15:4:1) (B) propanol–water–ammonia (d=0.88) (6:3:1) and (C) ethanol–water–ammonia (d=0.88) (16:3:1). All samples were applied after being treated with IR-120.

9) H.S. Forrest and A.R. Todd, *J. Chem. Soc.*, **1950**, 3295; J. Baddiley and E.M. Thain, *ibid.*, **1952**, 3783.

10) On paper electrophoresis of the mixture of the phosphorylation products (30 volt/cm for 100 min in 0.06M $Na_2B_4O_7$ buffer, pH 10), the relative mobilities of the four phosphates are as shown in Fig. 5. If compound V was the substance represented by the structure VIII, its relative mobility should be lower than that of compound IV considering from the number of ionizing groups.

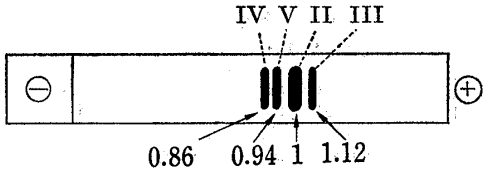


Fig. 5. Electrophoresis of the Mixture of Phosphorylated L-Ascorbic Acids

11) The phosphoryl group migration in sugar chemistry tends to occur in a direction away from the carbonyl group. C.E. Ballou and H.O.L. Fischer, *J. Am. Chem. Soc.*, **76**, 3192 (1954); T. Ukita and K. Nagasawa, *Chem. Pharm. Bull.* (Tokyo), **9**, 544 (1961).

Calcium Salt of bis(L-ascorbic acid-3,3′)phosphate (IV)——Chromatographic separation of the mixed phosphates was carried out under the same conditions as described in Part II, with the exception that 0.7M sodium bicarbonate was used at the beginning of the elution. The combined IV fractions were evaporated *in vacuo*. After being kept in a refrigerator, a precipitate of sodium bicarbonate was filtered. The filtrate was passed through a column of IR-120 (H–form). The effluent was neutralized with magnesium oxide and evaporated to dryness under reduced pressure. The residue was extracted with ethanol and the extract was precipitated by addition of acetone. The precipitate thus obtained was filtered and dissolved in water The resulting solution was freed from magnesium by means of another column of IR–120. The eluate was neutralized with calcium hydroxide and concentrated *in vacuo* to a small amount below 25°. Addition of ethanol to this concentrate yielded a colorless precipitate. 2.5 g. Recrystallization of the precipitate from water by addition of ethanol afforded 1.5 g of a colorless crystalline powder. Homogeneous on PPC: $Rf=0.27$, $R_{As.A}$[12]$=0.55$ in solvent A. $Rf=0.23$ in solvent B. $[\alpha]_D^{22}$ +67.9 ($c=1.0$, H_2O). *Anal.* Calcd. for $C_{12}H_{12}O_{14}P\cdot Ca^{3}/_{2}\cdot 2H_2O$: C, 28.31; H, 3.56; P, 6.08. Found: C, 28.13; H, 3.68; P, 6.24. IR ν_{max}^{KBr} cm^{-1}: 1731 (C=O), 1250 (P=O). UV $\lambda_{max}^{0.1N\ HCl}$ (ε) 235 mμ (1.82×10^4), $\lambda_{max}^{0.1N\ NaOH}$ (ε) 260 mμ (2.79×10^4).

Magnesium Salt of L-Ascorbic Acid 2-Phosphate (V)——The fractions corresponding to Peak IV in Fig. 1 were collected. The solution was treated with IR-120 (H–form) and neutralized with magnesium oxide. The solution was condensed to approx. 20 ml *in vacuo*. After repeated recrystallization from water–ethanol, a colorless crystalline powder (2.2 g) was obtained. PPC; $Rf=0.18$, $R_{As.A}=0.37$ (solvent A). $[\alpha]_D^{22}=+11.5°$ ($c=1.0$, H_2O). *Anal.* Calcd. for $C_6H_7O_9P\ Mg\cdot 4H_2O$: C, 20.55; H, 4.32; P, 8.84; Mg, 6.94. Found: C, 20.74; H, 4.46; P, 8.99; Mg, 6.67. IR ν_{max}^{KBr} cm^{-1}: 1736 (C=O), 1250 (P=O). UV $\lambda_{max}^{0.1N\ HCl}$ (ε) $=235$ mμ (0.87×10^4), $\lambda_{max}^{0.1N\ NaOH}$ (ε) 259 mμ (1.4×10^4).

Table II. Hydrolysis of bis(L-ascorbic acid-3,3′)phosphate (IV) and Ascorbic Acid 2-Phosphate (V) at 100° [a,b]

	0.5N Hydrochloric acid		0.1N Sodium hydroxide	
	IV	V	IV	V
0′	1.00*	1.00	1.00	1.00
10′	0.505	0.563	0.33	0.95
20′	0.052	—	0.17	—
30′	0.026	0.238	0.08	0.94
60′	—	0.104	—	0.91

a) Relative values of the color intensities (absorbances at 480 mμ) are tabulated.
b) Color was developed by the addition of 10 ml ferric chloride reagent (1%) and 10 ml 1N hydrochloric acid to 5 ml of the hydrolysate. After being diluted accurately to 50 ml with water, color intensity of the resulting solution was measured.

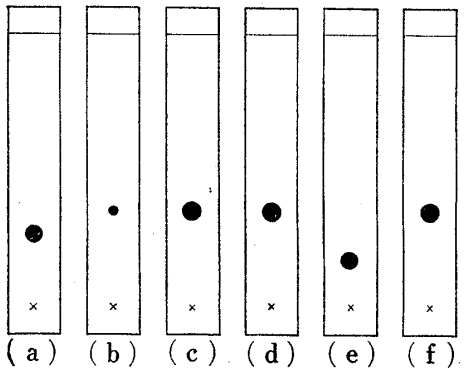


Fig. 6. Paper Chromatogram of the Acid Hydrolysates

(a) bis(L-ascorbic acid-3,3′)phosphate
(b) hydrolysate of bis(L-ascorbic acid-3,3′) phosphate (100°, 10 min in 0.5N HCl)
(c) hydrolysate of bis(L-ascorbic acid-3,3′) phosphate (20°, Dowex-1-Cl, in 1N HCl)
(d) L-ascorbic acid 3-phosphate (standard)
(e) L-ascorbic acid 2-phosphate (standard)
(f) hydrolysate of L-ascorbic acid 2-phosphate (100°, 10 min in 0.5N HCl)

Methylation of L-Ascorbic Acid 2-Phosphate (V) and Subsequent Ammonolysis and Ozonization——1N methylcyclohexylammonium salt of L-ascorbic acid 2-phosphate (0.3 g) was dissolved in 20 ml of methanol and treated with excess ethereal diazomethane under ice–cooling. After being kept overnight in a refrigerator, the solvent was evaporated *in vacuo* to afford 0.4 g of the methyl ether as a pale yellow syrup. No coloration with ferric chloride was observed. The amidation of the methyl ether and the subsequent ozonization were performed similarly as described in the corresponding reactions of II.

After addition of water and subsequent removal of the solvent, the paper chromatographic analysis of the mixture indicated the presence of oxamic acid. [$Rf=0.23$, solvent C, spraying reagent 1% $KMnO_4$–2% Na_2CO_3 in water). On the other hand, the chloroform extraction of the solution and subsequent chromatography of the extract on silica gel was performed, however, no proof for the existence of methyl oxamate was obtained.

Hydrolysis of bis(L-ascorbic acid-3,3′)phosphate (IV) and Ascorbic Acid 2-Phosphate (V)——i) Acid or Alkaline

12) Relative mobility to L-ascorbic acid.

Hydrolysis: The salt (300 mg each) of the phosphate was dissolved in 50 ml of 0.5N hydrochloric acid or 0.1N sodium hydroxide, and the aliquots (8 ml) were sealed in glass ampoules and heated in a boiled water. At the selected intervals, ampoules of the reaction mixture were withdrawn from the bath and cooled. The precise amounts (5 ml) were pipetted into 50 ml volumetric flasks respectively. Analytical procedures for the colorimetric determination of the phosphate was performed according to the method described in Part II. Absorbance at 480 mμ gave a measure of the original ester plus hydrolytic intermediates in which the enediol-monophosphate system are still present.[13] The results are shown in Table II and Fig. 6.

ii) Catalytic Hydrolysis of bis(L-ascorbic acid-3,3′)phosphate (IV) on Dowex-1-chloride: A solution of IV (50 mg as the calcium salt) was placed on the top of a column of Dowex-1-chloride (1.5×15 cm, 200—400 mesh). The column was washed with a small amount of water. Elution with 1N hydrochloric acid was run at a rate of 0.5 ml per minute. The eluate from the column was collected in a volumetric flask up to 200 ml. Paper chromatography indicated that the conversion of the starting material to II was almost quantitative. The colorimetric determination as well as the iodometric analysis showed the production of the stoichiometric amounts of II and ascorbic acid, both of which were liberated from the starting material.

Potentiometric Titration for Ascorbic Acid 2-Phosphate (V)——The titration was performed according to the method reported by Parke.[14] Precisely 20.0 g of the solution,[15] containing 0.27 mM of V (free acid), 4.00 g of 1.5N HCl and 0.743 g of KCl, dissolved in water was weighed in a double-walled beaker, and during the titration, thermostated water (22 ±0.3°) was circulated through a jacket of the beaker. The solution was titrated potentiometrically with 0.5N KOH. A Hitachi-Horiba model M-4 pH-meter was used for pH measurements. In Fig. 3 the curve marked by solid circles is a conventional titration curve with volume of titrant plotted against pH. The curve marked by –x–x– is a blank titration of a solution like the sample solution omitting only the sample itself. When the blank is subtracted volumewise from the sample curve, the resulting curve indicates the presence of three inflections. Since the acidity of the phosphate (V) is so strong that the titration inflection for pK_1 is not observed in the range of reliable pH values, the approximate value for pK_1 can be estimated by producing the theoretical curve below pH 1, as in Fig. 3.

Acknowledgement The authors wish to express their sincere thanks to Dr. S. Tatsuoka, Messrs. J. Manaka and K. Tsuchihashi for their encouragement and to Mr. M. Yasumatsu and Drs. Y. Abe, H. Morimoto and M. Nishikawa for their valuable suggestions throughout the present work. Thanks are also due to Mr. K. Maeda for his technical assistance.

13) This will be discussed in detail in our forthcoming paper.
14) T.V. Parke and W.W. Davis, *Anal. Chem.*, **26**, 642 (1954).
15) μ (ionic strength)=0.8