[Chem. Pharm. Bull. 17(2) 381—386 (1969)] UDC 615.356.011.5 : 577.164.2

Studies on L-Ascorbic Acid Derivatives. II.[1] L-Ascorbic Acid 3-Phosphate and 3-Pyrophosphate[2]

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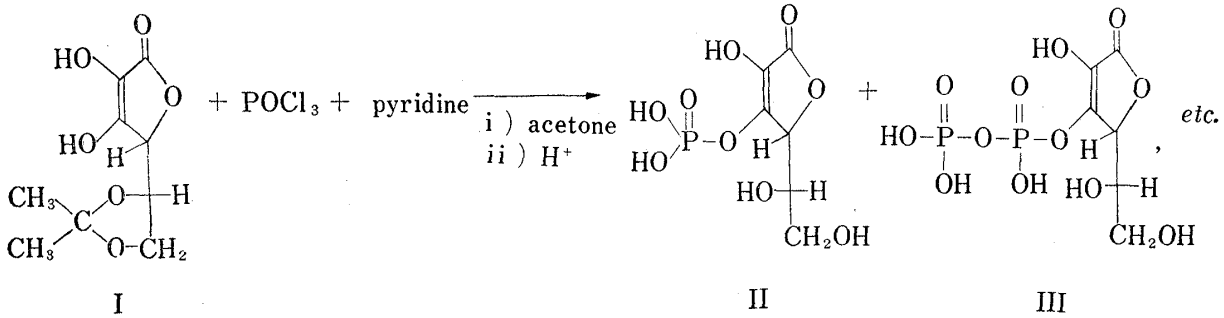
(Received September 2, 1968)

Phosphorylation of 5,6-isopropylidene-L-ascorbic acid with phosphorus oxychloride gave mainly a mixture of four phosphates. Column chromatography on Dowex-1-bicarbonate with a solution of sodium bicarbonate as a developer was found suitable for the separation of these phosphates for the preparative purpose. Consequently, L-ascorbic acid 3-phosphate and 3-pyrophosphate were isolated as the magnesium salts. Treatment of the former with diazomethane, followed by successive amidation and ozonization gave methyl oxamate, and this provided evidence that the starting ester was the 3-phosphate. The latter ester, on treatment with acid or alkali, afforded quantitatively the same product, *i.e.*, L-ascorbic acid 3-phosphate. These facts, together with the elemental analysis and potentiometric analysis, determined the structure of the latter to be L-ascorbic acid 3-pyrophosphate.

In connection with our earlier work on the synthesis of L-ascorbic acid derivatives which are anti-scorbutically active and are stabilized against oxidation, we have attempted the preparation of some phosphorylated ascorbic acids in which the phosphoryl residue is attached to the hydroxyl of the enediol-system. This report deals with the synthesis, characterization and properties of L-ascorbic acid 3-phosphate and 3-pyrophosphate.

Hitherto the syntheses of 2- and 3-phenylphosphoryl esters of 5,6-isopropylidene-L-ascorbic acid[4] and L-ascorbic acid phosphate of unknown structure[5] have been reported.

We have found that the phosphorylation of 5,6-isopropylidene-L-ascorbic acid (I) with an equivalent amount of phosphorus oxychloride and excess of pyridine in acetone yielded a mixture of four L-ascorbic acid phosphates, all of which were made visible on a paper strip of the paper-partition chromatography.[6] Attempts at utilization of several chromatographic conditions developed for nucleotides thus far met with failure for the separation of the phosphorylated ascorbic acids. In general, these esters have a strong affinity with anion-exchange resins and, therefore, require increased concentrations of the competing ion of a salt or acid,



I II III

Chart 1

1) Part I: H. Nomura and K. Sugimoto, *Chem. Pharm. Bull.* (Tokyo), **14**, 1039 (1966).
2) Presented at the 88th Annual Meeting of the Pharmaceutical Society of Japan, Tokyo, April 1968.
3) Location: *Juso, Higashiyodogawa-ku, Osaka.*
4) V.M. Clark, J.W.B. Hersley and D.W. Hutchinson, *Experientia*, **22**, 425 (1966).
5) E. Cutolo and A. Larizza, *Gazz. Chim. Ital.*, **1961**, 964.
6) The positions of the phosphates were detected by spraying with an ethanolic ferric chloride solution.

which may, in the course of the chromatography, hydrolyze the phosphates. A satisfactory separation and isolation of a group of these phosphates could be accomplished using Dowex-1-bicarbonate column and a solution of sodium bicarbonate as the eluant. Fig. 1 shows the elution curve obtained. A phosphate ester isolated from the fractions making up Peak I showed an elemental analysis which fits the formula $C_6H_6O_9PMg3/2 \cdot 5H_2O$, corresponding to the magnesium salt of L-ascorbic acid monophosphate (II). An intense red coloration with ferric chloride and lack of the iodine reducing power of the compound indicate the phosphoryl group being attached to the hydroxyl either on C_2 or C_3 of L-ascorbic acid.

In order to determine the position where the phosphate group is attached, the following degradation reactions were performed as shown in Chart 2.

On methylation of the phosphate (II) with diazomethane, the corresponding methyl ether (IV) was obtained. The compound, by treatment with alcoholic ammonia, gave the corresponding amide (V), which in turn, on ozonolysis, gave colorless needles identical with authentic methyl oxamate (VI) (Fig. 2). The overall yield from the starting phosphate (II) was 39.2%. These facts establish that the starting phosphate (II) is L-ascorbic acid 3-phosphate. This identification was further supported by the NMR analysis. The spectrum

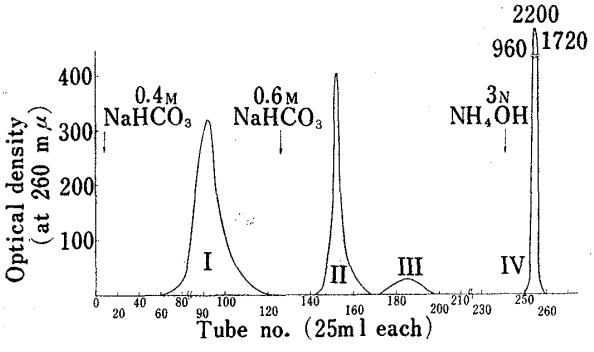


Fig. 1. Ion-Exchange Chromatography of the Phosphorylated Ascorbic Acids

material: 10 g of phosphorylated ascorbic acid
column: Dowex-1×8 bicarbonate (200—400 mesh) 4×25 cm
flow rate: 1.8 ml/min, 25 ml/tube

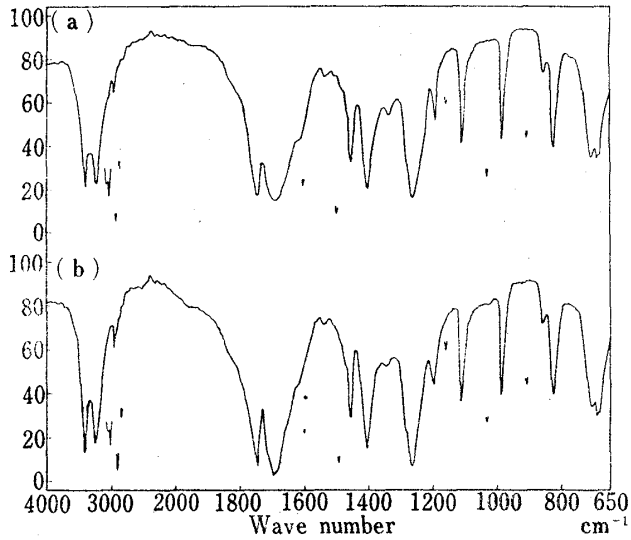


Fig. 2. Infrared Spectra [7] of the Authentic Sample (VI) (a) and Isolated Methyl Oxamate (b) in KBr Disk

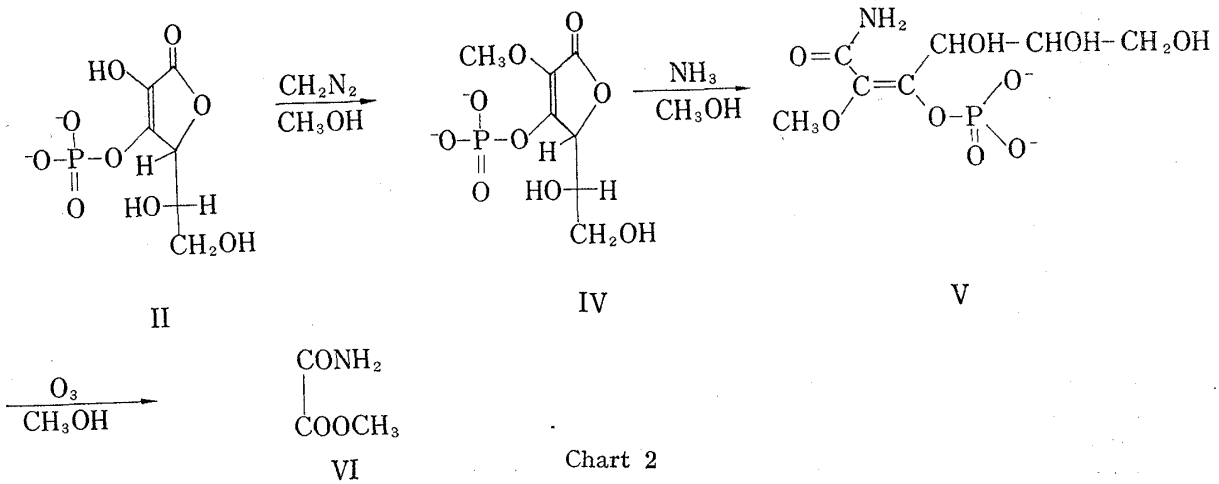


Chart 2

7) The spectra were measured by the infrared spectrophotometer, Hitachi Model EPU-2.

of compound II measured in D_2O showed resonance signals which were interpreted as an AB_2X pattern. Chemical shifts and coupling constants of the individual signals were calculated on the basis of the transition energies described by Pople, *et al.*[8] The signals at 4.16δ (1H, J_{AB}=6.64 and J_{AX}=1.6 cps) and 3.83δ (2H, J_{AB}=6.64 cps) are assigned as the C_5 and C_6 protons, respectively. The signals for C_4 proton appear at 4.64δ as quartet (J_{Ax}=1.6 and $^4J_{PHX}$=0.75 cps). Comparison with other organophosphorus compounds[9] suggests that the coupling constant of 0.75 cps is due to the four bond long range coupling of C_4 proton to the phosphorus, as is the configuration P–O–C–C–H (Chart 3).

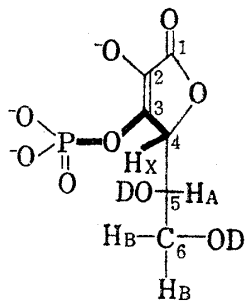


Chart 3

The second phosphate, isolated from the fractions making up Peak II in Fig. 1 was evidently demonstrated by the analysis and potentiometric titration together with the following hydrolysis studies to be L-ascorbic acid 3-pyrophosphate (III). On mild acid hydrolysis (0.5N HCl, 100°, 10 min) III was partially hydrolyzed with the liberation of one mole each of phosphoric acid and II, almost quantitatively. On treatment with 0.1N sodium hydroxide (100°, 30 min), III was similarly hydrolyzed to II quantitatively. These results clearly indicate that the pyrophosphate group is located on the 3 position of L-ascorbic acid.

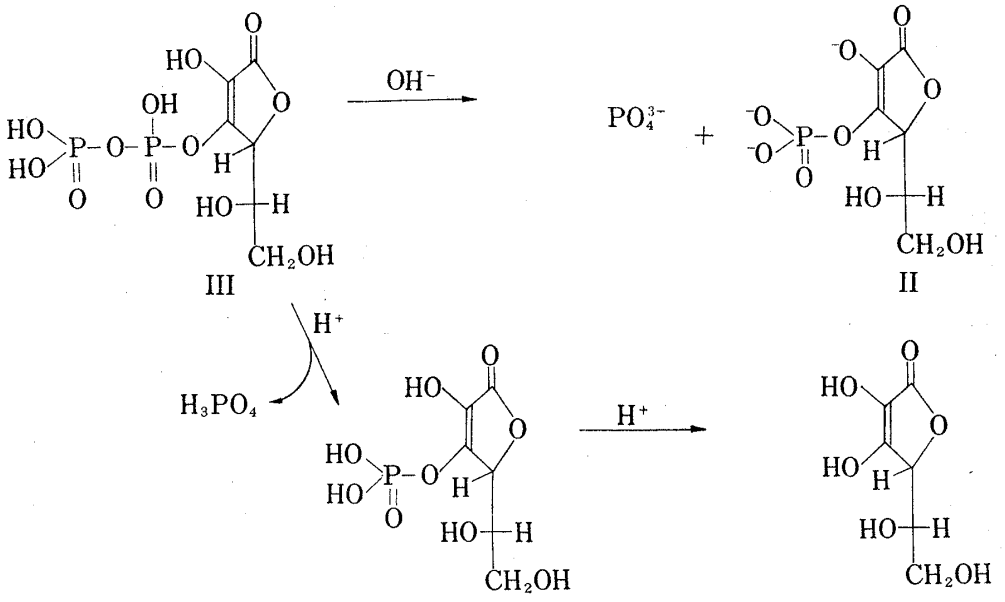
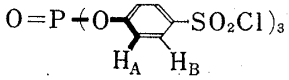


Chart 4

As contrasted with III, L-ascorbic acid 3-phosphate (II) is fairly stable towards acid. When the partial hydrolysis of the mixture of four L-ascorbic acid phosphates was carried out, the yield of the 3-phosphate (II) was improved. The relative stability of each phosphate towards acid can be visualized on the paper chromatograms as shown in Fig. 3. It should be noted that relatively labile phosphates in the mixture are either prone to the phosphate migration or hydrolyzed readily to give the 3-phosphate (II) or L-ascorbic acid. By this

8) J.A. Pople, W.G. Schneider and H.J. Bernstein, "High-resolution Nuclear Magnetic Resonance," McGraw-Hill Book Company, New York, 1959, p. 124.

9) *a*) An analogous four-bond long range coupling has been observed, for example, in *tris*(*p*-chlorosulfonylphenyl)phosphate.
J. Herweh, *J. Org. Chem.*, 31, 2422 (1966).



δ_A 7.58 δ_B 8.18 $^4J_{PH_A}$= 1 cps

b) C.F.Griffin, R.B. Davison and M. Gordon, *Tetrahedron*, 22, 561 (1966).

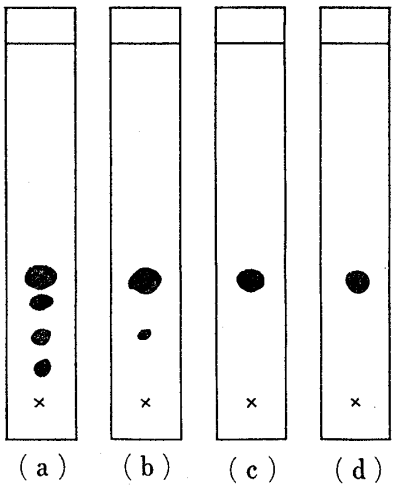


Fig. 3. Paper Chromatograms of the Acid Hydrolysates of the Mixed Phosphorylated Ascorbic Acid

conditions:
0.5_N hydrochloric acid at 100°
(a) starting substance
(b) 10 min
(c) 30 min
(d) authentic II
PPC: solvent A

procedure II can be obtained approximately in 30% yield based on I used. The hydrolysis of the ester (II) is now being studied and will be reported elsewhere. Further studies on the products other than II and III obtained in the phosphorylation of I will be reported in the following paper.

Experimental

Reagents——Ferric Chloride Reagent: One g of ferric chloride ($FeCl_3 \cdot 6H_2O$) was dissolved in distilled water to make 100 ml.

Paper Chromatography——Paper chromatograph (PPC) was carried out with Toyo Roshi No. 51 filter paper (2×40 cm) by the ascending development with solvent systems (A) trichloroacetic acid–PrOH–H_2O (5:75:20), (B) PrOH–H_2O–ammonia (28%) (60:30:10), (C) EtOH-ammonia (28%)–water (85:5:10). All samples of the phosphates were used after being treated with IR-120 (H-form). For the detection of spots an ethanol solution of ferric chloride (0.5%) was employed.

Phosphorylation of 5,6-Isopropylidene-L-ascorbic Acid (I)——To a solution of 5,6-isopropylidene-L-ascorbic acid (10 g, 0.046 mole) and pyridine (18.2 g, 0.23 mole) in acetone (200 ml) containing a small amount of water (0.36 ml) was added dropwise phosphorus oxychloride (7.4 g, 0.048 mole) under ice-cooling. The mixture was stirred for 3 hr at 0°. On removal of the solvent a pale brownish residue remained. This was dissolved in water and the resulting solution was passed through a column of Amberlite IR-120 cation exchanger (H–form, 300 ml). The effluent was neutralized with magnesium oxide, filtered from the solid material and concentrated to 70 ml. The solution was adjusted to pH 4.5 and to this was added ethanol (200 ml) under stirring to give a colorless precipitate of the mixed phosphates; Yield: 12 g. The paper partition chromatography (rolvent A, B) showed the presence of four enol–phosphates of L-ascorbic acid.

Ion-Exchange Chromatography——A solution of the mixed phosphates of L-ascorbic acid (10 g), obtained as described in the preceeding section, was passed through a column of IR-120 (H–form, 3×20 cm). The eluate was concentrated under reduced pressure to a small volume (25 ml), to which was added some amount of Dowex-1-bicarbonate (40 ml). The resulting mixture was continued stirring until carbon dioxide evolution had ceased. The slurry war placed on top of a Dowex-1×8-bicarbonate column (200—400 mesh, 4×25 cm). The column was washed with water (500 ml) and the elution with sodium bicarbonate was begun (zero volume was taken as this point), the flow rate being approx. 1.8 ml/min. The elution with 0.4M sodium bicarbonate (3.1 liters) gave peak I. The elution with 0.6M sodium bicarbonate (2.7 liters) afforded a mixture of peaks II and III. The final elution with 3N ammonia (0.6 liter) gave peak IV. The elution diagram is as shown in Fig. 1.

Preparation of L-Ascorbic Acid 3-Phosphate (II) by Ion-exchange Chromatography——The fractions corresponding to peak I were collected, evaporated *in vacuo* to about 50 ml. Sodium bicaronate was removed by filtration after being left standing at 0° for 1 hr. The filtrate was again evaporated to about 15 ml. This was cooled and filtered. The obtained solution was passed though a column of IR-120 (H–form, 50 ml). After washing the column with water, the combined effluent and washing was neutralized with magnesium oxide and allowed to stand for a day. The solution was filtered and concentrated to 30 ml. Methanol was added slowly to the solution with stirring to give a colorless precipitate (3.2 g). Recrystallization of the precipitate from water–methanol gave 1.95 g of a colorless crystalline powder. mp 300° (decomp.). $[\alpha]_D^{22}$

10) Relative mobility to L-ascorbic acid.

+42.0 (c=1.0, H_2O). *Anal.* Calcd. for $C_6H_6O_9PMg^3/_2 \cdot 5H_2O$: C, 19.0; H, 4.22; P, 8.18; Mg, 9.63. Found: C, 18.88; H, 4.15; P, 8.14; Mg, 9.81. The paper chromatographic analysis indicated a single spot (Rf 0.35, $R_{As.A}$[10]0.71 in solvent A and Rf 0.15 in solvent B). UV $\lambda_{max}^{0.1N\ HCl}$ (ε)=237 mμ (0.98×10^4), $\lambda_{max}^{0.1N\ NaOH}$ (ε)=261 mμ (1.61×10^4). IR ν_{max}^{KBr} cm^{-1}: 3400 (OH, broad), 1726 (C=O), 1605 (C=C), 1410.

Methylation of L-Ascorbic Acid 3-Phosphate (II)——An aqueous solution of magnesium L-ascorbic acid 3-phosphate (1 g) obtained by ion-exchange chromatography was passed through a column of IR-120 (20 ml). The eluate was adjusted with N-methylcyclohexylamine to pH 4.2. The solution was evaporated *in vacuo* to give 1.26 g of a syrup, which was dissolved in 30 ml of methanol and treated with excess of diazomethane at −5° for 18 hr. On removal of the solvent there was obtained 2-O-methyl ether of L-ascorbic acid 3-phosphate as a pale yellow syrup. This substance did neither react with an iodine solution nor give the characteristic coloration with ferric chloride. Yield 1.0 g. IR ν_{max}^{liquid} cm^{-1}: 1785 (C=O).

Ammonolysis of N-Methylcyclohexylammonium-2-O-methyl-L-ascorbic Acid 3-Phosphate (IV) and Subsequent Ozonization——The 2-O-methyl ether (IV) (1.0 g) was dissolved in 50 ml of methanol. Treatment of the solution with dry ammonia at 0° for one hour afforded an amide (V) which was subsequently ozonized by stirring in an atmosphere of ozone at 0° for 1.5 hours. A white precipitate gradually separated. To the reaction mixture was added a small amount of water and the solution was evaporated to 10 ml and diluted with 50 ml of water. The resulting solution was extracted with chloroform (25 ml×5) and the combined extracts was washed with water, dried over Na_2SO_4 and evaporated to give a residue which was chromatographed on SiO_2 (11 g). The elution with ethyl acetate–methanol (8:2) afforded colorless needles (VI). After recrystallization from methanol, 80.3 mg of crystalline methyl oxamate was obtained. mp 115—117°. The mixed melting point with an authentic sample[11] was 116—120°. NMR ($CDCl_3$): 3.90 (–COO$\underline{CH_3}$). IR ν_{max}^{KBr} cm^{-1}: 3400, 3325 (–NH_2), 1745 (–$\underline{C}OOCH_3$), 1692 (–N$\underline{C}OH_2$). The infrared spectra, as shown in Fig. 2, was identical with that of an authentic sample. In the aqueous layer, after the chloroform extraction, no oxamic acid[12] was detected on PPC (solvent C).

Acid Hydrolysis of the Mixed Phosphates and Preparation of L-Ascorbic Acid 3-Phosphate (II)——Magnesium salt of the mixed phosphates (10 g) described above was passed through a column of Amberlite IR-120 cation exchanger (2.7×17 cm). The eluate was concentrated to 20 ml *in vacuo* and to this was added 0.7 ml of 12N HCl. The mixture was heated for 30 min at 70°. Examination of the reaction mixture by paper chromatography (solvent A) showed a single spot (Rf=0.36). In order to remove L-ascorbic acid present, the reaction mixture, after being diluted with 1.2 liters of water, was placed on the top of a column of Dowex-1×8-chloride (2.8 cm diameter×11 cm high). The column was washed with 0.02N hydrochloric acid, and the washing was discarded. The elution with 0.3N HCl gave a fraction (about 0.5 liter) in which L-ascorbic acid 3-phosphate was contained. The solution was adjusted to pH 8 with MgO and allowed to stand for 18 hr. After the precipitate had been removed by filtration the filtrate was concentrated to 20 ml *in vacuo*. EtOH (35 ml) was added to this concentrate, and the resulting precipitate was filtered and dried. Yield 4.5 g. Recrystallization from water-ethanol gave a colorless crystalline powder. Yield 2.5 g. *Anal.* Calcd. for $C_6H_6O_9PMg^3/_2 \cdot 5H_2O$: C, 19.0; H, 4.22; P, 8.18; Mg, 9.63. Found: C, 18.6; H, 4.16; P, 8.35; Mg, 9.77. PPC. Rf 0.34 (solvent A).

Magnesium Salt of L-Ascorbic Acid 3-Pyrophosphate (III)——The pyrophosphate fractions making up Peak II of the ion-exchange chromatography mentioned above were collected and worked up as described for the 3-phosphate. 1.6 g. $[\alpha]_D^{22}$ +24.5° (c=1.0, H_2O). Rf=0.13, $R_{As.A}$0.26 (solvent A). A wine red coloration with ferric chloride. *Anal.* Calcd. for $C_6H_6O_{12}P_2Mg_2 \cdot 8H_2O$: C, 13.74; H, 4.19; P, 11.82. Found: C,

Table I. Hydrolysis of Ascorbic Acid 3-Phosphate (II) and 3-Pyrophosphate(III) at 100° [a]

Time (min)	II		III	
	0.5N HCl	0.1N NaOH	0.5N HCl	0.1N NaOH
0	1.00	1.00	1.00	1.00
10	0.71[b]	1.00	0.95[c]	0.94
20	0.495	—	0.69	—
30	—	0.996	0.49	0.92
60	0.124	0.991	—	0.91

a) Relative value of the color intensities (Absorbances at 480 mμ) are tabulated.
b) the value of the rate constant, K=5.87×10^{-4} sec^{-1} (100°)
c) The PPC of the hydrolysate at this time showed a single spot, the Rf value of which was identical with that of II.

11) P.P.T. Sah and S.L. Chein, *J. Am. Chem. Soc.*, 53, 3901 (1931).
12) An authentic oxamic acid showed a Rf value 0.23 (spray reagent: 1% aq. solution of $KMnO_4$).

13.47; H, 4.06; P, 11.41. IR ν_{max}^{KBr} cm^{-1}: 1730 (C=O), 1610 (C=C), 1245 (P=O), 1115 (–P(=O)–OR). UV $\lambda_{max}^{0.1N\ HCl}$ 235 mμ (0.98×10^4), $\lambda_{max}^{0.1N\ NaOH}$ 259 mμ (1.7×10^4).

Hydrolysis of L-Ascorbic Acid 3-Phosphate (II) and L-Ascorbic Acid 3-Pyrophosphate (III)——a) Acid Hydrolysis: Magnesium salts of the mentioned phosphates (0.10 g) were separately dissolved in 40 ml of of 0.5N hydrochloric acid, and aliquots (7 ml) of the solutions sealed in glass ampoules. These were placed in a boiling water and individuals removed at given periods of time and stored at 0° until analysed. Each solution (5 ml) of the hydrolysates was pipetted into 50 ml volumetric flasks. To each flask, 10 ml of 1% ferric chloride solution was added and the resulting solution was diluted with water to the mark. The absorbance at 480 mμ was determined by means of Hitachi Model EPU-2A spectrophotometer. The relative values are shown in Table I.

b) Alkaline Hydrolysis: Samples of the phosphates (0.15 g) were dissolved in 0.1N sodium hydroxide (40 ml) respectively. The aliquots (about 7 ml) were sealed in glass ampoules (20 ml) and heated in a boiling water bath. At the appropriate intervals, the ampoules were taken out and cooled in ice water. Five ml of the hydrolysate, 10 ml of ferric chloride solution and 0.5N hydrochloric acid were pipetted into a 50 ml volumetric flask, diluted with water to the mark and measured by Hitachi Model EPU-2A spectrophotometer. The results are shown in Table I. After 30 min, almost complete conversion of the pyrophosphate to L-ascorbic acid 3-phosphate was confirmed by the examination with PPC (solvent A).

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