Studies on L-Ascorbic Acid Derivatives. IV.¹⁾ The Ferric Chloride Reaction of L-Ascorbic Acid 3-Phosphate and Its Application for the Colorimetric Determination²⁾

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(Received August 5, 1970)

The wine red complex of iron (III)-L-ascorbic acid 3-phosphate was investigated spectrophotometrically. The color which showed a maximum absorption at 480 m μ was observed in aqueous solution over the acidity range from -2.0 (Ho) up to pH 5. The maximum intensity of the color was approximately at pH 0.9. Addition of an organic solvent prevented the coloration. The composition of the chelate was observed to be 1:1 in the molar ratio, L-ascorbic acid 3-phosphate/iron (III). Similarly, L-ascorbic acid 2-phosphate, 3-pyrophosphate, bis (L-ascorbic acid-3,3') phosphate, L-ascorbic acid 2- and 3-phenylphosphates gave the corresponding iron (III)-chelates. Whereas, L-ascorbic acid 2- and 3-diphenylphosphate and L-ascorbic acid 3-sulfate gave no coloration. As a result of the test, acid stable colorations in the former group of compounds are probably due to the presence of the enolic group which can take part in the chelate formation with the vicinally located primary or secondary phosphoryl group. The coloration was applied to a colorimetric method in the determination of L-ascorbic acid 3-phosphate.

In a previous paper¹⁾ of this series, we have reported the preparation and some chemical properties of L-ascorbic acid 2- and 3-phosphates, L-ascorbic acid 3-pyrophosphate and bis-(L-ascorbic acid-3,3') phosphate. These phosphates showed characteristic wine red colors in the presence of ferric ion in an acid solution. The present studies were undertaken to investigate the nature of the colored complex formed by the reaction of ferric ion with L-ascorbic acid 3-phosphate. As a result, a new technique for the colorimetric determination of L-ascorbic acid 3-phosphate has been found. The technique was proved to be useful for the kinetic studies of the hydrolysis of this phosphate.

Experimental

Instruments—Perkin-Elmer IR Spectrophotometer Model 21, Hitachi Spectrophotometer Model EPU-2A, Hitachi Horiba pH-meter Model M-4 and Varian's A-60 NMR Spectrophotometer were used.

Paper Partition Chromatography—Ascending chromatography on Toyoroshi No. 51 (2 $\times$ 40) was carried out with propanol-water-trichloroacetic acid (15:4:1).

Ferric Chloride Solution (1%)—One gram of ferric chloride (FeCl₃·6H₂O) was dissolved in water to make up to 100 ml.

L-Ascorbic Acid 3-Phosphate Solution (0.1%)—Accurately weighed 100 mg of magnesium ascorbic acid 3-phosphate (C₆H₆O₉·PMg·3/2 5H₂O) was placed in a 100 ml volumetric flask and diluted with water to the make.

L-Ascorbic Acid Solution (0.1%)—Precisely weighed 100 mg of L-ascorbic acid was dissolved in 100 ml of water using a volumetric flask.

Magnesium Salts of L-Ascorbic Acid 2- and 3-Phosphates, L-Ascorbic Acid 3-Pyrophosphate and Bis-(L-Ascorbic Acid-3,3')-phosphate—These compounds used here were prepared as described in Part II and III.¹⁾

Barium L-Ascorbic Acid 3-Sulfate—This compound used in the test of the ferric chloride coloration was prepared according to the method reported by Ford.⁴⁾

1) Part II and III: H. Nomura, T. Ishiguro and S. Morimoto, *Chem. Pharm. Bull.* (Tokyo), **17**, 381, 387 (1969).

2) Presented at the 88th Annual Meeting of the Pharmaceutical Society of Japan, Tokyo, April 1968.

3) Location: *Juso, Higashiyodogawa-ku, Osaka.*

4) a) E.A. Ford and P.M. Ruoff, *Chem. Comm.*, **24**, 630 (1965); b) C.G. Mead and F.J. Finamore, *Biochemistry*, **8**, 2652 (1969).

5,6-Isopropylidene-L-ascorbic Acid 3-Diphenyl Phosphate (II)—To an acetone solution (50 ml) containing 5 g of 5,6-isopropylidene-L-ascorbic acid and 7.3 g of pyridine was added slowly 5.0 g of diphenylphosphorochloridate at 0°. The mixture was allowed to react for 4 hr and evaporate *in vacuo*. The residue was dissolved in methanol–water and passed through a column of Amberlite IR-120 (150 ml). The eluate was extracted with chloroform and after evaporation of the extract, 2.5 g of yellow powder was obtained. One gram of this compound was chromatographed on silica gel (2.5 × 12 cm) with acetone to give an amorphous powder (0.5 g). Successive elution with ethanol gave another phosphate as an amorphous powder. The former compound was purified by recrystallization from ethanol–benzene to yield 0.3 g of a pale yellow amorphous powder. UV $\lambda_{\text{max}}^{0.1N \text{ NaOH}}$ m μ : 261. This was soluble in ethanol, chloroform, acetone and ethyl acetate but insoluble in ether, benzene and hexane. The methanol solution did not reduce the iodine solution nor did it show a positive ferric chloride coloration in acid, but the solution gave a transient blue color in the presence of a small amount of pyridine. The compound thus obtained exhibits a single spot on thin-layer chromatogram (Silica gel GF 254; butanol: acetic acid: water = 6:4:1; *Rf* = 0.84). *Anal.* Calcd. for $\text{C}_{21}\text{H}_{21}\text{O}_9\text{P}$: P, 6.92. Found: P, 6.36. UV $\lambda_{\text{max}}^{\text{ethanol}}$ (ϵ) = 254 m μ (1.30×10^4). IR $\nu_{\text{max}}^{\text{CHCl}_3}$ cm $^{-1}$: 3430 (broad, –OH), 3010 (CH), 3100–2900 (CH and chelated OH), 1788 (C=O), 1695 (C=O), 1630 (C=C), 1600 (C=C), 1180 (P–O–aryl). NMR (60 Mc, CDCl_3): 1.20 (6H, singlet, $>\text{C}(\text{CH}_3)_2$), 3.85–4.0 (2H, multiplet, $-\text{CH}_2$), 4.0–4.3 (2H, multiplet, two kinds of methine), 7.17 (10H, singlet, phenyl proton).

On the chromatographic separation of the mixed phosphate described above the successive elution with ethanol and following working up gave another phosphate. This was negative in ferric chloride test and showed a single spot on TLC. This was left unidentified.

L-Ascorbic Acid 3-Phenyl Phosphate (III)—i) The compound II (0.27 g) was dissolved in 6 ml of 1N sodium hydroxide–dioxane (1:1) and left to stand for a day to remove one phenyl group. The reaction mixture was treated with cation exchanger resin, IR-120 (H-form), and washed with ether. The solution was neutralized with sodium hydroxide and evaporated to a small portion. To this was added ethanol to give a precipitate. By the examination with NMR and PPC analysis this was shown to be identical with the sample reported in the following section.

ii) To a solution of 5,6-isopropylidene-L-ascorbic acid (2.5 g, 0.0115 mole) and pyridine (3.65 g, 0.046 mole) in acetone (25 ml) was added phenyl phosphordichloridate (2.4 g, 0.0115 mole) under ice-cooling. The mixture was stirred for 2 hr at 0° and left to stand overnight in a refrigerator. The reaction mixture was evaporated and the residue was dissolved in water and passed through a column of Amberlite IR-120 (H-form, 100 ml). The eluate was adjusted to pH 4 with sodium bicarbonate and concentrated to a small portion. Acetone was added to this and the precipitate was removed, and the filtrate was evaporated to dryness. The residue was purified by chromatography on silica gel (3.6 × 15 cm) using acetone. After evaporation of the solvent and the following recrystallization from dioxane–ether, a colorless hygroscopic powder was obtained (0.8 g). *Anal.* Calcd. for $\text{C}_{12}\text{H}_{13}\text{O}_6\text{P} \cdot 3/2 \text{ H}_2\text{O}$: C, 40.1; H, 4.46; P, 8.64. Found: C, 39.94; H, 4.42; P, 8.23. UV $\lambda_{\text{max}}^{0.1N \text{ HCl}}$ m μ (ϵ): 239 (0.79×10^4); $\lambda_{\text{max}}^{\text{pH } 7}$ m μ (ϵ): 258 (1.21×10^4). NMR (D_2O) δ : 3.82 (2H, defused doublet, $J_1 = 5.4$ cps, $-\text{CH}_2\text{OH}$), 4.16 (1H, multiplet, $J_2 = 5.4$ cps, $J_3 = 2.0$ cps, $-\text{CHOH}$), 5.04 (1H, quartet, $J_2 = 2.0$, $J_3 = 0.5$ cps), 7.36 (5H, broad singlet, phenyl proton). A single spot on paper partition chromatography (*Rf* = 0.8).

Fig. 1. Infrared Spectrum of L-Ascorbic Acid 3-Phenyl Phosphate (KBr)

L-Ascorbic Acid 2-Phenylphosphate (VI)—

Further elution with acetone–ethanol (1:1) of the silica gel column described in the preceding section (III-ii) gave another phosphate (VI). Neutralization with sodium hydroxide and the successive evaporation gave a brownish powder. Recrystallization from ethanol–water gave an amorphous powder. Positive in the FeCl_3 test. *Anal.* Calcd. for $\text{C}_{12}\text{H}_{11}\text{O}_6\text{PNa}_2 \cdot \text{H}_2\text{O}$: C, 36.55; H, 3.33; P, 7.87. Found: C, 36.32; H, 3.93; P, 7.31. UV $\lambda_{\text{max}}^{0.1N \text{ HCl}}$ m μ (ϵ): 236 (0.77×10^4); $\lambda_{\text{max}}^{0.1N \text{ NaOH}}$ m μ (ϵ): 259 (1.46×10^4). IR $\nu_{\text{max}}^{\text{KBr}}$ cm $^{-1}$: 3380 (broad, OH), 2950 ($-\text{CH}$), 1740 (C=O), 1600, 1498, 1420.

L-Ascorbic Acid 3-Phosphate (IV)—The compound III (100 mg) was catalytically reduced over Pt in 0.3N sodium hydroxide solution (2 ml). The reaction mixture was filtered. Paper chromatography indicated that the reduction product was identical with the authentic sample (IV).¹⁾

L-Ascorbic Acid 2-Phosphate (VII)—Similarly the catalytical hydrogenation of VI was performed as described above. The reaction product was confirmed to be VII on paper chromatography in the comparison with the authentic sample.

Calibration Curve—Exactly weighed 200 mg of L-ascorbic acid 3-phosphate ($\text{C}_6\text{H}_8\text{O}_6\text{P} \cdot 3/2 \cdot 5\text{H}_2\text{O}$) was placed in a 200 ml volumetric flask and diluted with water to the mark. Aliquots of 1.0, 2.0, 5.0, 10.0, 15.0, 25.0 and 35.0 ml of the solution were pipetted into 50 ml volumetric flasks, respectively. Then, 10 ml

of 1N hydrochloric acid and 10 ml of the ferric chloride solution were added to each flask. The resulting mixture was diluted with water to the 50 ml mark and shaken. The characteristic wine red color appeared immediately. The apparent absorbance at 480 m μ was subtracted by the reagent blank. By plotting the true absorbance against the concentration of L-ascorbic acid 3-phosphate, a straight line was obtained for the range of the concentration as shown in Fig. 4.

Analytical Procedure—Ten ml of an aqueous solution containing 8×10^{-3} to 6×10^{-4} moles of L-ascorbic acid 3-phosphate is pipetted into a 50 ml volumetric flask. Water (10 ml) is added into another flask (50 ml) to be used as a blank. Both the ferric chloride reagent (10 ml) and 1N hydrochloric acid (10 ml) are subsequently pipetted into each flask. The resulting mixture is diluted to the 50 ml mark with water and shaken. The absorbance at 480 m μ of the sample solution is determined using 1 cm cells *vs.* the blank solution. The concentration of L-ascorbic acid 3-phosphate is determined by reference to the calibration curve (Fig. 4).

Result and Discussion

Color Reaction of the Phosphorylated Ascorbic Acids with Ferric Chloride

The color reaction with ferric chloride solution is typical of phenols and enols, but many of them give color only in a narrow pH range and a suitable solvent system. Since the color is in most cases acid labile, the addition of a small amount of weak base⁵⁾ often promises a good result in the qualitative test.

In contrast to common phenols or enols the colored principles derived from L-ascorbic acid 3-phosphate and the related isomers are extremely acid stable as shown in Fig. 2. Thus, L-ascorbic acid 3-phosphates show the ferric chloride coloration over the pH range from -0.5 up to 5.

Similar coloration has been observed on the reaction of ferric ion with *o*-hydroxyphenyl phosphate or its alkyl esters.⁶⁾ Phenylphosphoryl- and diphenylphosphoryl esters of L-ascorbic acid, prepared by phosphorylating 5,6-isopropylidene-L-ascorbic acid with mono- and diphenylphosphoryl chlorides were investigated as to their color reactions. Consequently it was found that the former compounds which Clark and his coworkers⁷⁾ have previously synthesized by DCC method was positive in the color reaction, whereas the latter was negative in the test. These results are shown in Table I.

The remarkable acid stabilities of the color are probably due to the presence of the enolic group which can take part in the chelate formation with the vicinally located phosphoryl group as shown by the following partial structure, VIII.

Fig. 2. Absorbance of Ferric Chloride Complex of L-Ascorbic Acid 3-Phosphate at 480 m μ *vs.* Acidity Function (Ho) of Hydrochloric Acid^{a)}

a) The solution containing 2.9×10^{-5} mole of magnesium L-ascorbic acid 3-phosphate and 1.3×10^{-5} mole of potassium ferric sulfate was delivered into a volumetric flask and diluted with an appropriate concentration of hydrochloric acid which contains a calculated amount of KCl to accurately make up to 50 ml. The final ionic strength of the resulting solution was adjusted to $\mu=2.0$ with KCl. The same hydrochloric acid solution as used for the preparation of the optical solution was employed as control. The measurement of the absorbance at 480 m μ was performed at a constant temperature (20°) immediately after the sample preparation.

5) a) S. Soloway and S.H. Wilen, *Anal. Chem.*, **24**, 979 (1952); b) S. Soloway and P. Rosen, *ibid.*, **25**, 595 (1953).

6) a) T. Ukita and K. Nagasawa, *Chem. Pharm. Bull. (Tokyo)*, **9**, 544 (1961); b) K. Nagasawa and T. Ukita, *ibid.*, **7**, 397, 401 (1959).

7) V.M. Clark, J.W.B. Hershey and D.W. Hutchinson, *Experientia*, **22**, 425 (1966).

hydroxyphenylsulfonate⁹⁾ reportedly gave a similar coloration with ferric chloride. These findings suggest that one of the prime factors for the stabilization to acid is an existence of proton releasing group of large ionization constant which locates in a suitable position to take part with the enolic hydroxyl in the formation of ferric chelate.

By a spectrophotometric analysis of the coloration, the following information was obtained. In an acidic solution, a maximum intensity was obtained at the molar ratio, ferric chloride/L-ascorbic acid 3-phosphate, being not less than one (Fig. 3). Similar results have been reported on sulfosalicylic acid,⁸⁾ and on salicylic acid.¹⁰⁾ Since no color was formed by the change of water for methanol or other organic solvents, water molecules are essential for the coloration of L-ascorbic acid 3-phosphate. Probably water molecules coordinate with the iron (III) in the chelate. In analogy with an other iron complex which in general constructs an octahedral structure the present complex may tentatively be formulated as.

Colorimetric Method

L-Ascorbic acid 3-phosphate is conveniently determined by a colorimetry based on the color reaction with ferric ion. Fig. 4 shows a proportionality between the color intensity and the concentration of the phosphate. The procedure may be applied to the determination of individual L-ascorbic acid phosphates but not to a mixture of them. Reducing agents such as L-ascorbic acid may interfere because of the reduction of ferric to ferrous ion. This type of interference can be prevented by the prior removal of the reductant from a test solution or by use of excess iron (III). In fact, as shown in Table III, the determination of L-ascorbic acid 3-phosphate in the aqueous solution containing 3 molar equivalent of L-ascorbic acid could be quite satisfactorily performed.

Fig. 3. Color Intensity vs. Molar Ratio of Fe³⁺ to L-Ascorbic Acid 3-Phosphate (AP-3)

procedure: Ten ml of a stock solution containing 8.8 mg of the present phosphate (C₆H₆O₆P Mg 3/2·5 H₂O) was placed in a 50ml volumetric flask to which a given amount of FeCl₃ was added. The resulting mixture was made up to the mark with water and the absorbance at 480 mμ was measured.

9) V.I. Kuznetsov, *Chem. Abstr.*, **45**, 1454 d (1951).

10) a) A.K. Babko, *J. Gen. Chem. (USSR)*, **15**, 745 (1945); b) H. Broumand and J.H. Smith, *J. Am. Chem. Soc.*, **74**, 1013 (1952).

TABLE II. Effect of pH on the Color Intensity and on the Periodical Stabilities

Experiment run.	1N hydrochloric acid added ml	pH value of the solution	Absorbance ^{a)} (480 m μ)		
			Initial.	After 5 hr	After 24 hr
1	10.0	0.72	0.565	0.565	0.543
2	7.0	0.89	0.568	0.566	0.549
3	5.0	1.02	0.552	0.551	0.538
4	2.5	1.32	0.552	0.554	0.535
5	—	2.40	0.540	0.542	0.537

a) Ten ml of the 0.1% L-ascorbic acid 3-phosphate solution was pipetted into a 50 ml volumetric flask to which 10 ml of the ferric chloride reagent and the given amount of hydrochloric acid were added. The combined solution was diluted with water accurately up to 50 ml. The pH value and color intensity of the resulting solution were measured. Each solution was left standing for the given periods of time at room temperature ($17 \pm 1^\circ$) and periodical stability of the color intensity was measured.

TABLE III. Effect of L-Ascorbic Acid on the Color Intensity of Ferric Chloride Complex of L-Ascorbic Acid 3-Phosphate

Experiment No.	The ascorbic acid solution ml.	The Molar ratio, L-ascorbic acid/L-ascorbic acid 3-phosphate	Absorbance ^{a)} 480 m μ
1	0	0	0.553
2	5	1.1	0.553
3	10	2.2	0.552
4	15	3.2	0.552

a) The given volume of the 0.1% L-ascorbic acid solution and 0.1% L-ascorbic acid 3-phosphate solution (10 ml) were pipetted into 50 ml volumetric flasks. Then, 1% ferric chloride solution (10 ml) and 1N hydrochloric acid (5 ml) were successively added and the resulting mixture was diluted with water to the mark. The absorbancy of the solution was measured at 480 m μ .

The intensity of the color was varied with the pH of the solution and showed the maximum at pH 0.89. However, the variation was rather small at the pH range 0.7–2.4. The periodical stability of the color was similarly dependent on the pH of the solution, but decrease of the color intensity during the analytical manipulation was actually negligible as exemplified in Table II.

Fig. 4. Calibration Curve

Disturbance caused by impurities which undergo the color reaction competitively with iron (III) or L-ascorbic acid phosphate will make this method being fruitless.

Since L-ascorbic acid 3-phosphate is relatively unstable to an oxidizing agent such as ammonium molybdate,¹¹⁾ the molybdenum blue method for the determination of inorganic phosphate in the presence of L-ascorbic acid 3-phosphate is prone to be less accurate.

In consequence, notwithstanding the limitation caused by interferences as mentioned above, the present colorimetric analysis is quite useful for kinetic studies of the hydrolysis of L-ascorbic acid 3-phosphate. This will be reported in the following paper.

11) B.M. Blackburn and J.S. Cohen, "Topics in Phosphorus Chemistry," Interscience Publishers, division of John Wiley & Sons, Inc., New York, 1969, p. 197.