SYNTHESIS AND PROPERTIES OF L-ASCORBATE 2-SULFATE

by 613-8302

CHEN-HSIUNG LEE

B.S., CHUNG HSING UNIVERSITY, 1967

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

in

FOOD SCIENCE

Department of Grain Science and Industry

KANSAS STATE UNIVERSITY

Manhattan, Kansas

1973

Approved by:

Paul A Seib

Major Professor

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION AND LITERATURE REVIEW

Although the biosynthesis of organic sulfates has been shown to involve 3'-phosphoadenosine-5'-phosphosulphate as a sulfating agent (1), other unknown cofactors have been implicated as sulfate carriers (2). Because of a number of indirect relationships between L-ascorbic acid deficiency and sulfate incorporation into biologically important compounds (3), it has been postulated that L-ascorbic acid may be involved in biological sulfation. Ford and Ruoff (4) observed the chemical behaviour of 5,6-O-isopropylidene-L-ascorbic acid 2-sulfate[1] and suggested that L-ascorbate 2-sulfate (I) may be biologically important as a sulfating or hydroxylating agent. Mumma (5) prepared I from the 5,6-O-isopropylidene derivative and demonstrated that in the presence of a number of oxidizing agents, including air, the compound is an excellent sulfating agent. Compound I has been also shown to be a good sulfating agent *in vitro* and *in vivo* (5,6). Rats injected with ^{35}S-ascorbic acid 2-sulfate were shown to accelerate the excretion of ^{35}S-cholesterol sulfate by a factor of 50 compared to rats injected with $^{35}SO_4^=$ (6,7).

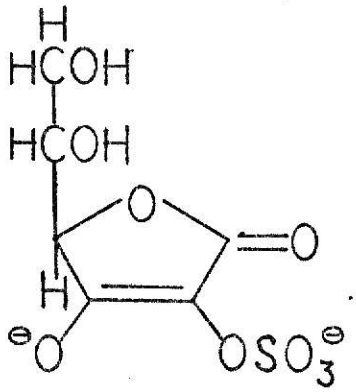
The discovery of L-ascorbate 2-sulfate (I) in brine shrimp (8,9), which contained no L-ascorbate, and the isolation of the sulfated compound from the urine of the guinea pig, trout, rat, and man (10)

---

[1] This compound has previously been reported as the 3-sulfate derivative. However, recent X-ray studies have proven the structure to be the 2-sulfate ester (11,12). For more information on the structure, see Appendix I.

demonstrated that I is a compound of widespread biological distribution. It is not just a laboratory curiosity but is biologically important. Compound I is perhaps a biological sulfating agent, or it serves some other undefined metabolic function.

In the literature prior to 1972, L-ascorbate 2-sulfate (I) was incorrectly assigned the structure of L-ascorbate 3-sulfate. Recent X-ray crystallographic determination of structure has proven the compound which occurs naturally and which has been synthesized chemically in several laboratories is L-ascorbate 2-sulfate (11,12).



I

Thus, the sulfated derivative of L-ascorbic acid most people have isolated and worked with is probably L-ascorbate 2-sulfate (I). To the author's knowledge, no one has ever isolated and characterized L-ascorbate 3-sulfate.

L-ascorbate 2-sulfate (I) has recently been reported (13) to have antiscorbutic activity in guinea pigs equal to L-ascorbate (II). The sulfate derivative (I) has also been reported to prevent scurvy in rainbow trout and coho salmon (14). If these reports are confirmed, the sulfate derivative (I) would indeed be a valuable source of vitamin C in processed foods since it is more stable than II (5,15,16) and since it appears to be a normal metabolite in animals (10). The lability of

II to oxygen, especially in alkaline media, is well known (17-20). Furthermore, compound II is destroyed even more rapidly when a food, such as wheat flour (21), contains the enzyme L-ascorbic acid oxidase. The enzyme converts II to dehydro-L-ascorbic acid, a labile (22) compound whose irreversible destruction corresponds directly to the loss of antiscorbutic activity in a food. Thus, a major portion of II is destroyed in cereal products manufactured at elevated temperature (23). Quadri (24) reported the salts of I have excellent stability in such yeast-leavened baked goods as bread (15% loss) and in chemically leavened products such as pancakes (4% loss). Simulated pasteurization and storage of milk also caused no destruction of I.

The first chemical synthesis of I was accomplished in 1965 by Ford and Ruoff (4,25) who sulfated 5,6-O-isopropylidene-L-ascorbate with an amine-sulfur trioxide complex in N,N-dimethylformamide and reported the isolation of barium 5,6-O-isopropylidene-L-ascorbate 2-sulfate. However, it is most likely the compound isolated was instead barium L-ascorbate 2-sulfate since the sulfated 5,6-ketal derivative was purified on an Amberlite IR-120 (H^+) resin followed by neutralization with barium hydroxide. In the author's experience, this resin treatment has been found to remove the 5,6-O-isopropylidene group very rapidly. Chu and Slaunwhite (26) prepared I using sulfation of 5,6-O-benzylidene-L-ascorbic acid with pyridine-sulfur trioxide in N,N-dimethylformamide followed by hydrolytic removal of the isopropylidene group. They reported isolation of the potassium salt of I in 41% yield as an amorphous monohydrate not having a sharp melting point. Mumma and coworkers (15) employed the same synthesis scheme to sulfate 5,6-O-isopropylidene-L-ascorbic acid, however these workers isolated only 15% yield of potassium

L-ascorbate 2-sulfate which contained one mole of water of crystallization. Both these groups used a tedious isolation technique. Tolbert et al. (16) have announced the preparation of the barium salt by way of sulfation of 5,6-O-benzylidene-L-ascorbic acid with a mixture of sulfuric acid and dicyclohexyldicarbodiimide in N,N-dimethylformamide (27). The barium salt of I was purified by ion-exchange chromatography, but details of the procedure have not been published. Bond et al. (11) reported an approximately 50% conversion of 5,6-O-cyclohexylidene-L-ascorbic acid to compound (I). Others (28) have stated that column chromatography with diethylaminoethyl cellulose was the only method of purification.

Thus, there is a need for improved methods of preparation and isolation of I, if this compound is to become of commercial importance as a stable source of vitamin C. In addition, more information is needed on the physical and chemical properties of I, so that its behaviour in food systems can be predicted. For these reasons several salts of I were prepared, and the solubility of the potassium and barium salts of I at various temperatures was determined. The stability of I to acid and alkali was also examined.

RESULTS AND DISCUSSION

1. The Role of Pyridine in Improved Synthesis of L-Ascorbate 2-Sulfate in N,N-Dimethylformamide

Recently Quadri (24) improved the conventional procedure of preparing L-ascorbate 2-sulfate (I). The conventional procedure involved reaction of a 5,6-acetal derivative of L-ascorbic acid with an amine-sulfur trioxide complex in N,N-dimethylformamide. He simplified the isolation procedure and was thereby able to increase preparative yields from 15% (15) to approximately 50%.

It was found that, by adding a tertiary amine together with the 5,6-acetal of L-ascorbic acid prior to addition of the amine-sulfur trioxide complex, the isolated yields of compound I could be further improved from 50% to 75%. The reaction sequence is shown in Fig. 1 (p.6).

Several experiments were done to explain the role of the added tertiary amine, but, before discussing these experiments, it is necessary to present a short discussion of the chromatographic and ultraviolet spectrophotometric methods used to analyze the products of the sulfation reaction.

Spray reagents used to detect L-ascorbic acid and several of its derivatives are summarized in Table 1 (p.7). Silver nitrate is sensitive for reducing compounds. Neutral silver nitrate spray detects L-ascorbic acid, whereas 5,6-O-isopropylidene-L-ascorbic acid cannot be detected in trace amounts. The ferric chloride spray was used to detect enolic hydroxyl groups. Previous reports concerning L-ascorbate sulfate have clearly

THIS BOOK CONTAINS NUMEROUS PAGES WITH DIAGRAMS THAT ARE CROOKED COMPARED TO THE REST OF THE INFORMATION ON THE PAGE.

THIS IS AS RECEIVED FROM CUSTOMER.

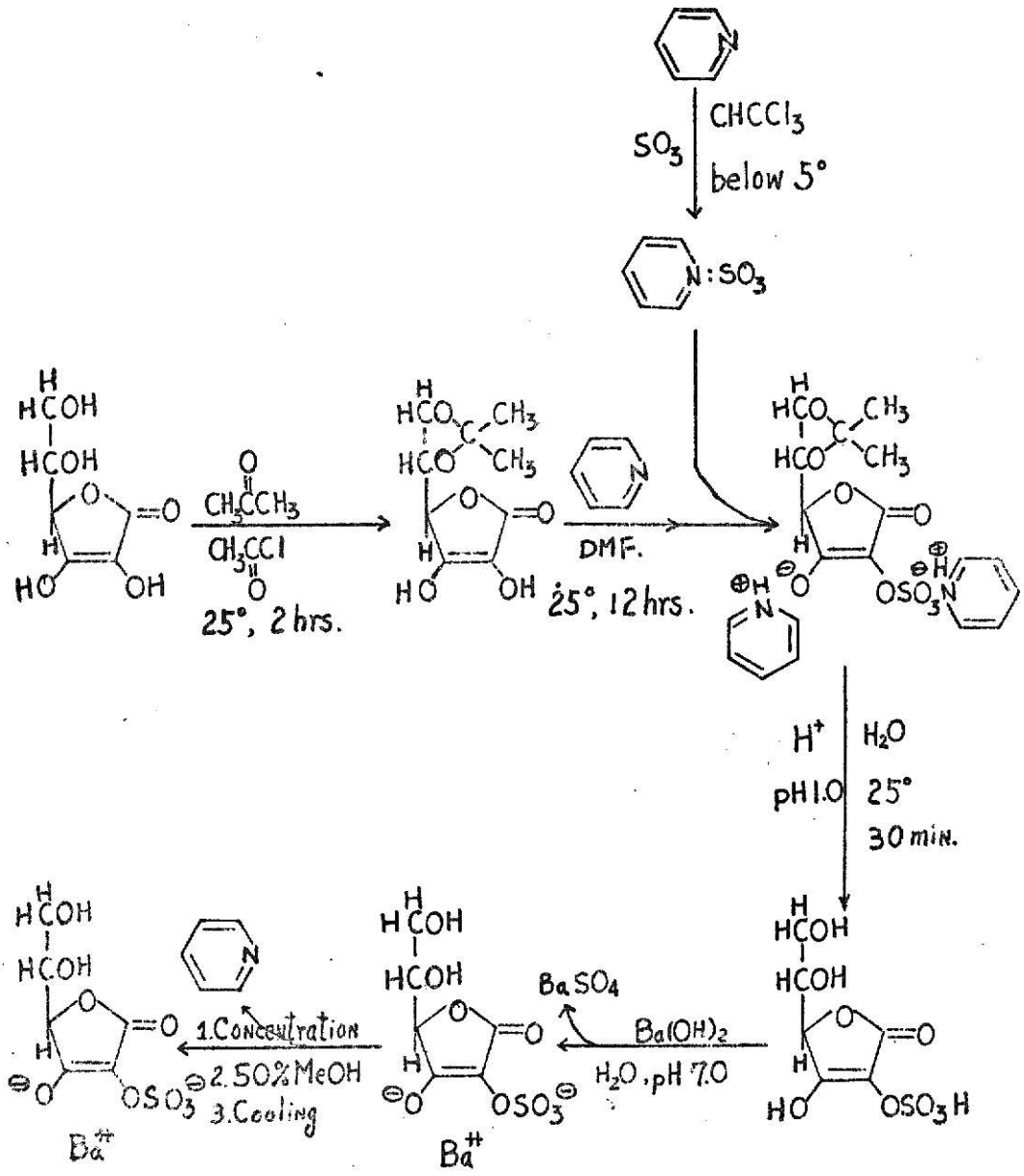


Fig. 1. Synthesis of L-Ascorbate 2-Sulfate in N,N-Dimethylformamide.

TABLE 1. Reaction of Various Compounds with Sprays on Chromatography

Compounds	Sprays		
	Silver nitrate (neutral)	Silver nitrate (KOH)	Color with $FeCl_3$
L-Ascorbic acid	+	+	purple
5,6-O-Isopropylidene-L-ascorbic acid	+	+	purple
L-Ascorbate 2-sulfate	-	+	red
5,6-O-Isopropylidene-L-ascorbate 2-sulfate	-	+	red
L-Ascorbate 2,6-disulfate	-	+	red

" + " Detected.

" - " Not detected.

demonstrated that the sulfate must reside either at the 2- or 3- position. Based on indirect evidence, the sulfate was assigned to the 3- position by Ford and Ruoff (4,25), and every subsequent report repeated this assignment. However, the ability to form a ferric chloride complex is not restricted to the 3-isomer as supposed (29,30). The mobilities of L-ascorbic acid and several of its derivatives in two paper chromatographic developing systems are given in Table 2 (p.9).

Ultraviolet spectrophotometry can be used to differentiate between the 2- and 3- sulfate esters of L-ascorbic acid. In analogy with the phenylphosphate esters, the 3-sulfate should have λmax. at 238 nm at neutral pH (Table 3, p.10), whereas the 2-sulfate should have λmax. of 257 nm. Substitution at the C-6 or C-5 position would result in the compound having λmax. of 265 nm.

To determine the improving role of added pyridine in the sulfation reaction, two sulfation reactions were run and the products formed during sulfation were analyzed by paper chromatography and ultraviolet spectrophotometry (Table 4, p.11). In reaction A, one molar equivalent of pyridine, based on 5,6-O-isopropylidene-L-ascorbic acid, was added prior to sulfation, whereas in reaction B, no free pyridine was added prior to sulfation. The developing solvent system for preparative paper chromatography was n-propanol-water-trichloroacetic acid (15:4:1, V/V/W).

Aliquots were removed from reaction A with time, and paper chromatography showed two principal components were formed with Ra0.71 and 0.10 during partial and after complete disappearance of the starting material (5,6-O-isopropylidene-L-ascorbic acid). After complete disappearance of 5,6-O-isopropylidene-L-ascorbic acid in reaction A, the component with Ra 0.71 was hydrolyzed by acid, then neutralized with sodium bicarbonate.

TABLE 2. Paper Chromatographic Mobilities of Derivatives of L-Ascorbic Acid.

Compound	Ra values[a] in solvent system[b] given	
	A	B
5,6-O-Isopropylidene-L-ascorbic acid	1.56	1.60
5,6-O-Isopropylidene-L-ascorbic acid 2-sulfate	0.71	0.73
L-Ascorbic acid 2-(hydrogen sulfate)	0.50	0.52
Barium L-ascorbic 2-sulfate	0.48	0.50

(a) Ra value = $\frac{\text{distance migrated by derivative}}{\text{distance migrated by L-ascorbic acid}}$

(b) Solvent systems; A. n-propanol-water-trichloroacetic acid (15:4:1, V/V/W); B. n-butanol-water-acetic acid (5:3:2, V/V).

TABLE 3. Ultraviolet Spectral Characteristics of Substituted L-Ascorbic Acids (11).

	Acid		Neutral		Base	
Compound	λmax.	$\varepsilon \times 10^3$	λmax.	$\varepsilon \times 10^3$	λmax.	$\varepsilon \times 10^3$
L-Ascorbic acid	245	12.2	265	16.5	--	--
3-Methyl-L-ascorbic acid	244	9	244	9	276	9
2,3-Dimethyl-L-ascorbic acid	235	9	--	--	--	--
3-Phenylphosphoryl-L-ascorbic acid	237	8	238	8	263	8
2-Phenylphosphoryl-L-ascorbic acid	233	9.6	257	14.5	258	15
L-Ascorbate 2-sulfate	232	14.1	255	21.7	255	21.7

TABLE 4. Paper Chromatographic and Ultraviolet Spectrophotometric Analysis of the Sulfation Products of 5,6-O-Isopropylidene-L-Ascorbic Acid with Free Pyridine (Reaction A) and Without Free Pyridine (Reaction B).

Component	Sulfation Products[a]				Sodium Salt of Sulfation Products[b]	
	Reaction A		Reaction B		Reaction A	Reaction B
	Ra[c]	Amount (%)	Ra[c]	Amount (%)	Ra[c]	Ra[c]
1. 2-Sulfate ester	0.71	92	0.71	35	0.37	0.37
2. 2,6-Disulfate ester	X[d]	X[d]	0.21	57	X[d]	0.21
3. Unknown	0.10	8	0.10	8	0.11	0.11

(a) Sulfation products of 5,6-O-isopropylidene-L-ascorbic acid in N,N-dimethylformamide prior to hydrolysis.

(b) The extracted sulfation products were hydrolyzed by acid, then neutralized with sodium bicarbonate.

(c) Ra value = $\frac{\text{distance migrated by derivative}}{\text{distance migrated by L-ascorbic acid}}$

(d) Not detected.

The neutralized component showed the same Ra (0.37) as analytically pure sodium L-ascorbate 2-sulfate. It indicated the component with Ra 0.71 was 5,6-O-isopropylidene-L-ascorbate 2-sulfate. The materials with Ra 0.71 and Ra 0.10 were eluted from the paper and, from the absorbance values of the components, it was estimated the mixture contained 92% 2-sulfate ester and 8% unknown.

When no extra pyridine was added prior to sulfation (Reaction B), paper chromatography showed the sulfation products contained three principal components with Ra values of 0.71, 0.21 and 0.10. The extra component at 0.21 was assigned the 2,6-disulfate structure based on the following facts.

(1) It gave a red color with ferric chloride spray indicating the 2 or 3 position was esterified. The ultraviolet spectrum of the compound showed the 2-hydroxyl group was substituted. At pH 7.0, the derivative gave λmax 255 nm and at pH 2.0, 232 nm. These ultraviolet spectrophotometric data are consistent only with a 2-substitution (Table 3, p.10).

(2) The slower mobility of the compound on paper chromatography indicated a more polar molecule than a mono-sulfate ester of L-ascorbate (Ra 0.37). Thus, the compound is most likely a disulfate ester of L-ascorbate.

(3) Aliquots were removed from reaction B at various times, and paper chromatography showed that the intensity of the extra spot (Ra 0.21) increased with time. The intensity of the spot with Ra 0.71, namely 5,6-O-isopropylidene-L-ascorbic acid 2-sulfate, decreased at the same time. That indicated the 5,6-O-isopropylidene group was removed and then the free 6-hydroxyl group sulfated.

(4) The 6-hydroxyl group of underivatized L-ascorbic acid is known to be more reactive than the 2-, 3-, or 5-hydroxyl groups towards pyridine-sulfur trioxide (28). Therefore, the most likely disubstituted derivative is the 2,6-disulfate ester.

These results clearly show why improved yields of compound I were obtained by adding pyridine prior to sulfation. With added pyridine more I is formed and crystallization of I from the reaction mixture is also aided because of the fewer components in the crystallization medium.

The formation of L-ascorbate 2,6-disulfate means that the 5,6-O-isopropylidene group is being cleaved somehow during sulfation reaction B. Even in the early stages of sulfation, the 2,6-disulfate (Ra 0.21) was observed along with the desired product with Ra 0.71. The 5,6-O-isopropylidene group is easily removed from 5,6-O-isopropylidene-L-ascorbic acid if the latter molecule comes into contact with water (31). The acidic 3-hydroxyl group of 5,6-O-isopropylidene-L-ascorbic acid is capable of catalyzing the hydrolysis of its own 5,6-acetal group. Thus, water must be rigorously excluded during sulfation of 5,6-O-isopropylidene-L-ascorbic acid, otherwise L-ascorbic acid of L-ascorbic acid 2-sulfate will be formed by hydrolysis. The L-ascorbic acid or L-ascorbic acid 2-sulfate will then be sulfated to give the 2,6-disulfate. Apparently very little L-ascorbic acid 6-sulfate is formed.

The role of the pyridine added prior to sulfation (Reaction A) is to neutralize any acid which may be present in the original mixture prior to sulfation and which may be formed during the sulfation reaction. In this way if any water is introduced into the sulfation reaction, the 5,6-acetal will not be hydrolyzed because the reaction medium is basic and not acidic.

One molar equivalent of pyridine added prior to sulfation increased the yields of L-ascorbate 2-sulfate to 75%. However, the added pyridine slowed down the reaction time which now required 12 hours for complete sulfation. For complete sulfation, reaction B required only half the time that reaction A required.

2. The Synthesis of L-Ascorbate 2-Sulfate in an Aqueous Medium

Smith and his colleagues (32) sulfated wheat flour in 80 to 90% yield with trimethylamine-sulfur trioxide in an aqueous sodium hydroxide system. They reported the extent of sulfation, yield, and properties of the flour sulfates were influenced by reaction time, temperature, catalyst concentration, and the amount of trimethylamine-sulfur trioxide.

The C-3 and C-2 hydroxyl groups of L-ascorbic acid (II) are much more ionized in aqueous solvents, especially under alkaline conditions, than that in aprotic solvents (for example, N,N-dimethylformamide). The C-3 hydroxyl group is the more acidic (pK_{a1} 4.17) and would be expected to be sulfated before the C-2 hydroxyl group (pK_{a2} 11.5). However, resonance of one pair of electrons with the enone structure results in the following two resonance forms (Fig. 2, p.15). Hence, sulfation of the 3-hydroxyl group would not yield a stable sulfate ester. The 3-sulfate ester is a mixed anhydride and, if formed in the reaction mixture, this mixed anhydride would react further by sulfating other hydroxyls on 5,6-O-isopropylidene-L-ascorbic acid or L-ascorbic acid to produce more stable sulfate esters.

L-ascorbate (II) reacted rapidly at 70° with trimethylamine-sulfur trioxide in alkali at pH 9.5-10.5 to produce L-ascorbate 2-sulfate (I) nearly quantitatively. Trimethylamine was removed by ion-exchange

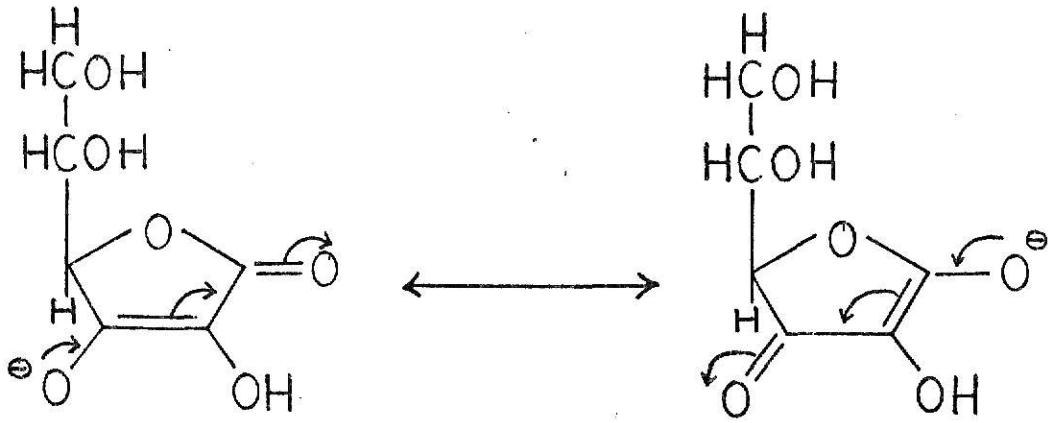


Fig. 2. Resonance forms of L-ascorbate (neutral pH).

chromatography, and the crystalline, analytically-pure barium salt of I was easily isolated in high yield (80%). The reaction sequence used to prepare I is shown in Fig. 3 (p.17).

After sulfation of L-ascorbic acid in an aqueous medium, ultraviolet absorption spectroscopy at 255 nm indicated 99.2% sulfation of the 2-hydroxyl had occurred at pH 9.5-10.5 (Table 5, p.18). Preparative paper chromatographic separation and ultraviolet spectrophotometric analysis indicated the reaction products consisted of a 40:1 mixture of the 2-sulfate (I) and the 2,6-disulfate ester of L-ascorbate. The time required for disappearance of starting material (II) also depended on pH (Table 5, p.18). The data indicate the formation of L-ascorbate 2-sulfate (I) accelerates as alkalinity increases, probably because ionization of the 2-hydroxyl is increased. Conditions that form I faster give higher yields because I is more stable in alkali than is L-ascorbate (II). Conversions are lower and reaction periods longer when the pH exceeds 10.5 because the higher hydroxyl ion concentration destroys the amine-sulfur trioxide (33), and L-ascorbate (II) is slowly degraded by alkali. It is likely the pH range at which II is nearly quantitatively converted to I could be extended by using larger amounts of trimethylamine-sulfur trioxide. The synthesis of compound I by the above procedure represents a vast improvement over other methods of preparation. The advantages include the following: a. The starting material is L-ascorbate (II); b. the solvent is water; c. conversion of II to I is nearly quantitative and the reaction period is only 30 minutes; d. the isolation scheme is simple; and, e. isolated yields of 80% of analytically-pure salts of I are obtained.

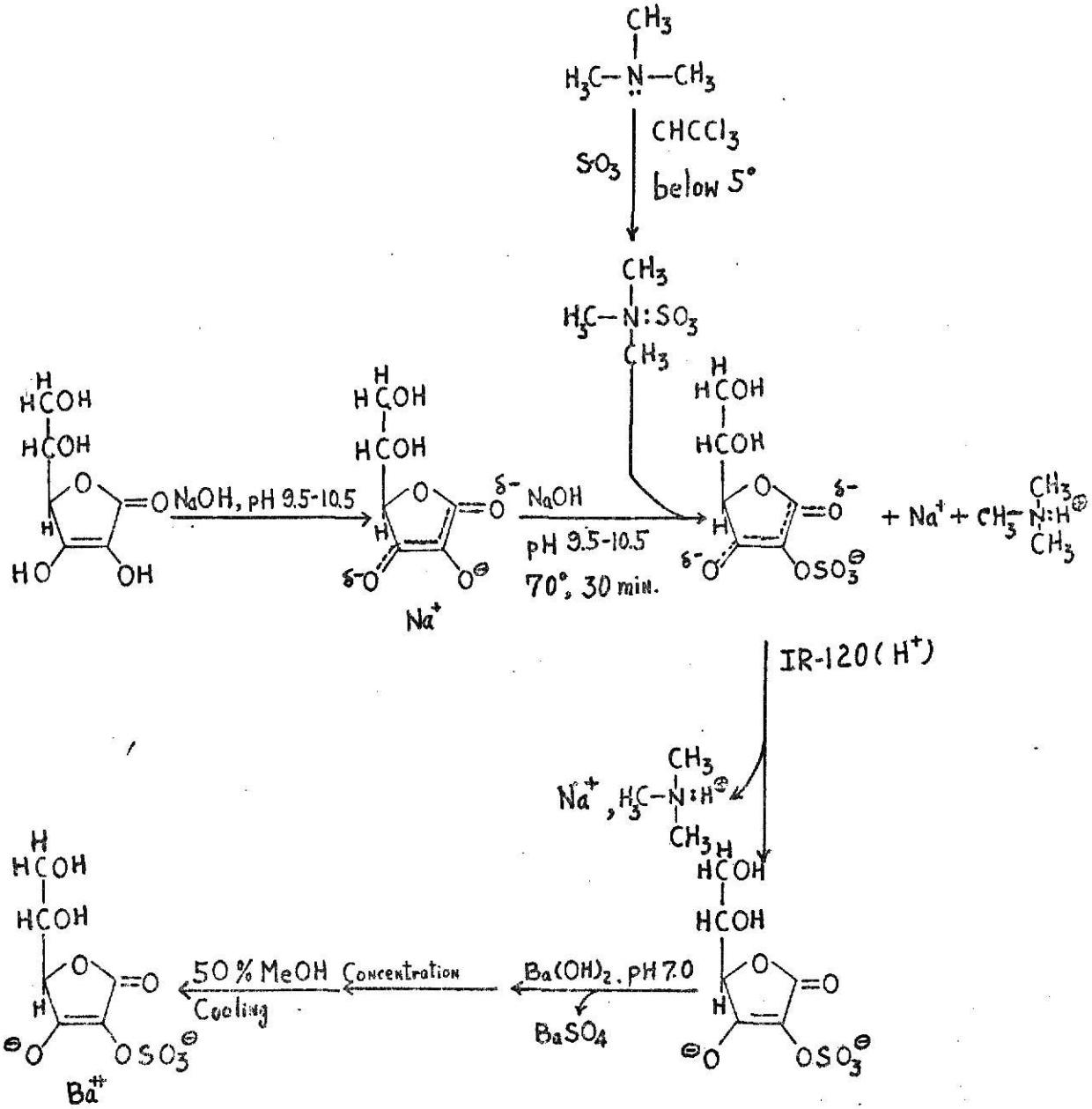


Fig. 3. Synthesis of L-Ascorbate 2-Sulfate in an Aqueous Medium.

TABLE 5. Sulfation of the 2-Hydroxyl Group of L-Ascorbate[a].

pH	Reaction Period, Minutes	Conversion, %
8.5	630	77
9.0	360	83
9.5	45	99
10.0	30	99
10.5	35	99
11.0	60	76
11.5	180	35

(a) See Materials and Methods.

3. Comparison of Sulfated Derivative of L-Ascorbic Acid with Authentic L-Ascorbate 2-Sulfate

Bond et al. (11,12) reported that X-ray crystallographic studies have demonstrated the sulfated derivative of L-ascorbic acid which occurs naturally and which has been synthesized chemically is L-ascorbate 2-sulfate. Comparison of our synthetic compound with that found in brine shrimp (11) showed both to have identical physical and spectral properties. The m.p. and specific rotation of the barium salts isolated from brine shrimp by Bond et al. (11) and synthesized in our laboratory by both methods (in N,N-dimethylformamide and in aqueous media) gave identical properties. The infrared spectra of the barium salts from both sources were identical. Ultraviolet spectra of L-ascorbate 2-sulfate (I) from both sources had the same maximum at 255 nm in neutral solution, which shifted to 232 nm at pH 2.0. The Ra values of the ammonium and barium salts from both sources in developing solvent A, n-propanol-water-trichloroacetic acid (15/4/1, V/V/W), are shown in Table 6 (p.20). Various sprays on chromatography papers gave identical results. It was concluded that synthesized compound was L-ascorbate 2-sulfate (I).

4. Properties of L-Ascorbate 2-Sulfate

The sodium, potassium, ammonium, pyridinium, alanine, calcium, magnesium, zinc, and cobaltous salts of L-ascorbate 2-sulfate (I) were prepared by either ion-exchange chromatography (Method B) or treatment of barium L-ascorbate 2-sulfate with a sulfate salt carrying the desired cation (Method A) (see page 41). Comparison of the two methods shows that Method A is easier, and gives higher yields.

TABLE 6. Paper Chromatographic Mobilities of Derivatives of L-Ascorbic Acid.

Source	Salt	Ra values* in Solvent A
Brine shrimp	Ammonium	0.60
	Barium	0.48
Synthesis	Ammonium	0.60
	Barium	0.48

$$*\text{Ra value} = \frac{\text{distance migrated by derivative}}{\text{distance migrated by L-ascorbic acid}}$$

The barium, potassium, and zinc salts of L-ascorbate 2-sulfate (I) are found to recrystallize readily. The barium salt is the preferred salt for purification. Its solubility at different temperatures is shown in Fig. 4 (p.22). All the salts are hygroscopic solids; however, they can be stored in the open under normal temperature and humidity conditions without becoming a syrup.

Elemental analysis revealed that the barium and zinc salts of I contain two moles of water of crystallization, the other salts contain no solvent of crystallization. The pyridinium and alanine salts of I were shown to be monovalent salts by the integrated intensities of appropriate proton signals in their nuclear magnetic resonance spectra and by their elemental analyses. In the other salts the L-ascorbate moiety is divalent. Most salts of I give identical specific rotations, namely $[\alpha]_D^{25}+50^\circ$ (c 1.0 in water). Only the pyridinium and alanine salts are different with a rotation of $[\alpha]_D^{25}+40^\circ$ (c 1.0 in water).

The salts of I with univalent cations have similar chromatographic mobilities. The monovalent cation salts, such as sodium, potassium, and ammonium, possess different chromatographic mobilities than the divalent cation salts, such as barium, magnesium, calcium, zinc, and cobaltous.

The pKa of the 3-hydroxyl group of L-ascorbate 2-sulfate (I) is reported to be 2.75, which was determination by ultraviolet spectrophotometry (8) and by titration (11). The C-3 hydroxyl group of I is therefore more acidic than the C-3 hydroxyl group of L-ascorbic acid (pKa_1 4.17).

A. Spectrophotometric Properties

Ultraviolet spectra of L-ascorbate 2-sulfate (I) showed a maximum absorption (λmax) of 232 nm in acidic (pH 2.0) solution. The maximum

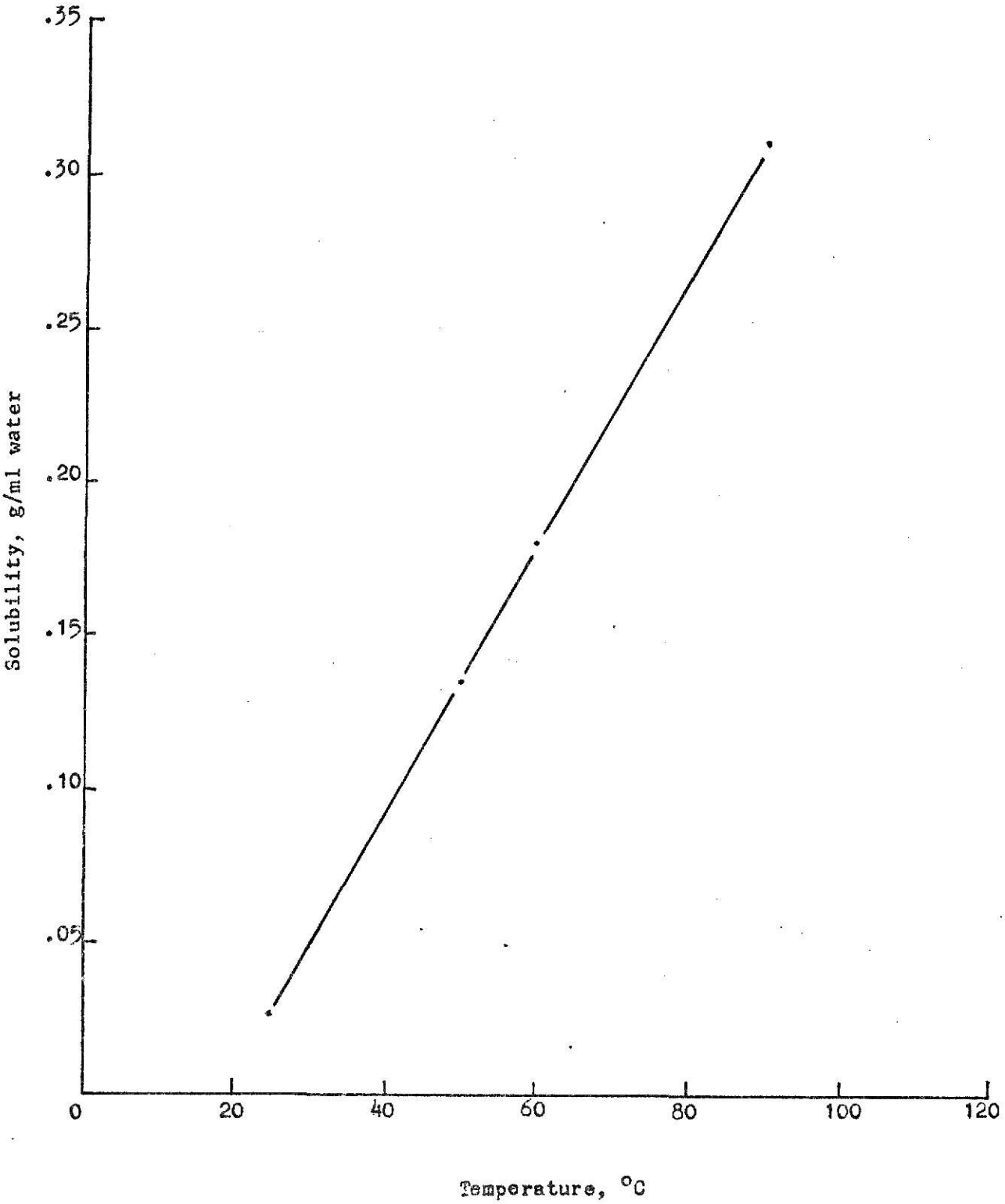


Fig. 4. Solubility of Barium L-Ascorbate 2-Sulfate in Water at Different Temperatures.

shifted to 255 nm in neutral or alkaline solutions with a concomitant increase (54%) in extinction coefficient from ϵ = 21,900 to ϵ = 29,350 (Fig. 5, p.24).

The infrared spectrum of potassium L-ascorbate 2-sulfate was measured and compared with that of potassium L-ascorbate (Fig. 6, p.25). It shows a typical pattern for a sulfate ester (34-36). Two very strong absorptions at 1265 cm^{-1} and 1230 cm^{-1} are asymmetric stretching vibrations of sulfate. Absorption at 1230 cm^{-1} is accompanied by a shoulder; this is a typical of organic sulfate esters. Also, a very strong and sharp absorption at 1050 cm^{-1} is due to the symmetric stretching vibration of sulfate. The C-O-S- vibration (medium) occurs at 830 cm^{-1} and 775 cm^{-1}.

B. Nuclear Magnetic Resonance (N.M.R.) Spectroscopic Properties

The nuclear magnetic resonance spectrum of potassium L-ascorbate 2-sulfate was examined (Fig. 7, p.26). The doublet at lowest field (τ 5.45) was assigned to H-4; the splitting ($J_{4,5}$ = 2.0 Hz) is due to vicinal coupling with H-5. The remaining signals in the spectrum result from an ABX system comprised of the C-6 methylene protons and H-5, and can be analyzed to a good approximation by first-order rules, since the chemical shifts of H-6 and H-6' are almost identical. The outer signals of the AB (H-6 and H-6') pattern were so weak that they could not be resolved above background noise; therefore, geminal couplings could not be measured. The three lines at highest field, which resulted from overlap of the four most intense lines of the AB pattern, were assigned to H-6 and H-6' (τ = 6.26). The septet at τ 5.94 was assigned to H-5; the major splittings are attributable to coupling of H-5 with H-6 and H-6' ($J_{5,6}$ = 5.5 Hz and $J_{5,6'}$ = 7.5 Hz) and the minor splittings to

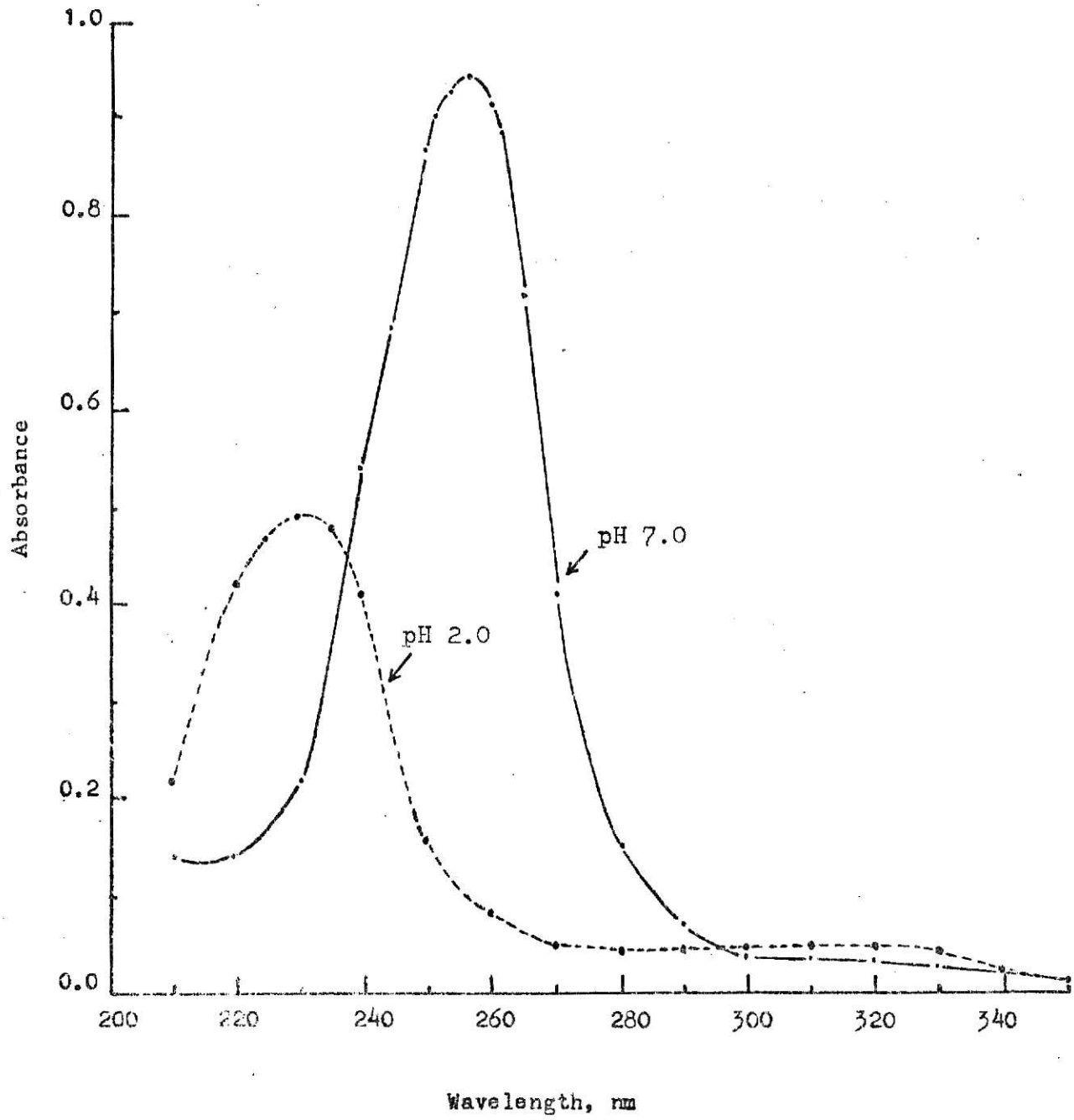


Fig. 5. U.V. Spectra of L-Ascorbate 2-Sulfate at pH 2 and pH 7.

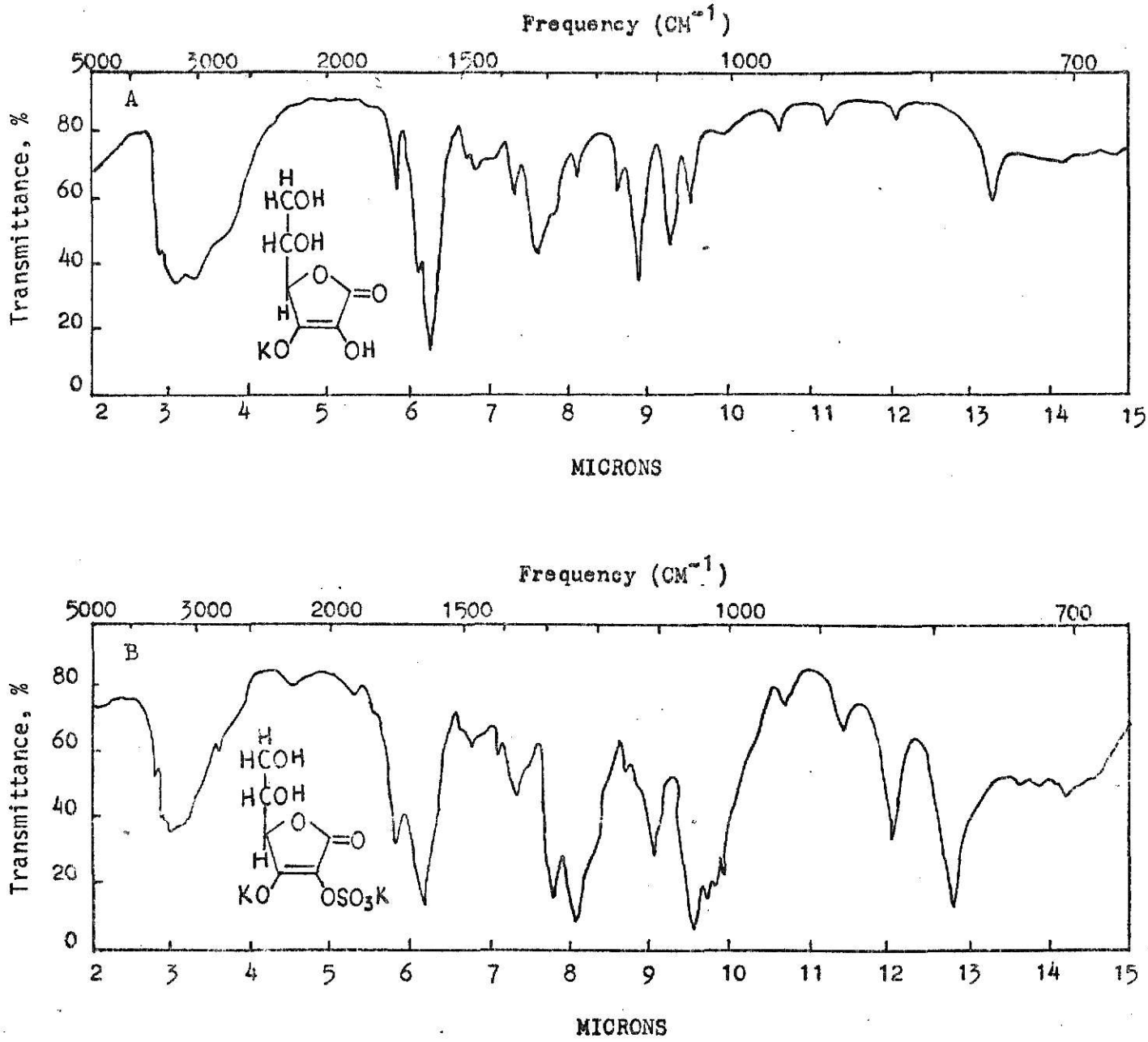
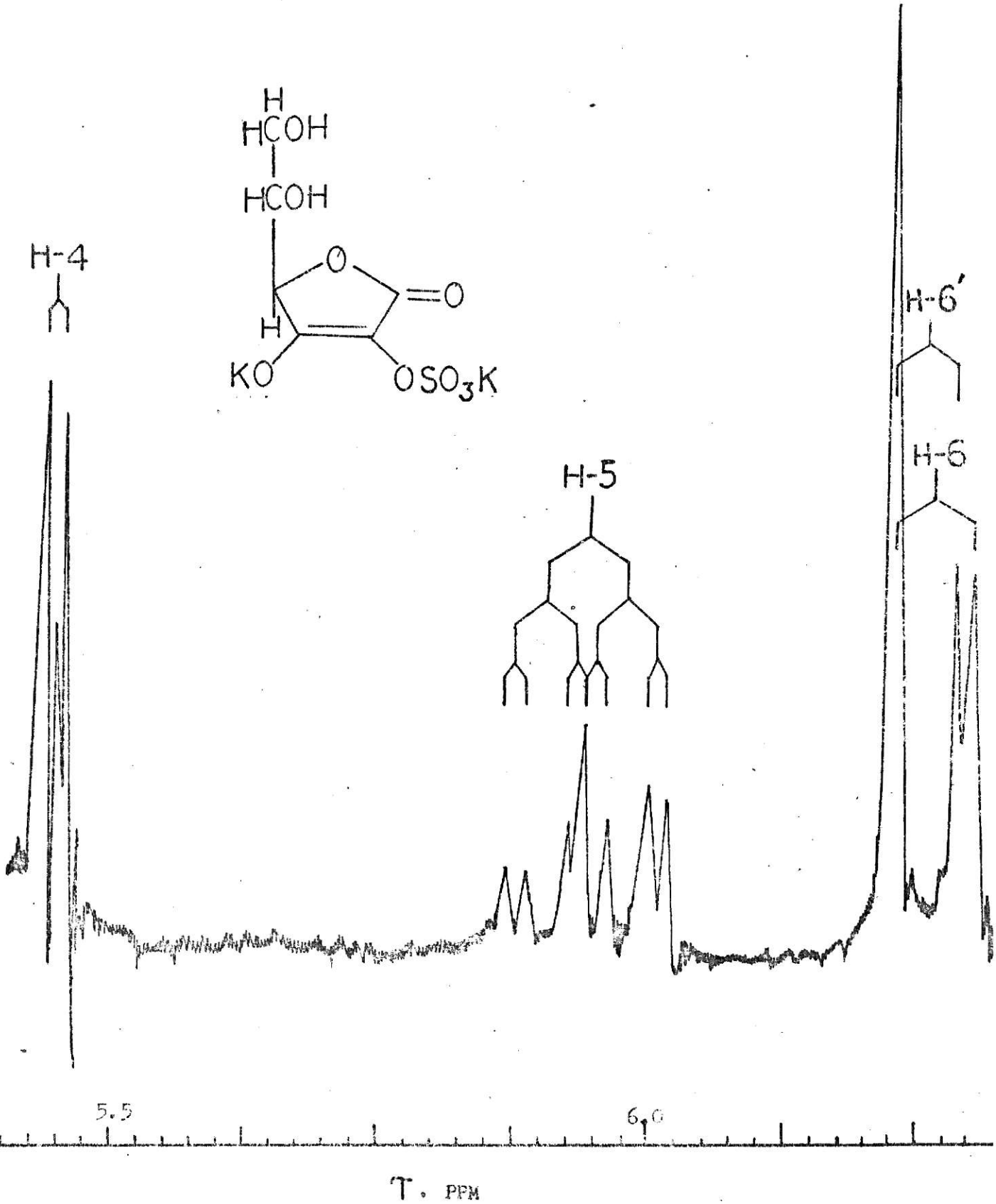


Fig. 6. Infrared Absorption Spectra of (A) Potassium L-Ascorbate and (B) Potassium L-Ascorbate 2-Sulfate.

Fig. 5. 100 MHz N.M.R. Spectrum of Potassium L-Ascorbate 2-Sulfate.



coupling with H-4.

C. Non-Oxidative Sulfate Transfer from L-Ascorbic Acid 2-(Hydrogen Sulfate) by Alconolysis

Several investigators have proposed L-ascorbate 2-sulfate (I) may transfer sulfate to biological compounds under oxidative conditions. The mechanism first proposed by Ford and Ruoff (4) is shown in Fig. 8 (p.28). Mumma (5) found that 1-octanol and 3β-cholestanol were sulfated by 5,6-O-isopropylidene-L-ascorbate 2-sulfate in N,N-dimethylformamide in the presence of bromine, oxygen, *m*-chloroperbenzoic acid, and 2,3-dichloro-5,6-dicyanobenzoquinone. Chu and Slaunwhite (26) found that I served as a sulfating agent for both equatorial and axial hydroxyls of steroids in the presence of a condensing agent and an oxidizing agent. Most recently Bond (11) also demonstrated oxidative transfer of sulfate *in vitro* from I to a variety of acceptors in anhydrous dimethylsulfoxide.

However, it has been found in this work that oxidation of compound I may not be necessary for transfer of the sulfate group. If compound I is converted to its free acid form and then placed in a solvent of low-ionizing power, such as ethanol or methanol, the sulfate group is rapidly lost.

The rate of loss of sulfate from L-ascorbic acid 2-(hydrogen sulfate) in various mixtures of methanol-water was studied. In all mixtures the sulfate was lost by a zero-order reaction which greatly accelerated as the water content of the solvent decreased (Table 7, p.29). Apparently, decreased ionization of the sulfate group in a solvent like methanol, of lower dielectric strength than water, facilitated the acid-catalyzed removal of the sulfate ester group.

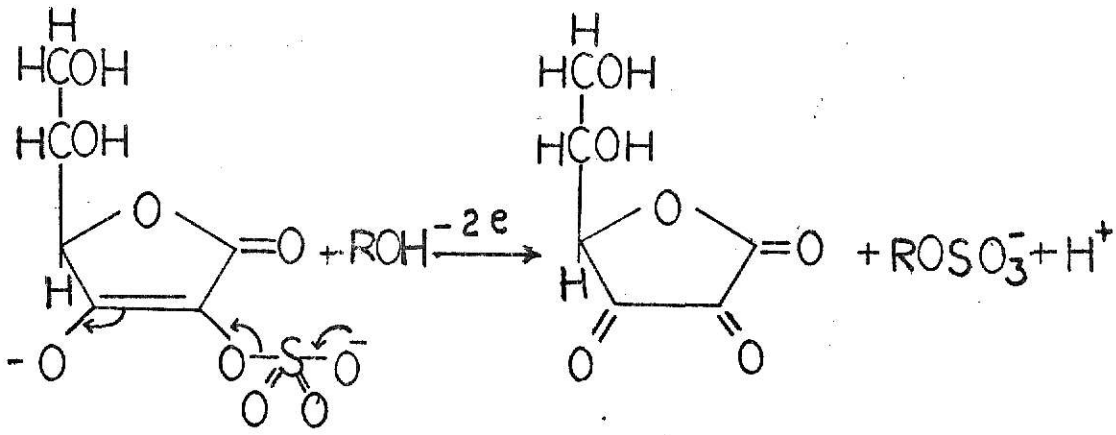


Fig. 8. Mechanism of Oxidative Sulfate Transfer from L-Ascorbate 2-Sulfate.

TABLE 7. Stability of L-Ascorbic Acid 2-(Hydrogen Sulfate) in Methanol-Water

Methanol Volume %	Velocity μ-moles/liter-min.	Relative Velocity
0	0.0254	1.00
50	0.0488	1.92
75	0.0894	3.52
95	1.63	64.1
99	19.5	770.

Information concerning the methanolysis of sulfate esters of carbohydrates in general is meager (37). Several polysaccharide sulfates have been desulfated at room temperature with hydrogen chloride (< 0.1 mole) in methanol. However, the reactions were run several days because the polysaccharides were insoluble in the reaction media (37). It can be concluded that in vivo transfer of sulfate from I could be effected by an enzyme which protonates I in proximity to the hydroxyl acceptor.

D. Comparison of the Stability of L-Ascorbate 2-Sulfate and L-Ascorbate to Oxygen, Alkali, and Acid

In boiling water and a stream of air, Quadri (24) reported that potassium L-ascorbate 2-sulfate and potassium L-ascorbate had half-lives of 140 hours and 7.6 hours, respectively. Therefore, the former is approximately eighteen times more stable than the latter. However, in a stream of nitrogen, it was found that potassium L-ascorbate 2-sulfate was only three times more stable than potassium L-ascorbate (Fig. 9, p.31). Hence, L-ascorbate 2-sulfate (I) is much more stable than L-ascorbate (II) towards oxygen at neutral pH.

In boiling, deaerated alkali (pH 13.0), L-ascorbate 2-sulfate (I) and L-ascorbate (II) had half-lives of 21 hours and 15 hours, respectively. (Fig.10, p.32). Therefore, the sulfate derivative (I) was only 1.4 times stabler than II. This result is surprising in that Quadri (24) found 96% recovery of L-ascorbate 2-sulfate (I) from pancakes, but 0% for L-ascorbate (II). Apparently the abundance of oxygen in the air in chemically leavened flour products is mainly responsible for the loss of II in these products.

In strong hot acid (pH 1.0, 100°) it was found that L-ascorbate 2-sulfate (I) had a half-life of only 4.7 minutes (Fig. 11, p.33).

Fig. 9. Thermal Stability of L-Ascorbate 2-Sulfate and L-Ascorbate in Aqueous Solution at 100° with and without Air.

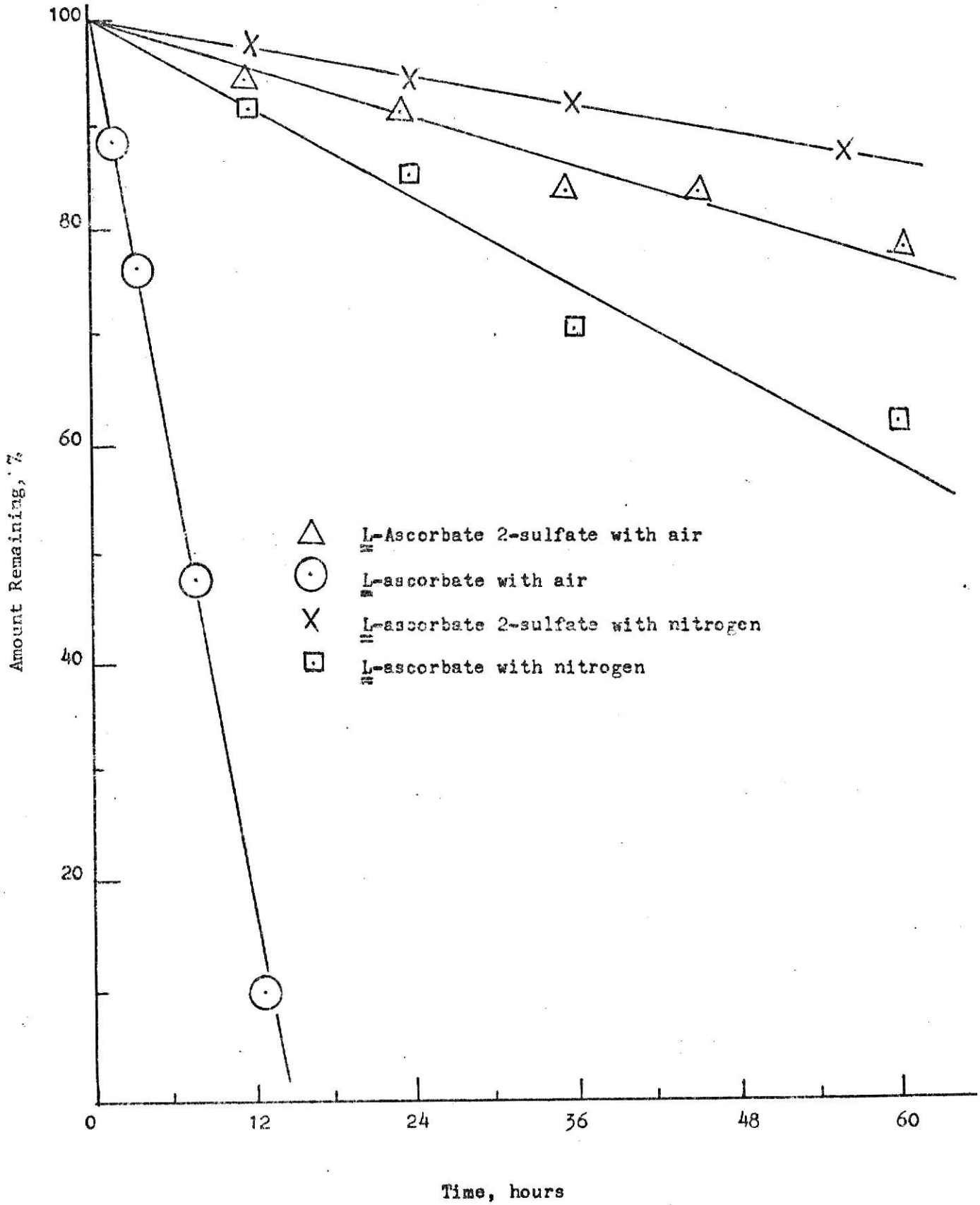


Fig. 10. Stability of L-Ascorbate 2-Sulfate and L-Ascorbate in Boiling, Deaerated Alkali (pH 13.0).

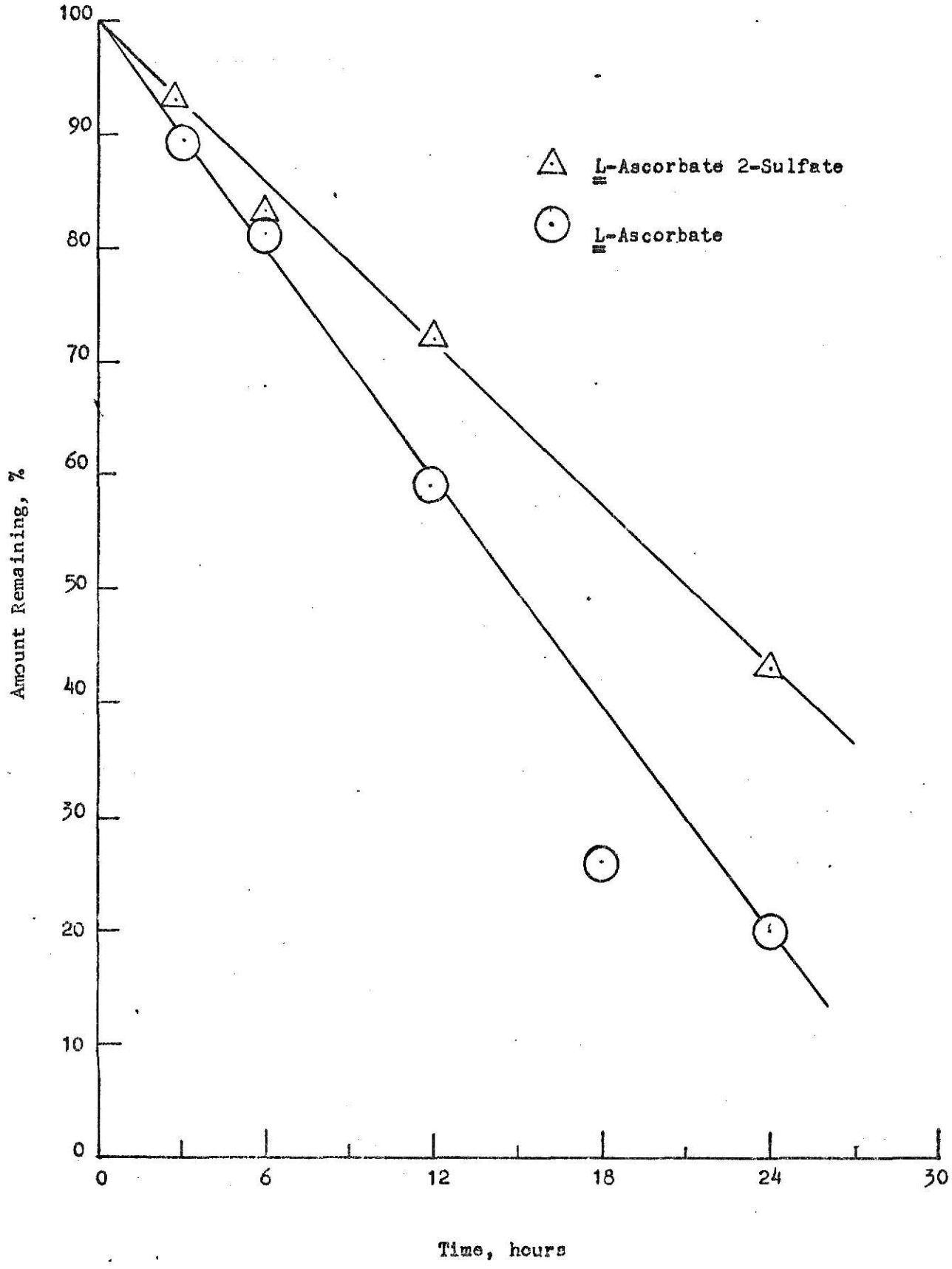
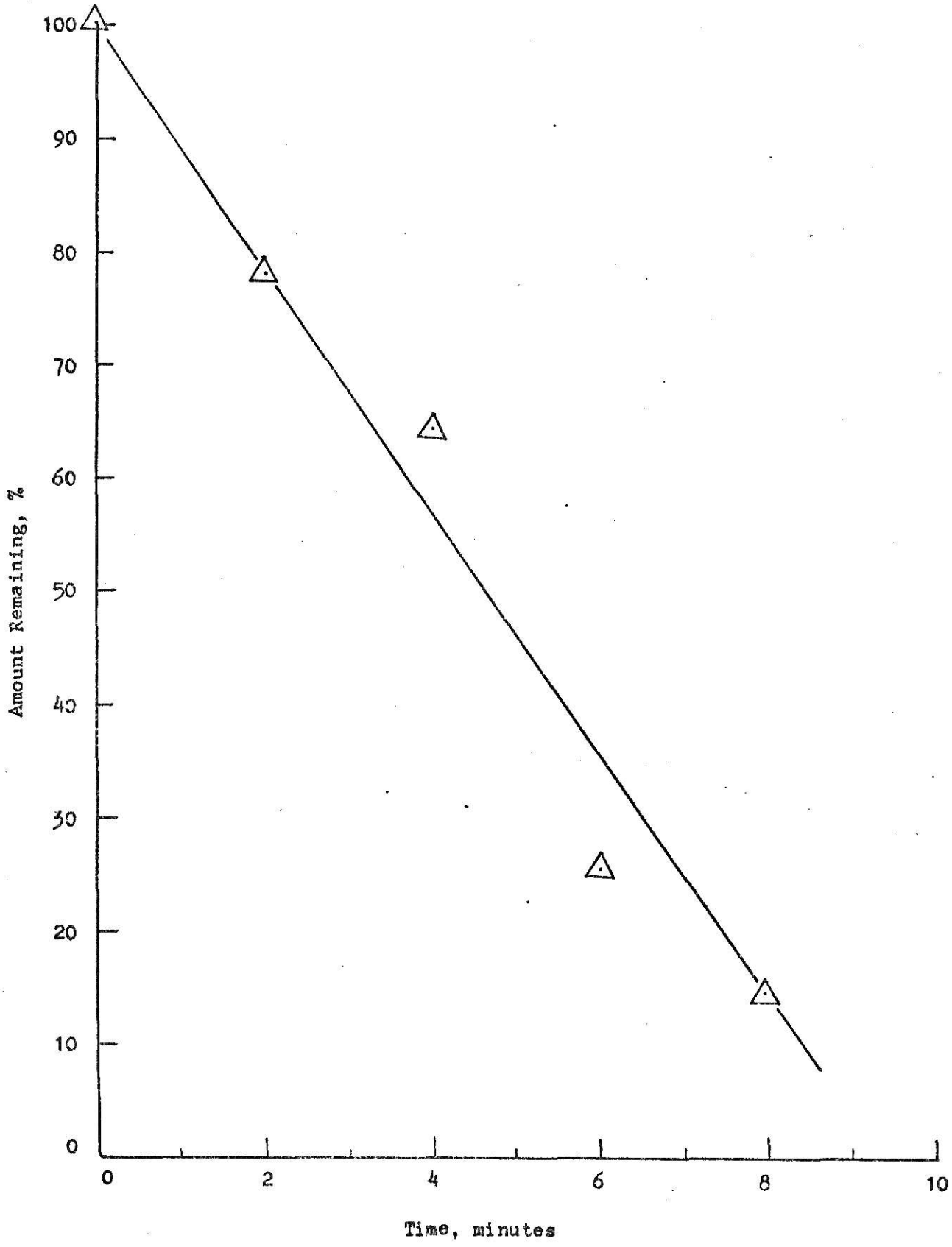
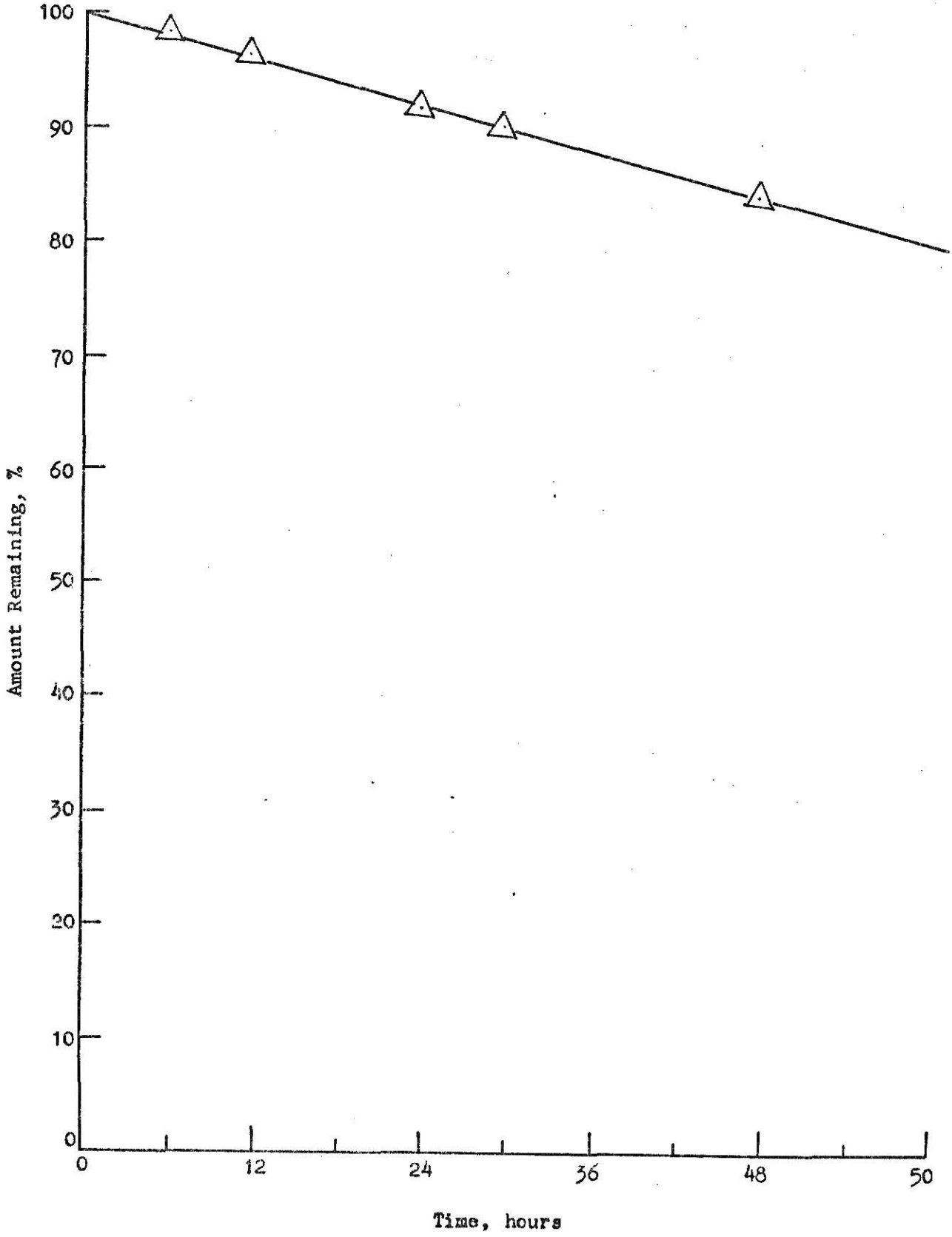


Fig. 11. Stability of L-Ascorbate 2-Sulfate in Boiling, Deaerated Acid (pH 1.0).



Mumma previously reported (15) that I possessed considerable acid stability at room temperature; only 20% of the sulfate ester group was hydrolyzed to sulfate in 6.5 hours at pH 1. In our laboratory, it was also found that I had a half-life of 150 hours in strongly acidic solution (pH 1.5) at 37° (Fig. 12, p.35). Thus, even though the sulfate group on compound (I) is labile to acid under physiological conditions that exist in the stomach of a monogastric animal, the rate of hydrolysis is too slow to convert significant amounts of L-ascorbate 2-sulfate (I) to vitamin C (II). It can be concluded that pathways other than hydrolysis of I in the stomach must be present in an animal to convert I to vitamin C (II).

Fig. 12. Stability of L-Ascorbate 2-Sulfate in Acid (pH 1.5) at 37°.



MATERIALS AND METHODS

All evaporation were under reduced pressure below 50°. M.p.s. were determined with a Thomas-Hoover apparatus. Optical rotations were obtained on a Swiss-made Kern polarimeter. A Beckman Expandomatic SS-2 pH meter was used for all pH determination. Elemental analyses were performed by Huffman Laboratories, Inc. (Wheatridge, Colorado). The analytical samples were transferred and weighed in an atmosphere of dry nitrogen, since most samples are hygroscopic.

Thin layer chromatography was performed on plates coated with silica gel G (Brinkman Instruments, Inc., Westbury, New York); developing solvents (V/V) are quoted in parentheses. The compounds were located by spraying with 50% aqueous sulfuric acid followed by charring on a hot plate. Compounds containing enolic hydroxyl groups were located by spraying with 1% ferric chloride in 95% ethanol.

Paper chromatography was performed on Whatman No. 1 paper at 25° with either of two developing solvents: (A) n-propanol-water-trichloroacetic acid (15:4:1, V/V/W); (B) n-butanol-water-acetic acid (5:3:2, V/V). Two sprays (15) were used to locate components; Spray (A) Chromatograms were dipped in a solution of 0.7% silver nitrate in acetone (5 ml saturated $AgNO_3$, 5ml concentrated NH_4OH, and 5 ml water in 1 l acetone), followed by 0.05M sodium hydroxide in methanol. After reduction of silver was adequate, the papers were washed consecutively with 5% sodium thiosulfate and water. Spray (B) A ferric chloride spray (1% ferric chloride in 95% methanol) was used to detect compounds containing enolic hydroxyl group.

Ultraviolet spectra were obtained for various salts in aqueous solutions using a Beckman model DB-G spectrophotometer. The infrared spectra of salts of L-ascorbate 2-sulfate and L-ascorbate were obtained on a Perkin-Elmer model 457 spectrophotometer. The KBr pellet was prepared by grinding a mixture of 10 mg dry salts of L-ascorbate 2-sulfate or L-ascorbate and 1 g dry potassium bromide together until they appeared to be completely mixed, then pressing the mixture into a disc-shaped pellet.

Nuclear magnetic resonance spectra were recorded with a Varian XL-100 or T60 spectrometer. Chemical shifts are reported in τ-values from the reference signal of sodium 4,4-dimethyl-4-silapentane-1-sulfonate. Fifty milligrams of potassium or another salt of L-ascorbate 2-sulfate was dissolved in 0.5 ml 99% deuterium oxide, the solution heated for 2 minutes, then examined at the normal operating temperature of the n.m.r. instrument.

1. Synthesis of L-Ascorbate 2-Sulfate in N,N-Dimethylformamide. 5,6-O-Isopropylidene-L-Ascorbic Acid: The procedure used was identical to the one published by Jackson and Jones (38).

A mixture of L-ascorbic acid (300 g) in acetone (1350 ml) containing acetyl chloride (37.5 ml) was stirred vigorously at room temperature for 2 hours. Crystalline 5,6-O-isopropylidene-L-ascorbic acid separated from the solution was collected by filtration, and washed with cold acetone-hexane (4:7, V/V), then dried in vacuo over potassium hydroxide. Yield, 348 g (97%) of needle-shaped crystals with m.p. 217-223°. Thin layer chromatographic analysis of the product using ethyl ether-acetic acid (96/4, V/V) showed only one component (R_f = 0.7) as the reaction product. Pyridine-Sulfur Trioxide Complex: The method used to prepare the complex is essentially the method reported by Guiseley and Ruoff (39).

Eighty milliliters of stabilized liquid sulfur trioxide (Sulfan B, Allied Chemical Corp., Morristown, N.Y.) were added dropwise to a mixture of 2.2 liters of dry, alcohol-free chloroform and 210 ml of anhydrous pyridine in a 5-liter, 3-necked flask. The temperature inside the flask was maintained below 5°. After the addition was complete, the product was collected by filtration, and washed with cold, dry alcohol-free chloroform and aspirated under a rubber dam. The solid was dried over phosphorous pentaoxide in a vacuum desiccator. Yield of crystalline complex was 304 g with m.p. 150-152°.

Barium L-Ascorbate 2-Sulfate: To anhydrous N,N-dimethylformamide (20 ml) containing 10 g of 5,6-O-isopropylidene-L-ascorbic acid and anhydrous pyridine (4 ml) at 25° was added dropwise with stirring a mixture of pyridine-sulfur trioxide (14 g) in N,N-dimethylformamide (40 ml). The reaction mixture was stirred 12 hours, at which time thin-layer chromatographic analysis using methanol-ethyl acetate (85/15) showed the starting material (R_f = 0.7) was completely reacted. The solvent was recovered by evaporation below 50° and the reaction mixture was concentrated to a thick syrup. Addition of water (30 ml) dropped down the pH of the aqueous solution to 1.0. The mixture was kept for 30 minutes at 25°, its pH then adjusted to 7.0 by the addition of a saturated aqueous solution of barium hydroxide, and barium sulfate (8.38 g) was removed by filtration through a glass fiber filter pad (grade 984H, Arthur H. Thomas Co., Philadelphia, Pa.). The filtrate was decolorized with charcoal, and concentrated to a volume of 50 ml. Methanol (50 ml) was added, the mixture cooled, and the crystalline solid collected by filtration. The white solid was dried over phosphorus pentaoxide to give 14.8 g (75.0% yield) of barium L-ascorbate 2-sulfate dihydrate with m.p. 220-225° (dec.). Recrystallization gave

analytically pure barium L-ascorbate 2-sulfate dihydrate with m.p. 230-235° (dec.), $[\alpha]_D^{25}$ +50° (c 1.0 in water). Anal. Calc. for $C_6H_6BaO_9S \cdot 2H_2O$: C, 16.85; H, 2.36; S, 7.50. Found: C, 16.50; H, 2.32; S, 7.00.

Role of Added Pyridine in Sulfation of 5,6-O-Isopropylidene-L-Ascorbic Acid in N,N-Dimethylformamide: To anhydrous N,N-dimethylformamide (20 ml) containing 10 mg of 5,6-O-isopropylidene-L-ascorbic acid and 4 ml of anhydrous pyridine (Reaction A), or no free pyridine (Reaction B) was added 14 g of pyridine-sulfur trioxide. Both reaction mixtures were stirred. Aliquots were removed from Reaction A and B with time, and characterized with paper chromatography. Solvent A, n-propanol-water-trichloroacetic acid (15:4:1, V/V/W), was used as developing solvent. Each separated component was extracted with water, hydrolyzed with acid (pH 1.0, 30 minutes), then neutralized with sodium bicarbonate. The ultraviolet absorbance of each neutralized component was measured at 255 nm. The amount of each component was calculated from the ratio of the absorbtion intensities of these components. The components isolated after preparative paper chromatography were reexamined by paper chromatography.

2. Synthesis of L-Ascorbate 2-Sulfate in an Aqueous Medium. Trimethylamine-Sulfur Trioxide Complex: The method used to prepare the complex is essentially the method reported by Guiseley and Ruoff (39).

Eighty milliliters of stabilized liquid sulfur trioxide (Sulfan B, Allied Chemical Corp., Morristown, N.J.) were added dropwise to a mixture of 2.2 liters dry, alcohol-free chloroform and 232 ml of trimethylamine in a 5-liter, 3-necked flask. The temperature inside the flask was maintained below 5°. After the addition was complete, the product was collected by

filtration, washed with cold, dry, alcohol-free chloroform and aspirated under a rubber dam. The solid was dried over phosphorous pentaoxide in a vacuum desiccator. Yield of crystalline complex was 240 g with m.p. 235-239°.

Barium L-Ascorbate 2-Sulfate: To a solution of L-ascorbic acid (2 g, 11.5 mmoles) in water (25.0 ml) was added 10M aqueous sodium hydroxide until the pH of the medium reached 10.0. The mixture was heated to 70° and trimethylamine-sulfur trioxide (2.4 g, 16.2 mmoles) was added. The pH of the reaction medium was maintained at 9.5-10.0 by periodically adding 10M aqueous sodium hydroxide. The reaction mixture was stirred 30 minutes, at which time thin-layer chromatographic analysis using methanol-water (6/4) as developing solvent and spraying with 1% ferric chloride in 95% methanol, showed no starting material remained. The mixture was cooled and passed through a strong cation-exchange resin, Amberlite IR-120 (H^+). The effluent and washings from the column were combined and neutralized with a saturated aqueous solution of barium hydroxide. The mixture was filtered to remove insoluble barium sulfate and the filtrate was concentrated under diminished pressure to a small volume (15 ml). An equal volume of methanol was added, the mixture was cooled, and the solid was removed by filtration. The solid was dried in vacuum over phosphorous pentaoxide, yield 3.9 g (80%) with m.p. 212-219°. Recrystallization gave analytically pure barium L-ascorbate 2-sulfate dihydrate with m.p. 230-235°, $[\alpha]_D^{25}$ + 50° (c 1.0 in water). The compound gave infrared, ultraviolet, and nuclear magnetic resonance spectra, and chromatographic analysis identical with L-ascorbate 2-sulfate prepared in N,N-dimethylformamide.

Conversion of L-Ascorbate to L-Ascorbate 2-Sulfate at Different pH Levels: L-Ascorbic acid (2.0 g) was reacted in alkali (25.0 ml) of differing molarity (pH 8.5, 9.0, 9.5, 10,0, 10.5, 11.0, 11.5) with trimethylamine-sulfur trioxide (2.4 g) at 70° until the starting material disappeared as evidenced by thin-layer chromatography. Aqueous sodium hydroxide (10 M) was added periodically to each reaction to maintain its pH during reaction. Sulfation of the 2-hydroxyl of L-ascorbate (II) was determined by dilution of a reaction mixture to 100.0 ml and passage of an aliquot (500 μl) through a weakly basic anion-exchange resin (200 mg, Type AG3-X4A, chloride form, 200-400 mesh, Biorad Lab., Richmond, California). The resin was washed with water, and the sulfated derivatives were eluted with 1M aqueous ammonium sulfate (100.0 ml). After dilution absorbances were measured at 255 nm and pH 6.5. A control run in which L-ascorbic acid was degraded in alkali at pH 10.5 for six hours gave an effluent which showed no absorbance at 255 nm. The results are given in Table 5 (p.18). The conversion of L-ascorbate to the 2-sulfate (I) and the 2,6-disulfate ester of L-ascorbate at pH 9.5-10.5 was determined by preparative paper chromatography and ultraviolet spectrophotometric analysis as described before (p. 39).

3. Preparation and Properties of Salts of L-Ascorbate

The sodium, potassium, ammonium, pyridinium, calcium, magnesium, zinc, cobaltous, aluminum, and alanine salts of L-ascorbate 2-sulfate were prepared by either, or both, of the following two procedures. The procedures for the preparation of the magnesium salt illustrate the two methods.

Method A: Barium L-ascorbate 2-sulfate dihydrate (2.000 g) was dissolved in water (100 ml) and one equivalent of magnesium sulfate heptahydrate

(1.153 g) was added. The mixture was stirred one hour and the insoluble barium sulfate (1.270 g) removed by filtration. Evaporation of the water gave 1.269 g (97.5% yield) of a crystalline solid, m.p. 205-210° (dec.), $[\alpha]_D^{25}$ + 50° (c 1.0 in water). Elemental analysis: Calc. for $C_6H_6MgO_9S$; C, 25.88; H, 2.17; S, 11.51. Found: C, 26.62; H, 2.24; S, 10.86.

Method B: Barium L-ascorbate 2-sulfate dihydrate (3.0 g) was dissolved in water (100 ml) and the aqueous solution was passed through 12 ml of a cation-exchange resin, Amberlite IR-120 (Mg^{++}). The column was washed with two volumes of water, the effluents combined and concentrated to dryness. The solid crystalline product amounted to 1.48 g (76% yield), and it was identical in all respects to magnesium L-ascorbate 2-sulfate isolated by Method A.

Potassium L-Ascorbate 2-Sulfate

This compound was crystallized from 50% aqueous methanol as a crystalline solid which contained no solvent of crystallization; m.p. 87-89.5°. $[\alpha]_D^{25}$ + 50° (c 1.0 in water). Elemental analysis: Calc. for $C_6H_6K_2O_9S$; C, 21.05; H, 2.14; S, 9.60. Found: C, 21.05; H, 2.14; S, 9.60. Solubility in water (g/ml); 0.795 at 25°.

Pyridinium L-Ascorbic Acid 2-Sulfate

This compound is a crystalline solid with m.p. 133-134°, $[\alpha]_D^{25}$ + 40° (c 1.0 in water). The nuclear magnetic resonance spectrum of the compound gave the correct proportion of aromatic to ascorbic acid protons for a mono-pyridinium salt (24). Elemental analysis: Calc. for $C_6H_7(C_5H_5N)O_9S$; C, 39.52; H, 3.62; S, 9.59. Found: C, 39.69; H, 3.72; S, 9.57.

Sodium L-Ascorbate 2-Sulfate

This compound is a crystalline solid with m.p. 190-210° (dec.), $[\alpha]_D^{25}$ + 50° (c 1.0 in water). Elemental analysis: Calc. for $C_6H_6Na_2O_9S$: C, 24.01; H, 2.02. Found: C, 23.35; H, 2.09.

Calcium L-Ascorbate 2-Sulfate

This compound is a crystalline solid with m.p. 220-240° (dec.), $[\alpha]_D^{25}$ + 50° (c 1.0 in water). Elemental analysis: Calc. for $C_6H_6CaO_9S$: C, 24.49; H, 2.06; S, 10.90. Found: C, 24.23; H, 2.02; S, 10.86.

Zinc L-Ascorbate 2-Sulfate

This compound is a crystalline solid with m.p. 185-190° (dec.), $[\alpha]_D^{25}$ + 50° (c 1.0 in water). Elemental analysis: Calc. for $C_6H_6ZnO_9S \cdot 2H_2O$: C, 20.27; H, 2.84. Found: C, 20.26; H, 3.15.

Magnesium L-Ascorbate 2-Sulfate

This compound is a crystalline solid with m.p. 205-210° (dec.), $[\alpha]_D^{25}$ + 50° (c 1.0 in water). Elemental analysis: Calc. for $C_6H_6MgO_9S$; C, 25.88; H, 2.17; S, 11.51. Found: C, 25.62; H, 2.24; S, 10.86.

Cobaltous L-Ascorbate 2-Sulfate

This compound is a crystalline solid with m.p. 196-198° (dec.), $[\alpha]_D^{25}$ + 50° (c 1.0 in water). Elemental analysis: Calc. for $C_6H_6CoO_9S$: C, 20.62; H, 2.88. Found: C, 20.31; H, 3.18.

Alanine L-Ascorbic Acid 2-Sulfate

This compound was crystallized from cold aqueous acetone as a crystalline solid with m.p. 155-160°, $[\alpha]_D^{25}$ + 40° (c 1.0 in water).

Ammonium L-Ascorbate 2-Sulfate

This compound is a crystalline solid with m.p. 90-95° (dec.), $[\alpha]_D^{25}$ + 50° (c 1.0 in water).

4. Proof of Structure of L-Ascorbate 2-Sulfate

A sample of ammonium L-ascorbate 2-sulfate isolated from brine shrimp was kindly donated by Dr. A. D. Bond of Oak Ridge National Laboratory, Oak Ridge, Tennessee (11). The donated material was converted to barium salt by passing through a cation-exchange resin, Amberlite IR-120 (H+).

The effluent was neutralized with saturated aqueous barium hydroxide, filtered, and the filtrate concentrated under diminished pressure. Crystallization from 50% aqueous methanol (V/V) gave a white solid which was dried over phosphorous pentaoxide. The material had m.p. 225-230°, $[\alpha]_D^{25}$ + 50° (c 1.0 in water). The barium salt of L-ascorbate 2-sulfate was chromatographed with that prepared by the synthetic methods in our laboratory. The barium salts from both laboratories gave identical ultraviolet, infrared, and nuclear magnetic resonance spectra.

5. Stability of L-Ascorbic Acid 2-(Hydrogen Sulfate) in Methanol-Water

An aliquot (200 μl) of a solution of L-ascorbic acid 2-(hydrogen sulfate) (600 mg) in water (2.0 ml) was diluted with water and methanol at 50° to a total volume of 20.0 ml. The disappearance of L-ascorbic acid 2-(hydrogen sulfate) was followed using the same analytical scheme described under Conversion of L-Ascorbate to L-Ascorbate 2-Sulfate at Different pH Levels (p.41).

6. Stability of L-Ascorbate 2-Sulfate and L-Ascorbate

Thermal Stability of L-Ascorbate 2-Sulfate and L-Ascorbate in Aqueous Solution at 100°: Potassium L-ascorbate 2-sulfate (500 mg, 1.5 mMoles) or potassium L-ascorbate (322 mg, 1.5 mMoles) was dissolved in 100 ml of water, which had been previously boiled. A stream of nitrogen (24 ml/min. at 1 atmosphere and 25°) was bubbled through the solution while it was heated to reflux. Aliquots of the reaction mixture were withdrawn with time, and analyses for L-ascorbate 2-sulfate were performed as described before (p. 41). Analysis of L-ascorbate was accomplished by fluorometry (40).

Destruction of L-Ascorbate 2-Sulfate and L-Ascorbate with Strong Acid and Strong Base at 100°: Potassium L-ascorbate 2-sulfate (200 mg, 0.60 mMoles)

was dissolved in either 100 ml of aqueous hydrochloric acid (pH 1.0) or aqueous potassium hydroxide (pH 13.0), both of which had been previously boiled before adding the salts. Aliquots from the reaction mixture were withdrawn with time, and analyses for L-ascorbate 2-sulfate were performed as previously described using Biorad AG3-X4A resin. Analyses for L-ascorbate 2-sulfate were performed using ultraviolet spectrophotometry. An aliquot (1.0 ml) from one of the L-ascorbate reaction mixtures was quickly diluted to 100.0 ml with 5% metaphosphoric acid, and absorbances were measured at 245 nm at pH 2.0.

Destruction of L-Ascorbate 2-Sulfate with Strong Acid at 37°: Potassium L-ascorbate 2-sulfate (500 mg, 1.5 mMoles) was dissolved in 100 ml of aqueous hydrochloric acid (pH 1.5). The reaction mixture was maintained at 37° in a water bath. Aliquots of the reaction mixture were withdrawn at various times and analyzed as described before (p.41).

SUMMARY

Previous methods of preparing L-ascorbate 2-sulfate (I) were found inadequate for the isolation of large quantities of pure salts of I; yields were generally disappointing (<22%), the desired product (I) was frequently contaminated with inorganic sulfate, or compound I had to be purified by column chromatography. If compound I is to become attractive as a stable source of vitamin C, there is a need for improved methods of preparation and isolation of I. Two improved syntheses of I are described herein.

In one improved snythesis, 5,6-O-isopropylidene-L-ascorbic acid was first dissolved in N,N-dimethylformamide and neutralized by addition of a tertiary amine (for example, pyridine). The resulting salt of 5,6-O-isopropylidene-L-ascorbate was then sulfated using pyridine-sulfur trioxide complex. In this manner, yields of I could be increased to 75% as compared to less than 50% for direct sulfation of 5,6-O-isopropylidene-L-ascorbic acid. The added tertiary amine is believed to prevent the hydrolytic removal of the isopropylidene group before or during sulfation of 5,6-O-isopropylidene-L-ascorbate.

In the other improved synthesis of I, L-ascorbate (II) was reacted with trimethylamine-sulfur trioxide complex in alkali (pH 9.5-10.5) to give nearly quantitative yields of I. The reaction was complete in 30 minutes at 70°, and barium L-ascorbate 2-sulfate dihydrate was easily isolated in 80% yield. Other salts of I that were found to crystallize readily included potassium and zinc. Compound I, prepared by chemical synthesis, was shown to be identical to the L-ascorbate 2-sulfate (I) occurring naturally in brine shrimp.

L-Ascorbic acid 2-(hydrogen sulfate) breaks down quantitatively to L-ascorbic acid in less than six minutes in 99% methanol-water at 50°. It can be concluded that *in vivo* transfer of sulfate from I could be effected by an enzyme which protonates I in proximity to the hydroxyl acceptor.

In boiling water, L-ascorbate 2-sulfate (I) is more stable toward oxygen than L-ascorbate (II). In boiling, deaerated alkali (pH 13.0), I and II have half-lives of 21 hours and 15 hours, respectively. In strong hot acid (pH 1.0, 100°), I has a half-life of only 4.7 minutes. However, hydrolysis of the sulfate group on I was very slow at pH 1.5 and 37°, under which condition I has a half-life of 150 hours. It is therefore doubtful that I could be converted to vitamin C by acid hydrolysis in the stomach of a monogastric animal.

ACKNOWLEDGEMENTS

The author wishes to express his deep gratitude to Dr. Paul A. Seib, major professor, for his able guidance and valuable suggestions during studies, research and preparation of this manuscript.

Special acknowledgement is due to Dr. D. R. Lineback, Dr. R. C. Hoseney, and Dr. C. W. Deyoe for serving on the supervisory committee and reviewing this manuscript.

Appreciation is expressed to Dr. Y. T. Liang for her assistance during the course of this work.

For providing facilities, funds and materials needed to carry out this work, the author is grateful to Dr. W. J. Hoover, Head of the Department of Grain Science and Industry.

The author wishes to express his appreciation to his wife, Lisa, for her patience, encouragement, and understanding.

LITERATURE CITED

1. LIPMANN, F. Biological sulfate activation and transfer. Science 128: 575 (1958).

2. DODGSON, K. S. Synthesis and hydrolysis of sulfuric esters. Proc. Intern. Congr. Biochem. 13: 23 (1958) (pub. 1960).

3. ROBERTSON, W. VAN B. Ascorbic acid in connective tissues. Ann. N. Y. Acad. Sci. 92: 164 (1961).

4. FORD, E. A., and RUOFF, P. M. Synthesis and oxidation of 5,6-O-isopropylidene-L-ascorbic acid 3-sulfate. Chem. Commun. 24: 630 (1965).

5. MUMMA, R. O. Ascorbic acid sulfate as a sulfating agent. Biochem. Biophys. Acta. 165: 571 (1968).

6. MUMMA, R. O., and VERLANGIERI, A. J. In vivo sulfation of cholesterol by L-ascorbic acid 3-sulfate as a possible explanation for the hypocholestemic effects of ascorbic acid. Fed. Proceedings, Fed. American Soc. Exp. Biol. 30: 370 (1971).

7. VERLANGIERI, A. J., and MUMMA, R. O. In vivo sulfation of by L-ascorbic acid 2-sulfate. Atherosclerosis, in press (1972).

8. MEAD, C. G., and FINAMORE, F. J. The occurrence of ascorbic acid sulfate in the brine shrimp, artemia salina. Biochemistry 8: 2652 (1969).

9. GOLUB, A. L., and FINAMORE, F. J. Ascorbic acid sulfate metabolism in the brine shrimp. Federation Proceedings Fed. American Soc. Exp. Biol. 31(2): 2765 (1972).

10. BAKER, E. M., III, HAMMER, D. C., TOLBERT, B. M., and CANHAM, J. E. Ascorbate sulfate urinary metabolite of ascorbic acid in man. Science 173: 826 (1971).

11. BOND, A. D., McCLELLAND, B. W., EINSTEIN, J. R. and FINAMORE, F. J. Ascorbic acid-2-sulfate of the brine shrimp, artemia salina. Arch. Biochem. Biophys. 153: 207 (1972).

12. McCLELLAND, B. W., and EINSTEIN, J. R. Crystal and molecular structure of barium 2-sulfuryl-L-ascorbate dihysrate. Arch. Biochem. Biophys. 153: 207 (1972).

13. MUMMA, R. O., McKEE, E. E., VERLANGIERI, A. J., and BARRON, G. P. Anti-scorbutic effect of ascorbic acid 2-sulfate in the guinea pig. Nutrition Reports Internation 6(3): 122 (1972).

14. HALVER, J. E., JOHNSON, C. L., SMITH, R. R., TOLBERT, B. M., and BAKER, F. M. Vitamin C_3 reduces fish scurvy. Federation Proceedings, Fed. American Soc. Exp. Biol. 31(2): Abst. No. 1764 (1972).

15. MUMMA, R. O., VERLANGIERI, A. J., and WEBSER, W. W., II. L-ascorbic acid 3-sulfate. Preparation and characterization. Carbohydrate Res. 19: 127 (1971).

16. TOLBERT, B. M., ISHERWOOD, D. J., ATCHLEY, R. W., and BAKER, E. M. Ascorbic acid sulfate: synthesis and properties. Federation Proceedings, Fed. American Soc. Exp. Biol. 30(2), Abst. No. 1819 (1971).

17. OLLIBER, M., SEBRELL, W. H., Jr., and HARRIS, R. S. In "The Vitamins", Academic Press, New York, N. Y. eds., I, pp. 261-8 (1954).

18. OLLIBER, M., SEBRELL, W. H., Jr., and HARRIS, R. S. In "The Vitamins", Academic Pres, New York, N. Y. eds., I, p. 245 (1954).

19. CHANEY, M. S., and ROSS, M. L. "Nutrition", Houghton Mifflin Co., New York, N. Y. pp. 246-9 (1971).

20. HERBERT, R. W., HIRST, E. L., PERCIVAL, REYNOLDS, R. J. W., and SMITH, F. The constitution of ascorbic acid. J. Chem. Soc. p. 1270 (1933).

21. BAUERNFIEND, J. C., and PINNERT, D. M. Food processing with added ascorbic acid. Adv. in Food Research, pp. 272-277 (1970).

22. SMITH, F., SEBRELL, W. H., Jr., and HARRIS, R. S. In "The Vitamins", Academic Press, New York, N. Y. pp. 202-5 (1954).

23. BAUERNFIEND, J. C., and PINNERT, D. M. Food processing with added ascorbic acid. Adv. in Food Research, p. 275 (1970).

24. QUADRI, S. F. Improved method of synthesis of L-ascorbate 2-sulfate and its recovery from some cereal products. Ph.D. Dissertation, Kansas State University (1973).

25. FORD, E. A. Part II. Synthesis of sulfated derivatives of ascorbic acid. Ph.D. Dissertation, Syracuse University (1967), Dissertation Abstracts 28(B): Abst. No. 4059 (1968).

26. CHU, T. W., and SLAUNWHITE, W. R., Jr. The nonenzymic sulfation of androsterone. Steroids 12: 309 (1968).

27. MUMMA, R. O., HOIBERG, C. P., and SIMPSON, R. Preparation of sulfate esters. N,N'-Dicyclohexylcarbodiimide-mediated sulfation of carbohydrate derivatives. Carbohydrate Res. 14: 119 (1970).

28. TOLBERT, B. M., ISHERWOOD, R. W., ATCHLEY, R. W., and BAKER, E. M. Chemistry of the ascorbate sulfates. Federation Proceedings Fed. American Soc. Exp. Biol. 31(2), Abst. No. 2761 (1972).

29. CLARK, V. M., HERSHEY, J. W. B., and HUTCHINSON, D. W. The oxidative dephosphorylation of phosphoryl esters derived from L-ascorbic acid. Experientia 22: 425 (1966).

30. NOMURA, H., SHIMOMURA, M., and MORIMOTO, S. L-ascorbic acid derivatives. VI Phosphorylation of L-ascorbic acid and its isopropylidene derivatives. Chem. Pharm. Bull. (Tokyo) 19:335 (1971).

31. FIESER, M. and FIESER, L. Reagents for organic synthesis. Wiley-Interscience, Vol. 2, p. 6 (1969).

32. SMITH, H. E., RUSSELL, C. R., and RIST, C. E. Preparation and properties of sulfated wheat flour. Cereal Chem. 39: 273 (1962).

33. GILBERT, E. E. Reactions of sulfur trioxide, and of its adducts, with organic compounds. Chem. Rev. 61: 549 (1961), p. 553.

34. CHIHARA, G. Applications of infrared spectra to medicine and biology. Chem. Pharm. Bull. (Tokyo) 8: 988 (1960).

35. LLOYD, A. G., and DODGSON, K. S. Infrared studies on sulphate esters. II. Monosaccharide sulfates. Biochem. Biophys. Acta 46: 116 (1961).

36. NAKANISHI, K. Infrared absorption spectroscopy, Holden-Day, Inc., San Francisco, (1962), p. 55.

37. TURVEY, J. R. Sulfates of the simple sugars. VII. Other methods of desulfation. Adv. Carbohydrate Chem. 20: 203 (1965).

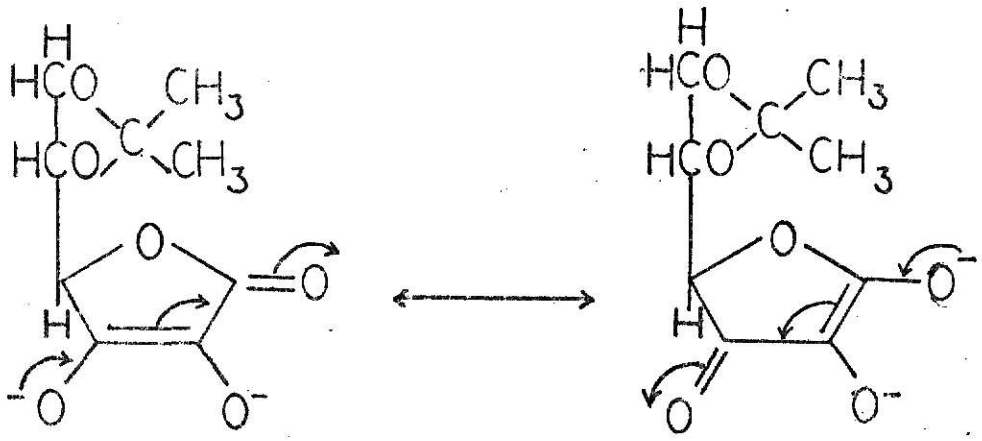
38. JACKSON, K. G. A., and JONES, J. K. N. Synthesis of 3-hexuloses. Can. J. Chem. 47: 2498 (1969).

39. GUISELEY, K. B., and RUOFF, P. M. Monosaccharide sulfates. I. Glucose 6-sulfate. Preparations, characterization of the crystalline potassium salt, and kinetic studies. J. Org. Chem. 26: 1248 (1961).

40. DEUTSCH, M. J., and WEEKS, C. F. Microfluorometric assay for vitamin C. J. Ass. Off. Agr. Chem. 48: 1248 (1965).

APPENDIX I

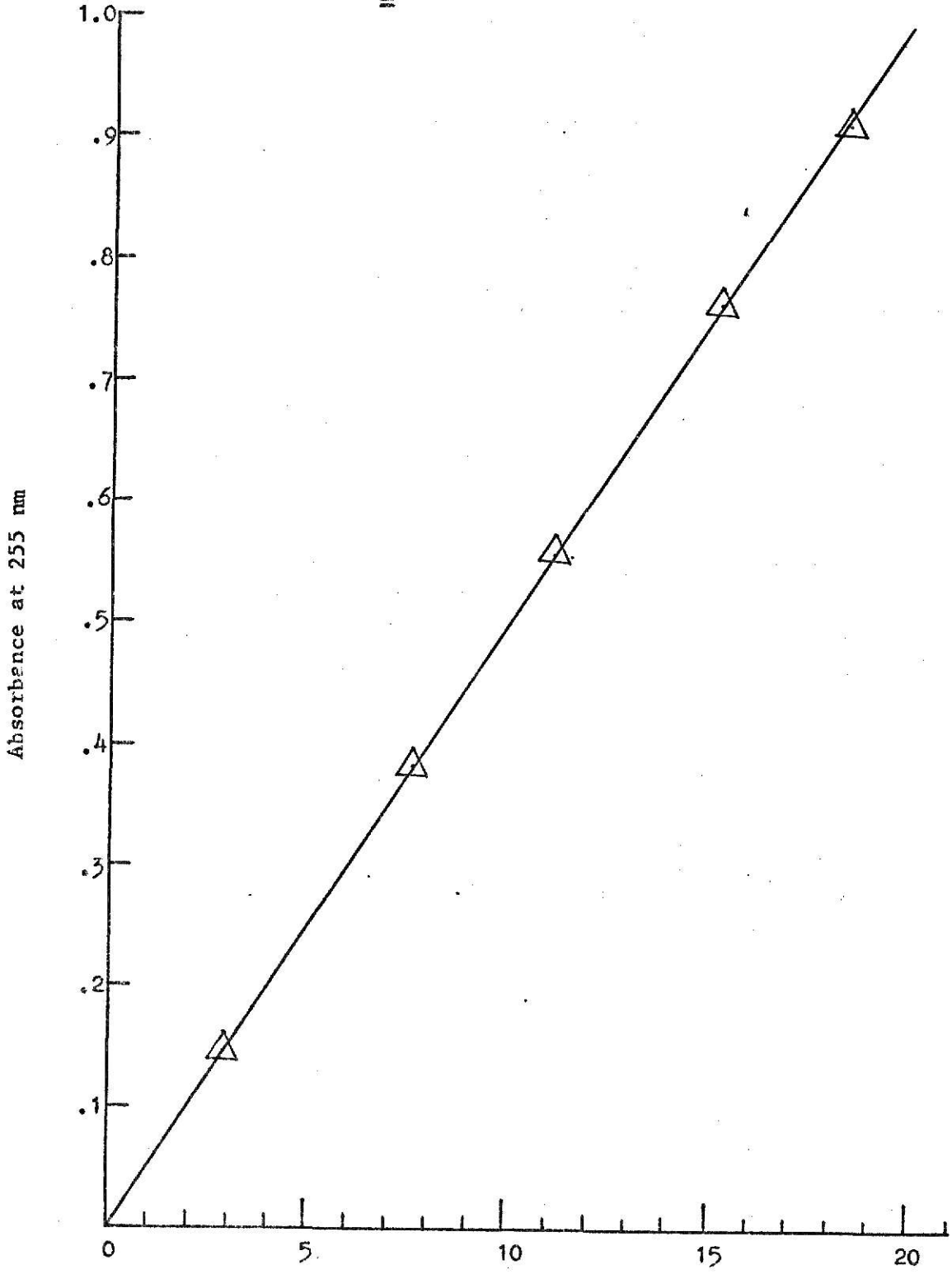
5,6-O-Isopropylidene-L-ascorbic acid possesses two enolic hydroxyl groups at C-2 and C-3. The C-3 hydroxyl group is the more acidic, and several investigators expected the 3-hydroxyl group would be sulfated before the 2-hydroxyl group. However, a 3-sulfate ester would not be expected to be stable in an ionizing medium, since delocalization of one pair of electrons between O-3, C-3, C-2, C-1 and O-1 would tend to keep O-3 unsubstituted (see the following figure).



Resonance Forms of 5,6-O-Isopropylidene-L-Ascorbate.

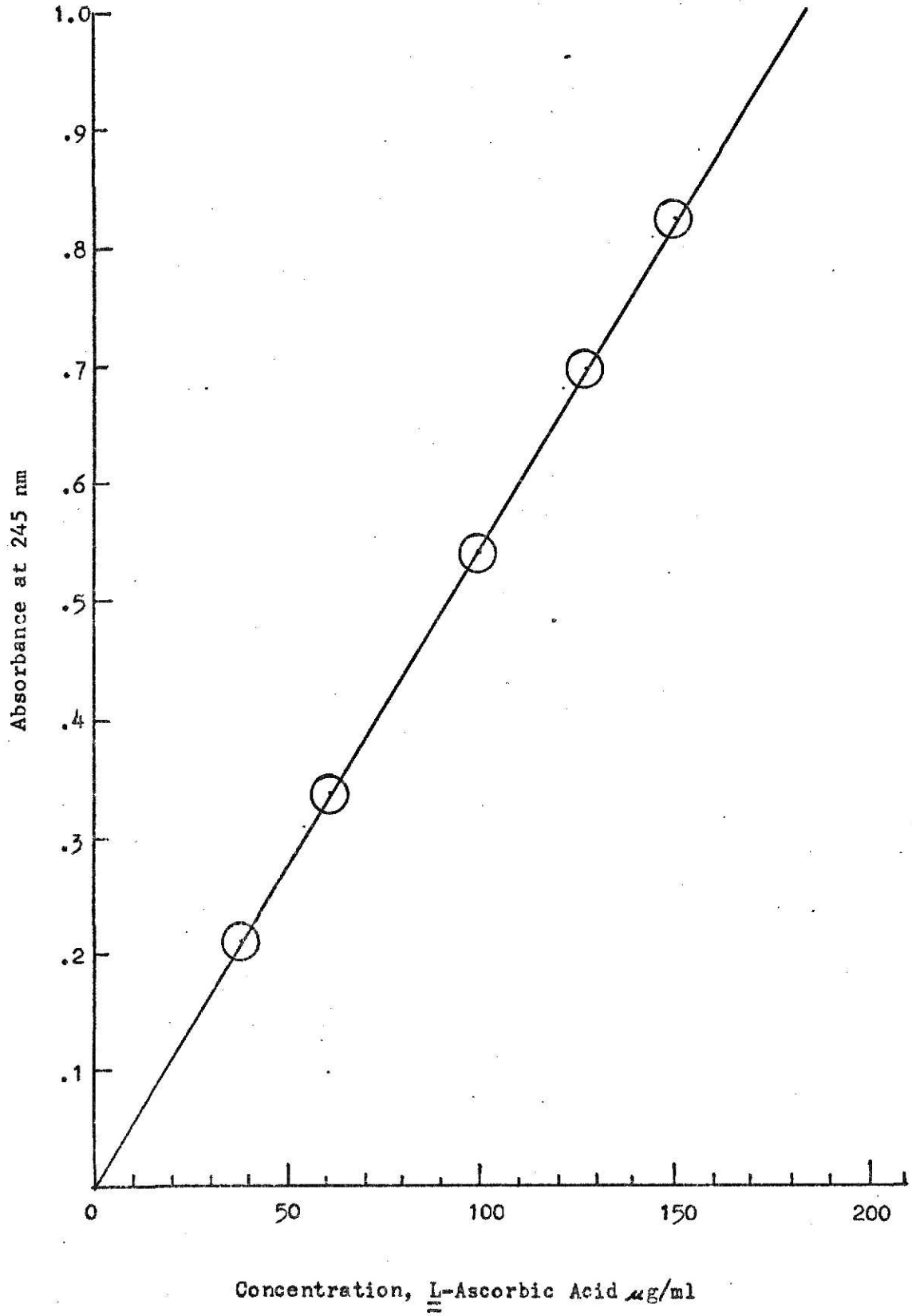
APPENDIX II

Standard Curve of Potassium L-Ascorbate 2-Sulfate in Water (pH 6.5)



APPENDIX III

Standard Curve of L-Ascorbic Acid in 5% Metaphosphoric Acid Solution (pH 2.0).



SYNTHESIS AND PROPERTIES OF L-ASCORBATE 2-SULFATE

by

CHEN-HSIUNG LEE

B.S., CHUNG HSING UNIVERSITY, 1967

AN ABSTRACT OF A THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

in

FOOD SCIENCE

Department of Grain Science and Industry

KANSAS STATE UNIVERSITY

Manhattan, Kansas

1973

L-Ascorbate 2-sulfate (I) has been shown to have antiscorbutic activity equal to that of L-ascorbate (II). The sulfate derivative (I) has also been shown to occur in nature. Previous methods of preparing I gave poor yields (below 50%). Two improved syntheses of I are described. Comparison of chemically-prepared compound (I) with the naturally occurring, sulfated derivative of L-ascorbic acid in brine shrimp show them both to be L-ascorbate 2-sulfate (I).

In one improved synthesis, 5,6-O-isopropylidene-L-ascorbic acid dissolved in N,N-dimethylformamide was first neutralized by addition of a tertiary amine (for example, pyridine). The resulting salt of 5,6-O-isopropylidene-L-ascorbate was then sulfated using pyridine-sulfur trioxide complex. In this manner, yields of I could be increased to 75% as compared to less than 50% for direct sulfation of 5,6-O-isopropylidene-L-ascorbic acid. Pyridine is believed to prevent the hydrolytic removal of the isopropylidene group before or during sulfation of 5,6-O-isopropylidene-L-ascorbate.

In the other improved synthesis of I, L-ascorbate (II) was reacted with trimethylamine-sulfur trioxide complex in alkali (pH 9.5-10.5) to give nearly quantitative yields of I. The reaction was complete in 30 minutes at 70°, and barium L-ascorbate 2-sulfate dihydrate was easily isolated in 80% yield. Other salts of I were found to crystallize readily, including potassium and zinc.

In boiling water, L-ascorbate 2-sulfate (I) is more stable toward oxygen than L-ascorbate (II). In boiling, deaerated alkali (pH 13.0), I and II have half-lives of 21 hours and 15 hours, respectively. In strong hot acid (pH 1.0, 100°), I has a half-life of only 4.7 minutes.

However, hydrolysis of the sulfate group on I was very slow at pH 1.5 and 37°; under these conditions I had a half-life of 150 hours. It is therefore doubtful that I could be converted to vitamin C by hydrolysis in the stomach of a monogastric animal.

L-Ascorbic acid 2-(hydrogen sulfate) was found to be converted quantitatively to L-ascorbic acid in less than six minutes in 99% methanol-water at 50°. This result indicates that in vivo transfer of sulfate from I to polysaccharides or steroids could be effected by an enzyme which protonates I in proximity to a hydroxyl acceptor.