L-ASCORBIC ACID IN CONCENTRATED SULFURIC ACID; IMPROVED SYNTHESIS OF L-ASCORBIC ACID 6-SULFATE

by

DONALD W. LILLARD JR.

B. S., BOISE STATE UNIVERSITY, 1974

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Grain Science and Industry

KANSAS STATE UNIVERSITY

Manhattan, Kansas

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Approved by:

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L-Ascorbic acid (I) does not dehydrate in concentrated sulfuric acid at room temperature, probably due to the formation of a hydroxyallyl cation structure. The c.m.r. spectrum of (I) dissolved in concentrated sulfuric acid showed that only two major components are present. The components were isolated by the following sequence. The reaction was quenched in cold ethyl ether ($\sim -65^{\circ}$), neutralized in the cold by addition of barium hydroxide, and the inorganic salts removed. The components were then separated and isolated using DEAE-cellulose ion-exchange column chromatography.

A total of four components were eluted from the column. The principal component (85%) was 6-sulfato-L-ascorbate (III), which was isolated in solid form as the barium salt in 48% yield. The other major component (5%) was thought to be 5-sulfato-L-ascorbate. The latter component was found to be unstable in base. When treated with alkali (pH 11.5 at 25°) the UV properties of the component changed, indicating elimination of the sulfate group to form a product thought to be 2,3,4,6-tetrahydroxy-2,4-hexadienoate-γ-lactone. This last compound was thought to be one of the two remaining minor components eluted from the column. The fourth component remains unidentified.

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Upon re-examination of the synthesis of L-ascorbic acid 6-palmitate (II)[a], Cousins et al.[1] reported L-ascorbic acid (I) was stable when dissolved in concentrated sulfuric acid. After an initial 5% decline, the ultraviolet absorbance of a 2.8×10^{-5} M solution of (I) in 95% to 98% sulfuric acid remained constant for 46 days at 25°. This result was surprising since L-ascorbic acid (I) rapidly dehydrates in hot aqueous acid to give almost quantitative yields of furfural.[2]

The stability of (I) in concentrated acid has been attributed to the formation of a hydroxyallyl cation[1] (Figure 1, p. 35). The cation structure is supported by c.m.r. data. Olah et al.[3] found upon protonation of trans-3-penten-2-one in SO_2ClF solution with HSO_3F-SbF_5, the C-2 and C-4 resonances of that ketone shifted markedly to lower field ($\Delta\delta$ = -23.3 and -29.0 p.p.m., respectively), while the C-3 signal shifted only slightly and to higher field (+2.9 p.p.m.). Dissolving (I) in 99% sulfuric acid produced six major ^{13}C signals. Chemical shifts of the C-1, C-2, C-3, and C-4 carbons of (I) in 99% sulfuric acid shifted downfield with reference to those observed in methyl sulfoxide solution, and the major shifts at C-1 and C-3 ($\Delta\delta$ = -12.3 and -14.7 p.p.m., respectively) were of the same magnitude and direction as observed by Olah et al.[3], in the model compound, trans-3-penten-2-one. The c.m.r. data suggest a delocalized cation ring structure for (I) in 99% sulfuric acid.

(a) Structures of L-ascorbate (I) and selected derivatives at neutral pH are given in Figure 2, p. 36.

The hydroxyallyl cation structure explains how the ring of (I) remains intact in concentrated sulfuric acid, but does not explain the fate of the C-5 and C-6 hydroxyl reaction sites. Polyols are known to undergo a number of reactions in concentrated sulfuric acid, including protonation, sulfation, racemization, elimination (dehydration), and polymerization.[4,5,6]

This work was undertaken primarily to determine which sulfation reactions occur at the C-5 and C-6 hydroxyls of L-ascorbic acid when it is dissolved in concentrated sulfuric acid. We have demonstrated by c.m.r. that polymerization and/or elimination of (I) in concentrated sulfuric acid is of minor importance, since there are only six major peaks and six minor ones in the c.m.r. spectrum of (I) in concentrated sulfuric acid. Also, L-ascorbic acid 6-palmitate (II) can be isolated in 85% yield by esterification of (I) in concentrated sulfuric acid,[1] which further suggests that polymerization and/or elimination of (I) in concentrated sulfuric acid is of minor importance. A secondary objective of this investigation was to show how c.m.r. spectroscopy can be used to identify esters of L-ascorbic acid (I).

Investigating reactions of (I) in concentrated sulfuric acid is also important to understanding the chemical synthesis of (II). L-Ascorbyl-6-palmitate (II) is currently used commercially (i) as an antioxidant in high fat foods to retard oxidative rancidity,[7,8] and (ii) as an emulsifying agent to produce food colorants from carotinoid pigments.[9,10] Although (II) has not been approved as a source of vitamin C in foods in the United States, a variety of uses in foods has been demonstrated; among these are prevention of nitrosamine formation during frying of bacon,[11] conditioning yeasted doughs,[12] and extending bread shelf-life.[12] L-Ascorbyl 6-palmitate (II) is known to have vitamin C potency[13] equivalent

to that of L-ascorbic acid (I).

Sulfation of (I) undoubtedly occurs in concentrated sulfuric acid.[14,15] The most likely products would be the 5- and 6- monosulfates and the 5,6-disulfates. The 2- and 3-sulfates of (I) would not be expected, since sulfation at those positions is not observed in a reaction mixture containing (I), concentrated sulfuric acid, and a fatty acid.[1] To date, the only known monosulfate esters of (I) are the 2- and 6-sulfate esters.[16]

Hatanaka et al.[17] synthesized L-ascorbic acid 6-sulfate (III) by sulfation of (I) with pyridine-sulfur trioxide in N,N-dimethylformamide. They characterized their product by ultraviolet and p.m.r. spectroscopy; however, purity and yield were not given.

Synthesis of (III) was also reported by Allaudeen et al.[18] Those investigators reacted 0.05 M of (I) in 100 ml. of dry pyridine with 0.10 M of pyridine-sulfur trioxide for 10 hr. at 37° with stirring, and then allowed the mixture to stand overnight at 25°. Pyridine was removed by evaporation and azeotropic distillation with water. The concentrate was neutralized with barium hydroxide, and barium sulfate removed by centrifugation. After evaporation, the material was purified using a DEAE-Sephadex A-50 column equilibrated with 0.01 M potassium phosphate buffer (pH 6.0). Unreacted (I) was eluted with the same buffer, while (III) was eluted with 0.05 M potassium phosphate buffer (pH 6.0) containing 0.05 M NaCl. Purity was determined by ascending-paper chromatography using two different solvent systems. Two components were detected, L-ascorbic acid (I) and L-ascorbic acid 6-sulfate (III). No other characterization was reported.

Tolbert et al.[19] prepared (III) by dissolving (I) in N,N-dimethylformamide and adding pyridine-sulfur trioxide. After neutralization with aqueous 2 M potassium hydroxide and concentration under reduced pressure,

the crude L-ascorbic acid 6-sulfate (III) was precipitated by pouring the concentrate into methanol. The solid was dissolved in aqueous potassium acetate and acetic acid, decolorized with charcoal, and reprecipitated in ethanol. The resulting solid was purified on a DEAE-cellulose column in the hydrogen sulfate form. The products were eluted using a linear gradient (0 to 0.009 M) of sulfuric acid. The eluted products were collected over barium carbonate, the mixture filtered, and the filtrate immediately lyophilized, to give 20% L-ascorbic acid 6-sulfate (III). Product purity was determined by ultraviolet and c.m.r. spectroscopy.

RESULTS AND DISCUSSION

Stability of L-ascorbic acid (I) in concentrated sulfuric acid.

The stability of (I) in concentrated sulfuric acid appears at first to be inconsistent with that compound's rapid dehydration in hot aqueous acid, to give almost quantitative yields of furfural.[2] However, UV and optical rotation properties of a solution of (I) in concentrated sulfuric acid showed the skeletal structure of (I) remained intact at 25° in that medium. After an initial 5% decline, the UV absorbance of a solution of (I) in concentrated sulfuric acid remained constant for 46 days at 25°. The absorbance maximum of the solution was at a longer wavelength (λ_{max} 265 nm) compared to that of unionized[1] L-ascorbic acid (pH 2, λ_{max} 245 nm). The observed optical rotation of two solutions of (I) (0.85 M) in 99% H_2SO_4 and in 100% H_2SO_4 plus 4% sulfur trioxide remained constant for 42 hr. at 25° (an initial 3% decline in observed rotation was observed in the solution containing added sulfur trioxide).

The c.m.r. spectrum of a solution of (I) in 99% H_2SO_4 gave one set of six major signals and one set of six minor signals (Figure 9, p. 44). The peak patterns of the two sets are very similar to those of (I) in water, indicating two main components are formed similar in structure to (I), each having an intact ene-diol ring structure and comprised of six carbons. The c.m.r. spectrum of a solution of (I) dissolved in 99% H_2SO_4 acquired at 18 min. and 3 hr. were identical.

The c.m.r. spectrum of the parent compound L-ascorbic acid (I) (Figure 10, p. 45) in water was studied as a first step in the identification and peak assignments of (I) and its derivatives. The carbon at lowest field (179.71 p.p.m.) which has a chemical shift similar to those

of carbonyl carbons in lactones[20] and which is weakly split into a doublet with a coupling of 2 Hz in the coupled spectrum[21] was assigned to C-1. Because the resonance at highest field (65.33 p.p.m.) appears as a triplet in the coupled spectrum, it was assigned to C-6, which is strongly coupled to the two protons on C-6. Since C-3 is a member of a conjugated ene-diol ring system, its hydroxyl group ionizes with pKa 4.1. The ionization of the C-3 hydroxyl results in a downfield shift (19.25 p.p.m.) due to the extended delocalization as the result of ionization. The carbon C-3 is weakly coupled to the proton on C-4 and the proton on C-5; the resonance at 177.87 p.p.m. which shows weak splitting into a doublet of doublets (J_{CCH}= 5 Hz, J_{CCCH}= 2 Hz)[21] in the coupled spectrum was assigned to C-3.

The carbon C-2 is also a member of the conjugated ene-diol ring system. Since the ionizable C-2 hydroxyl has a high pKa (11.8) and is distant from the nearest proton at C-4, one would expect at pH 5-10 a downfield shift but not as great as that of the C-3 resonance. Also, no splitting of C-2 would be expected in the coupled spectrum. Therefore, the resonance at 115.70 p.p.m. which does not exhibit splitting in the coupled spectrum was assigned to C-2.

The remaining two resonances at 80.96 and 72.26 p.p.m. correspond to either C-4 or C-5. Since both are coupled to one proton, and they each exhibit a doublet in the coupled spectrum, assignments based only on coupled and decoupled spectra are tenuous. However, the C-H coupling constant for C-4 should be larger than that of C-5 because C-4 is a member of a strained ring system. Mueller[21] found at pH 2.2 the resonance at 80.96 p.p.m. had a J_{C-H} of 153.3 Hz and the other resonance at 72.26 p.p.m. had a J_{C-H} of 145 Hz. Based on the J_{C-H}, we tentatively assigned the resonance at 80.96 p.p.m. to C-4 and the resonance at 72.26 p.p.m. to C-5.

This was confirmed by synthesizing 4-deuterio-L-ascorbate (IV) and examining the decoupled spectrum. The resonance centered at 78.44 in the spectrum of (IV) (Figure 11, p. 46) was a weak triplet, since C-4 is coupled to deuterium which has a spin of one.

The decoupled c.m.r. spectrum of (I) in concentrated sulfuric acid (Figure 9, p. 44) shows that the major and minor sets of six signals each are almost identical to the pattern of peaks in the c.m.r. spectrum of (I) in water (pH 2.0). This indicates that the ene-diol ring structure is intact and that each molecule is still comprised of six carbons. As a result of protonation in concentrated sulfuric acid, an average downfield chemical shift of $\sim$ 5.6 p.p.m. was observed for each resonance of (I) relative to their chemical shifts in water (pH 2.0). It is important to compare the spectrum of (I) in sulfuric acid to (I) in water at pH 2.0 since at pH 7.1 the resonance of C-3 shifts 19.8 p.p.m. downfield due to the ionization of the C-3 hydroxyl group.

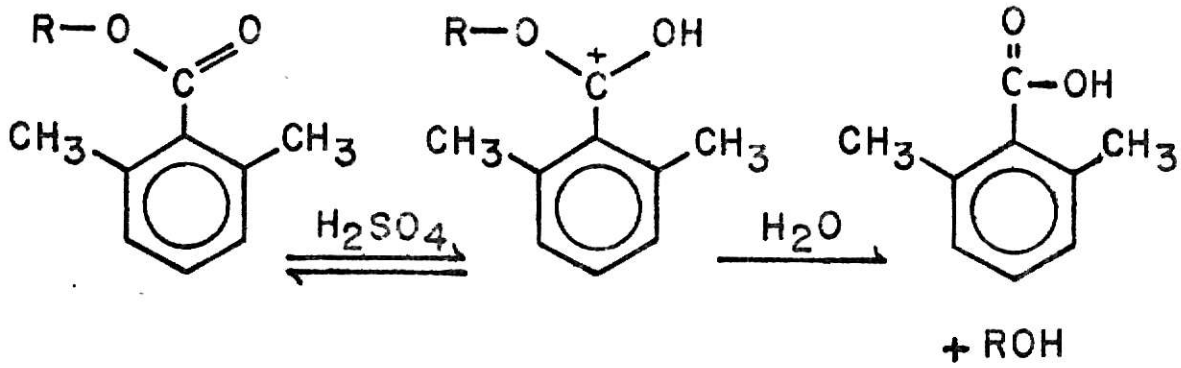
It was estimated by examination of the relative peak areas in the c.m.r. spectrum of (I) in concentrated sulfuric acid, that the ratio of the primary to the secondary component was $\sim$ 9/1. There is an indication in the carbonyl region of this spectrum that there may be a trace of a third minor component.

Quenching the reaction mixture of L-ascorbic acid in 99% concentrated sulfuric acid.

The reaction mixture of (I) dissolved in concentrated sulfuric acid was terminated or quenched by three different methods: Method 1. The reaction mixture was poured with rapid stirring by hand into chopped ice; Method 2. The reaction mixture was added dropwise, with rapid stirring by mechanical paddles, into ethyl ether cooled by addition of

dry ice to ∿ -65°; and Method 3. The reaction mixture was added dropwise with stirring, as in Method 2, to a mixture of ethyl ether and triethylamine cooled by addition of dry ice to ∿ -65°.

It is well known that mixing water with concentrated sulfuric acid produces heat, and that sulfate esters hydrolyze in hot acid. It is also known that in concentrated sulfuric acid, protonated esters of carboxylic acids hydrolyze when poured into water. This is the classic way of hydrolyzing a sterically-hindered ester as shown below. Thus, the



protonated lactone ring of L-ascorbic acid (I) in concentrated sulfuric acid may hydrolyze and the ring open upon quenching in water, if precautions are not taken. The important precautions that must be taken are cooling the quenching medium, and dispersing the reaction mixture as rapidly as possible during addition to the quenching medium. Both of these precautions limit excessive localized heating, which may lead to hydrolytic ring-opening.

The quenching medium is also important. Ethyl ether was chosen as a quenching medium because it is a non-protic solvent which can be readily protonated. Ethyl ether acts by competing with L-ascorbic acid or its derivatives for protons, thereby limiting ring-opening and elimination. We found 18% loss of the ene-diol groups (reducing power)

occurs if the reaction mixture is added to ethyl ether (Method 2.). However, using chopped ice as a quenching medium, also gave a 20% loss of ene-diol groups. But Method 2. is preferred to Method 1. because of the lower losses of reaction products in the subsequent neutralization step (74% versus 36% preservation of ene-diol groups).

Ethyl ether with enough triethylamine (0.07 M) to neutralize 19% of the sulfuric acid, cooled to $\sim -65^{\circ}$, gave the highest yields after quenching. But removal of the triethylamine during isolation was difficult. After removal of triethylamine by ion-exchange chromatography, yields were lower than when using ethyl ether alone.

Isolation Of 6-sulfato-L-ascorbate (III).

The best procedure developed to isolate the predominant reaction product, namely (III), may be summarized as follows. L-Ascorbic acid was reacted with 99% sulfuric acid for 4 hr. at room temperature. The reaction mixture was quenched by dropwise addition of the reaction mixture into ethyl ether maintained at $\sim -65^{\circ}$. The resulting mixture was brought to pH 1.5 by the addition of aqueous barium hydroxide (deaerated). After the temperature of the mixture reached $\sim -5^{\circ}$, the aqueous phase containing the reaction product and precipitated barium sulfate was drawn off using a separatory funnel. The ether layer was extracted with water, and the water extracts were combined, and adjusted to pH 7.0 using solid barium hydroxide. Barium sulfate was removed and the filtrate lyophilized. Prior to freeze-drying, a portion of the filtrate was titrated with iodine. The reducing power of the filtrate was 74% of that determined for the starting material (I).

The reaction products were separated on a DEAE-cellulose column using gradient elution (pH 0-1.7), followed by a constant pH wash (pH 1.7).

The components in the reaction mixture were detected by their UV absorbance. Four components were observed (Figure 3, p. 38). Each component was collected over deaerated aqueous barium hydroxide, and adjusted to pH 7.0 with dilute sulfuric acid. After removal of barium sulfate by filtration, portions of the filtrate were examined by UV spectroscopy and iodine titration. The remainder of the filtrate was lyophilized. A summary of the following data for each component is given in Table 4, p. 34; these include total elution volume, elution pH, percent of mixture by total UV and iodine titration, and the total recovery.

Component I was the first material eluted from the column at pH 6.0. This component comprises about 0.3 to 0.5% of the total products as measured by its UV absorption and iodine reducing power.

The structure of component I is unknown but its UV properties and chromatographic mobility suggests the structure to be 2,3,4,6-tetrahydroxy-2,4-hexadienoate γ-lactone. Component I was found to have the same column-chromatographic mobility as that of L-ascorbic acid (I), indicating both compounds have approximately the same pKa. The ultraviolet properties of component I (Figure 4, p. 39) indicate a structure with a possible extended conjugation to a total of three double bonds compared with the two of (I). At pH 2.0 and pH 7.0 the λ_{max} values are 15 nm and 20 nm higher than the corresponding absorbance maxima of (I). At pH 11.5 and 25°, component I decomposed since its UV absorption rapidly disappeared.

Component II eluted beginning at pH 6.0 and ending at pH 1.7. This component comprised ∿ 5% of the mixture applied to the column, based on iodine reduction power and UV absorbance. At pH 2.0 and pH 7.0 the λ_{max} values were 245 nm and 268 nm respectively (Figure 5, p. 40).

The structure of component II is unknown, but it appears to be 5-sulfato-L-ascorbate. When this component was subjected to treatment

with base, at pH 10.5 the λ_{max} of the component remained at 268 nm. But suddenly at pH 11.5 the λ_{max} shifted to 285 nm (Figure 6, p. 41). This sudden shift indicates that extended conjugation of the ene-diol system had occurred as a result of elimination at the carbon atoms C-4 and C-5. It is unlikely that this shift in λ_{max} is due to ionization, since one would expect a gradual shift in the λ_{max} to longer wavelength with increasing pH. These results are consistent with the structure of component II being 5-sulfato-L-ascorbate, which when treated with base (pH 11.5), eliminates to give component I (2,3,4,6-tetrahydroxy-2,4-hexadienoate γ-lactone). The presence of component I in the reaction products probably originates as a result of this elimination reaction occurring during neutralization and isolation of the reaction products. In one experiment, the eluent containing component II was neutralized with barium carbonate, filtered to remove barium sulfate, and lyophilized after addition of metaphosphoric acid. The resulting material had the same paper-chromatographic mobility as component I. All attempts to obtain component II in the solid form were unsuccessful.

Other experiments also indicate 5-sulfato-L-ascorbate (VIII) is a very labile compound. An attempt was made to synthesize (VIII) by the following sequence of reactions. 6-O-Valeryl-L-ascorbic acid was acetylated at its 2-O-position, followed by sulfation at the 5-O-position. The product 2-O-acetyl-5-sulfato-6-O-valeryl-L-ascorbate had the proper thin layer chromatographic properties. Attempts to deacylate that intermediate ester failed. Apparently the 5-sulfate gave the elimination product (component I), which decomposed in the basic medium.

However, it is possible to isolate a more stable elimination product. Sulfation of 6-O-palmitoyl-L-ascorbate gave 6-O-palmitoyl-2,5-disulfato-L-ascorbate (IV). The latter compound could be deacylated by careful

treatment with base at pH 11.0, forming compound (V). The structure of compound (V) was established by the red color it gave on chromatograms sprayed with ferric chloride, and by p.m.r. (Figure 16, p. 51) and c.m.r. (Figure 15, p. 50). The UV properties of compound (V) have not been determined. The formation of the 4,5-double bond in compound (V) confirms the ease of elimination of a 5-sulfato group from L-ascorbate.

Component III was the principal product and was found to be 6-sulfato-L-ascorbate (III). Component III was eluted beginning and ending at pH 1.7; this component accounted for ∿ 85% of the products based on UV absorbance and iodine titration. After removal of inorganic salts and lyophilization, barium 6-sulfato-L-ascorbate (III) was obtained in solid form (48% yield based on (I)). Characterization was done principally by c.m.r. spectroscopy in water (pH 6.7). The decoupled spectrum (Figure 13, p. 48) showed six major peaks similar to those of (I), which were assigned in the discussion previously. The peak furthest downfield in the spectrum of III at 179.37 p.p.m. was assigned to C-1, since this peak is not as broad as the resonance at 176.14 p.p.m. in the coupled spectrum and the former has a chemical shift similar to that of other ring carbonyl carbons.[20] As with the assignments for (I), the resonance at 176.14 p.p.m. which shows long range splitting to the C-4 proton in the coupled spectrum was assigned to C-3. Since this carbon has an ionizable group with a low pKa (4.2), a large downfield shift would be expected at pH 6.7. The carbon C-2 was assigned to the resonance at 116.06 p.p.m. It is a member of an ene-diol ring system and is not coupled to a proton, exhibiting a singlet in the coupled spectrum. Since the carbon C-2 has an ionizable hydroxyl with a high pKa (11.8), we would expect a downfield shift but not as great as that of C-3.

The resonances at 80.51 p.p.m. and 69.81 p.p.m. were assigned to C-4 and C-5 respectively, since in the coupled spectrum the doublet centered at 80.51 p.p.m. had a larger J_{C-H} than the doublet centered at 69.81 p.p.m. The carbon C-6 was readily assigned the resonance at 71.50 p.p.m. since this is the only resonance exhibiting a triplet in the coupled spectrum. Overall, the only major difference in chemical shifts of the carbons in compounds (I) and (III) is the chemical shift of C-6 (71.50 p.p.m.) in compound (III) with respect to the shift of C-6 (65.33 p.p.m.) in compound (I). This downfield chemical shift of the C-6 resonance after sulfation, is consistent with a similar shift observed for glucose-6-sulfate. Honda et al.[22] found that the C-6 of glucose resonating at 61.6 p.p.m. shifted downfield to 68.1 p.p.m. upon sulfation.

The structure of (III) was also confirmed using an alternate method to prepare (III). L-Ascorbic acid (I) was sulfated in pyridine with pyridine-sulfur trioxide as described by Allaudeen et al.[18] The main product was isolated by ion-exchange column chromatography. The c.m.r. spectrum of 6-sulfato-L-ascorbate prepared according to Allaudeen was identical to that of component III.

The identity of component IV is unknown. It comprises less than one half percent of the total components applied to the column. At pH 2.0 and pH 7.0 the λ_{max} values were 244 nm and 266 nm, respectively (Figure 8, p. 43). When this component was treated with base (pH 11.5), the λ_{max} did not shift to longer wavelength as did component II. That fact suggests that component IV is not 5,6-disulfato-L-ascorbate.

The UV absorptions and iodine-reducing values of components I, II, III, and IV, totaled 89% of the values for the reaction mixture applied to the column. Thus, the loss of material in the column was 11%.

The synthesis of 6-sulfato-L-ascorbate (III) compared to previously published methods.

When (III) was synthesized by the method of Allaudeen et al.[18], we found the conversion of (I) to (III) as measured by total UV and iodine titration on the crude reaction mixture was 64%. After isolation, the solids isolated from the reaction mixture were obained in 26% yield based on the starting compound (I). Besides low yield, the Allaudeen method has several other disadvantages; (i) the reaction is carried out in dry pyridine, (ii) pyridine-sulfur trioxide is used as the sulfating agent, and (iii) the reaction is conducted at 37° for 10 hr. and then has to stand at 25° for 12 hr. Pyridine is difficult to remove after the reaction is completed, while pyridine-sulfur trioxide gradually decomposes with time. Heating is unfavorable since it may facilitate degradation of (I) in the presence of an oxidant (sulfur trioxide). The other reported methods are those of Hatanaka et al.[17] and Tolbert et al.[19], which share the same problems associated with the use of pyridine-sulfur trioxide as in the Allaudeen method. The only other reported yield, that of Tolbert et al.[19] for the synthesis of (III) was 20%.

Our method is an improvement over previous methods for the following reasons: (i) (III) is synthesized from readily available and stable reagents, (ii) the reaction is fast (4 hr.), and (iii) the overall yield of (III) is higher (48%).

MATERIALS AND METHODS

General.

All evaporations were done under reduced pressure below 50°. Melting points 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 measurements. L-Ascorbic acid was obtained from ICN Pharmaceuticals, Inc., Cleveland, Ohio. Absolute sulfuric acid was prepared using the "fair and foggy" method described by Kunzler.[23] Other concentrations of sulfuric acid were prepared by dilution of absolute sulfuric acid or 99% sulfuric acid 37.0 ± 0.15 N, (Fisher Scientific, Fairlawn, New Jersey). Sulfuric acid containing ∿ 4% sulfur-trioxide was prepared by diluting 10.0 ml. (18.97 g.) of fuming sulfuric acid (20-23% SO_3) with 39.2 ml. (71.8 g.) of 100% sulfuric acid.

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. 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.[24,25]

Paper chromatography was performed on Whatman No. 1 paper at 25° with either of two solvents: (A) ethyl acetate, acetic acid, water (6:3:2, v/v/v); (B) N-propanol, ethyl acetate, water (6:1:3, v/v/v). Two developing procedures were used to locate components. Method A: Chromatograms were dipped in a solution of 0.7% silver nitrate in acetone (5 ml. saturated $AgNO_3$, 5 ml. concentrated NH_4OH, and 5 ml. of water in 1 liter of acetone), followed by 0.05 M sodium hydroxide in methanol. After reduction of silver was adequate, the papers were washed consecutively with 5% aqueous

sodium thiosulfate (w/v) and water.[26] Method B: Chromatograms were dipped in a solution of 1% ferric chloride in 95% methanol (w/v) to detect compounds containing an enolic hydroxyl group.[24,25]

Ultraviolet spectra were obtained in aqueous solutions using a Beckman, Model DB-G, spectrophotometer. Nuclear magnetic resonance spectra were recorded with a Varian Model T-60 or XL-100 spectrometer. For c.m.r., the Varian XL-100 was coupled to a Nicolet 1084 pulse Fourier transform in conjunction with a Diablo disk system. Chemical shifts are given, except where noted, in p.p.m. downfield from sodium-2,2-dimethyl-2-silapentane-5-sulfonate (DSS). Samples were placed in a 12 mm. I.D. sample tube with a concentric 5 mm. O.D. reference cell. Coupled and decoupled spectra of (I) in concentrated sulfuric acid were compared to those obtained in water. To aid in peak assignments, coupled and decoupled spectra of 4-deuterio-L-ascorbate (VI) were acquired in water and 99% sulfuric acid. The chemical shifts of these compounds and other selected derivatives of (I) are given in Table 1, p. 31.

L-Ascorbic acid (I) in concentrated sulfuric acid.

The stability of L-ascorbic acid in concentrated sulfuric acid was determined using optical rotation. Two solutions of (I) in different concentrations of sulfuric acid were prepared. Both solutions were 1.8 M with respect to (I) in either 99% H_2SO_4 or 100% H_2SO_4 plus 4% sulfur trioxide. The acid containing added sulfur trioxide was purged with nitrogen and stirred magnetically while (I) was added and dissolved. Each solution of (I) in concentrated sulfuric acid was then placed in a two decimeter polarimeter tube, and stored at 25°. After various periods of time, readings were taken. The results are given in Table 2, p. 32.

Quenching of the reaction between L-ascorbic acid (I) and concentrated sulfuric acid.

Method 1.

Compound (I) (9.00 g.) was added to a tared 50 ml. round bottom flask. Approximately 24 ml. of 99% sulfuric acid was added to the flask, and the weight (43.63 g.) of sulfuric acid was determined by difference. The flask was stoppered, and stirred with a magnetic bar at room temperature. After 4 hr., a portion (18.44 g.) of the syrupy reaction mixture was added dropwise with rapid stirring (by hand) to about 500 g. of chopped ice. A portion of the reaction mixture (34.19 g.) remained in the flask, and was determined by weighing. After the ice melted, the mixture was made to volume (1000 ml.) with deaerated water. A 10.0 ml. aliquot of the mixture was placed in a flask, to which was added five drops of 1% starch indicator and 50 ml. of a 6% aqueous solution of metaphosphoric acid. Titration with 0.1 N iodine solution gave 5.65 ml. of titer. A 5.0 ml. aliquot of a solution of L-ascorbic acid (1.024 g.) in 100 ml. of 6% metaphosphoric acid in water gave 11.5 ml. of titer. From those titers we calculated that 80% of the reducing power originally added to the sulfuric acid was still intact in the reaction products using Method 1. to quench.

Method 2.

A 15.22 g. portion of the reaction mixture remaining from Method 1. was added with rapid stirring to ethyl ether (300 ml.) at $\sim -65^{\circ}$. Stirring was done using a variable speed motor (Whirlozone 59880-10, Matheson Scientific, Kansas City, Missouri) coupled to a borosilicate-glass sleeve stirrer with two teflon paddles attached to the bottom (Cat. # 14-513-50A, Fisher Scientific Co., St. Louis, Missouri). After addition was complete, 500 ml. of distilled deaerated water was added with stirring, and the

temperature of the mixture allowed to rise to ∿ -5°. The water layer was removed using a separatory funnel, and the ether layer washed with water (2 x 200 ml.). The water extracts were combined and made to volume (1000 ml.). A 10.0 ml. aliquot was removed and titrated with 0.1 N iodine as previously described. The titer for the reaction mixture was 5.0 ml. as compared to 11.5 ml. for the control sample of L-ascorbic acid, 5.0 ml. aliquot of a solution of 1.024 g. of (I) in 6% metaphosphoric acid (100 ml.). Quenching by Method 2. gave 82% of the reducing power originally added to the sulfuric acid.

Method 3.

L-Ascorbic acid (2.90 g.) was added to 99% H_2SO_4 (18.40 g.) as described previously in Method 1. A 15.80 g. portion of that reaction mixture (total weight 21.30 g.) was added dropwise with rapid stirring, as described in Method 2., to a mixture of ethyl ether (300 ml.) and triethylamine (10 ml.) at ∿ -65°. Following aqueous extraction of the ether phase with water, the aqueous phase was made to volume (1000 ml.). A 10.0 ml. aliquot was removed and titrated with 0.1 N iodine as described in Method 1. The titer for the reaction mixture was 6.45 ml. compared to 7.95 ml. for the control sample of L-ascorbic acid,(5.0 ml. aliquot of a solution of (I) (0.995 g.) dissolved in 6% metaphosphoric acid in water (100 ml.)). The water phase reduced 94% of the theoretical amount of iodine. Titration of a control sample containing matching concentrations of ether, triethylamine, and sulfuric acid, treated in the same manner gave no reduction of iodine.

Neutralization of the quenched reactions with barium hydroxide.

Method 1A.

L-Ascorbic acid (6.02 g.) was added to 99% H_2SO_4 (35.95 g.) followed by removal of a 15.93 g. portion which was added to chopped ice as described in Method 1. The reaction was placed in an ice salt bath and neutralized with a deaerated saturated barium hydroxide solution to pH 7.0 using a pH meter. The mixture was then filtered by vacuum filtration to remove barium sulfate and the precipitate washed with deaerated water (2 x 50 ml.). The filtrate was made to volume (1000 ml.), and a 10.0 ml. aliquot was found to have an iodine titer of 1.85 ml. An aliquot (5.0 ml.) of a control sample containing (I) (1.015 g.) in 6% metaphosphoric acid (100 ml.) had a titer of 11.6 ml. Neutralizing by this method gave 36% of the reducing power originally added to the sulfuric acid.

Method 2A.

A portion (15.37 g.) of the reaction mixture remaining from Method 1A. was added with rapid stirring to ethyl ether (300 ml.) at $\sim -65^{\circ}$, as described in Method 2. While the temperature was still $\sim -65^{\circ}$, deaerated saturated barium hydroxide was added (200 ml.) and the temperature allowed to rise to $\sim -5^{\circ}$. The water layer containing barium sulfate was separated from the ether layer using a separatory funnel, and the ether layer was washed with water (2 x 150 ml.). The combined water fractions were cooled to $\sim 5^{\circ}$ using an ice salt bath. Using a pH meter, the mixture was raised from pH 1.5 to 7.0 by addition of solid barium hydroxide. The precipitated barium sulfate was removed by vacuum filtration, and the precipitate washed with deaerated water (2 x 50 ml.). The filtrate was made to volume (1000 ml.), and a 10 ml. aliquot was found to reduce 3.65 ml. of 0.1 N iodine solution. An aliquot (5.0 ml.) of a control sample containing L-ascorbic acid (1.015 g.) in 6% aqueous metaphosphoric acid (100 ml.)

reduced 11.6 ml. of 0.1 N iodine solution. The titers showed that 74% of the ene-diol groups originally present in the starting material had survived the strong acid solution, quenching, and neutralization with barium hydroxide.

L-Ascorbic acid 6-sulfate (III).

To obtain high yields of L-ascorbic acid 6-sulfate (III), it is essential that oxygen be excluded during the synthesis and isolation steps. All aqueous reagents, except concentrated sulfuric acid, were deaerated by boiling and cooling under a nitrogen atmosphere. In a stoppered round bottom flask, 3.000 g. of (I) was dissolved in 10 ml. of 99% sulfuric acid. After stirring magnetically 4 hr. (because of the solubility problem of (I) in 99% sulfuric acid), the syrup was added dropwise with rapid stirring (as in Method 2.) to 300 ml. of ethyl ether, which was maintained at $\sim -65^{\circ}$ by addition of dry ice. The reaction was then neutralized and the precipitate removed as described in Method 2A. The filtrate was made to volume (1000 ml.), and a 10 ml. aliquot was found to reduce 3.65 ml. of 0.1 N iodine solution. An aliquot (5 ml.) of a control sample containing 1.015 g. of L-ascorbic acid (I) in 6% aqueous metaphosphoric acid (100 ml.) reduced 11.6 ml. of 0.1 N iodine solution. The titers showed that 74% of the ene-diol groups originally present in the starting material had survived the strong acid solution, quenching, and neutralization with barium hydroxide.

The reaction mixture was evaporated to a thin syrup, which was made to volume (25.0 ml.) with water. An aliquot (10.0 ml.) was removed for ion-exchange column chromatographic separation, while the remaining 15.0 ml. was stored at room temperature. Diethylaminoethyl-cellulose (DE-52, Whatman, H. Reeve Angel Inc., Clifton, New Jersey) was converted

to the hydrogen sulfate form by washing with aqueous sulfuric acid (0.1 M). The column (1.6 x 40 cm.) was fitted with adjustable plungers (Pharmacia Fine Chemicals Inc., Piscataway, New Jersey) and the flow rate regulated by a peristaltic pump (Model Miniplus II, Gilson Medical Electronics, Inc., Middleton, Wisconsin). Ultraviolet absorbing compounds were detected and recorded using a UV monitor (254 nm., Model 260, Gilson Medical Electronics, Inc.) in conjunction with a strip chart recorder (Omniscribe, Model A-5111-2, Houston Instruments, Houston, Texas). Fractions were collected on a linear fraction collector (Model TL, Gilson Medical Electronics, Inc.). The column was washed 24 hr. to remove traces of air, and a 10 ml. portion of the thin syrup was added to the column. The components were eluted using a 200 ml. linear gradient (0 - 0.18 M) of aqueous sulfuric acid, followed by a 0.18 M wash to remove the remaining components. Four components were eluted from the column, which were collected over ∿ 6% metaphosphoric acid. The amount of each component was determined by the UV absorbance and iodine titration of the total effluent for each peak (Table 4, p. 34). L-Ascorbic acid 6-sulfate was identified as described in the paragraph below. The UV yield of each component was based on the assumption that all compounds had uneffected ene-diol groups with extinction coefficients equal to that of L-ascorbic acid.

A larger column (2.6 x 40 cm.) was used to isolate preparative amounts of barium L-ascorbate-6-sulfate (III). The column was developed as previously described, but during elution of (III) from the column, the effluent was directed into a stirred mixture of barium carbonate (10 g.) in water (50 ml.). After filtration, the filtrate was made to volume and titrated with 0.1 N iodine solution. The reducing power of the peak corresponded to a 54% yield of (III). Evaporation gave 2.78 g., 48% yield,

of the solid barium salt of (III). The solid material gave a single spot (Ra 0.27, Ra is mobility relative to that of (I)) on a paper chromatogram using solvent system (A). The spot rapidly reduced silver ions and did not form a red complex with ferric ions. This is consistent with the properties (silver reduction and complexing with ferric ions) of L-ascorbate (I), except for mobility. L-Ascorbic acid 6-sulfate prepared as described by Allaudeen, et al.[18] gave the same chromatographic properties on paper and on a DEAE-cellulose column as compound (III) isolated in this work. The structure of (III) was established using UV and c.m.r. spectroscopy. Barium 6-sulfato-L-ascorbate gave a λ_{max} of 245 nm at pH 2.0 and a λ_{max} of 265 nm at pH 7.0. The proton-decoupled c.m.r. spectrum of (III) in water at pH 7.0 gave six peaks (Figure 13, p. 48), which were identical to the signals produced by (III) prepared by the method of Allaudeen et al.[18] Peaks were assigned based on the proton-coupled and decoupled spectra of (III), L-ascorbate (Figure 10, p. 45), and 4-deuterio-L-ascorbate (Figure 11, p. 46). The chemical shifts of L-ascorbate and selected derivatives are given in Table 1, p. 31.

L-Ascorbic acid 4-d (VI).

Synthesis of (VI) was accomplished using a modification of the procedure of Brenner et al.[27] The hydrogens on the hydroxyl groups of L-ascorbic acid (0.05 M) were exchanged with deuterium by several evaporations of a solution of (I) in D_2O (10 ml.). Prior to each evaporation, the solution was purged with prepurified nitrogen to remove oxygen. After the last exchange, the solution was evaporated to dryness, and the residue dried thoroughly in a desiccator. To the dry residue was added sodium methoxide (4M) in methanol-d, and the mixture

was refluxed 24 hr. The mixture was added to water containing 45 g. of Amberlite IR - 120 (H^+), and stirred for 10 min. After removal of the resin, the filtrate was concentrated to a small volume and lyophilized. L-Ascorbic acid-4-d was recrystallized from hot acetonitrile to give white crystals with a melting point 192-193° (yield 2.8 g., 32%). The proton-decoupled c.m.r. spectrum of L-ascorbic acid-4-d gave eight peaks (Figure 11, p. 46), five of which are identical to those of L-ascorbic acid. However, the signal centered at 78.44 p.p.m. in the decoupled spectrum was a triplet, which was assigned to C-4.

Synthesis of 2-sulfato-2,3,4,6-tetrahydroxy-2,4-hexadienoate γ-lactone (V).

L-Ascorbic acid 6-palmitate (0.05 M) was dissolved in 100 ml. of dry pyridine, and pyridine-sulfur trioxide (0.1 M) was added. The progress of the reaction was followed with time by thin-layer chromatography using chloroform and acetic acid, 3/2 (v/v). Upon sulfation, the mobility of the starting compound (Rf 0.8) in System C. changed to (Rf 0.4) for the presumed monosulfate ester and to (Rf 0.1) for the disulfate. After a 4 hr. reaction period, the mixture was adjusted to pH 11 with saturated barium hydroxide solution, and the mixture evaporated to approximately 20 ml. to remove most of the pyridine. During evaporation, the pH of the mixture changed from 11.0 to 9.5. Sulfuric acid (1 M) was added to adjust the pH to 3.0, the precipitate removed by centrifugation, and the supernatant extracted with ethyl ether to remove fatty acid. This mixture was then adjusted to pH 7.0 with barium carbonate, centrifuged, and the supernatant concentrated under reduced pressure to give a solid residue.

The residue thought to be 2-sulfato-2,3,4,6-tetrahydroxy-2,4-hexadienoate γ-lactone (V) was characterized by paper chromatography, p.m.r., and c.m.r. Using paper chromatography with solvent system (A), we found

the residue contained one principal product which gave a red color with ferric chloride (Ra 0.55). The Ra values of L-ascorbic acid and selected derivatives of (I) are given in Table 3, p. 33.

The c.m.r. spectrum (Figure 15, p. 50) showed six major signals. The resonance furthest downfield at 172.71 p.p.m. was assigned to C-1, since it has a chemical shift similar to those of carbonyl carbons in other lactones,[20] and since it does not show any splitting in the coupled spectrum. Because the resonance at highest field (57.66 p.p.m.) appears as a triplet in the coupled spectrum, it was assigned to C-6, which is strongly coupled to the two protons of C-6. Surprisingly, the C-6 carbon does not appear to long range couple with H-5. The resonance at 114.20 p.p.m. was assigned to C-2. That signal does not show coupling in the coupled spectrum, and it is a member of a conjugated ene-diol ring system with an attached sulfate. It would not be expected to shift downfield as much as the carbonyl carbon C-1. The remainder of the peaks were assigned based on their splittings exhibited in an expanded (500 Hz) coupled spectrum of (V). The resonance centered at 171.15 p.p.m. in the expanded coupled spectrum was a doublet with a J_{CCCH} of 3 Hz, indicating long range coupling to one proton. Since C-3 is a member of a conjugated ene-diol ring system and has an ionizable hydroxyl group, a large downfield shift would be expected. Because of the large downfield shift and the long range coupling to one proton, this resonance was assigned to C-3. The resonance centered at 108.16 p.p.m. was split into a doublet in the coupled spectrum with a J_{C-H} of 162 Hz; this doublet was further split into a pair of triplets by long range coupling (J_{CCH} of 4.5 Hz). The J_{C-H} splitting of C-5 (162 Hz) is consistent with the J_{C-H} of similar alkene compounds. For example, the J_{C-H} in benzene is 159 Hz.[20] This indicates that carbon C-5 is an sp^2 hybridized carbon, since the J_{C-H} of sp^3 hybridized carbons is $\sim$ 125 Hz.[20]

The short and long range splittings of the signal at 108.16 p.p.m. are consistent for carbon C-5 in the structure postulated for compound (V). The resonance centered at 179.36 p.p.m. in the coupled spectrum is also a doublet split into a pair of triplets. However, in this case the couplings are both long range (J_{CCH} = ∿ 5.5 Hz and J_{CCCH} = ∿ 4.5 Hz). This is only consistent with carbon C-4 to which this resonance was assigned. A large downfield shift was observed, compared to that of the C-4 in compound (I) (Figure 10, p. 45), because the C-4 carbon of compound (V) is a member of a conjugated double bond system.

The p.m.r. spectrum of (V) (Figure 16, p. 51) in D_2O supports this assigned structure. The chemical shifts with reference to DSS were: a doublet at 4.23 p.p.m. with J_{5-6} = 7 Hz, and a triplet at 5.43 p.p.m. with a J_{5-6} = 7 Hz. These chemical shifts are within the range of those reported for aliphatic and alkene hydrogens.[28] This verifies that there are two protons attached to C-6 with an adjacent proton on C-5. No other protons are present. This is consistent with the assigned structure of (V).

Synthesis of 2,5-disulfato-6-O-valeryl-L-ascorbate.

The synthesis of the title compound was accomplished in two steps. The starting compound, 6-O-valeryl-L-ascorbic acid was prepared as follows. L-Ascorbic acid (0.1 M) was dissolved in 70 ml. of 99% sulfuric acid at room temperature and valeric acid (0.1 M) was added. After stirring magnetically for 4 hr., the mixture was poured slowly into ∿ 500 g. of chopped ice with stirring by hand and that mixture extracted with ethyl acetate (3 x 200 ml.). The ethyl acetate layer was washed with water (∿ 6 x 100 ml.) until sulfate ion in the water wash could not be detected by the addition of a small amount of barium chloride. After drying with

anhydrous sodium sulfate (200 g.), the salt was removed by filtration and the filtrate evaporated to a thick syrup by reduced pressure. The remaining residue was dissolved in ether and recrystalized from an ether-petroleum ether mixed solvent system. The white crystals were removed from the liquor by filtration and dried by vacuum in a desiccator, yielding 8.4 g. (0.03 M) of 6-O-valeryl-L-ascorbic acid, melting point 92-93°.

This material (6-O-valeryl-L-ascorbic acid, 0.01 M) was dissolved in 25 ml. of dry pyridine and pyridine-sulfur trioxide (0.02 M) was added to the mixture. The progress of the reaction was followed with time by thin-layer chromatography using solvent system (A) (ethyl acetate, acetic acid, and water, 6/3/2, v/v/v). Upon sulfation, the mobility of the starting material, 6-O-valeryl-L-ascorbic acid, Rf 0.9 in system (A) changed to Rf 0.5 for the presumed monosulfate ester and to Rf 0.2 for the disulfate ester. As detected by thin-layer chromatography after 5 hr., the starting material was no longer present and the disulfate was the predominate component. Pyridine-sulfur trioxide (0.005 M) was added to the reaction mixture to convert the remaining mono-ester to the disulfate ester. After a total of 8 hr., the only reaction product (by tlc) was the disulfate. Water (50 ml.) was added to react with any remaining pyridine-sulfur trioxide. The reaction mixture was adjusted to pH 9.5 with aqueous barium hydroxide and the mixture evaporated to $\sim$ 20 ml. to remove most of the pyridine. Sulfuric acid (1 M) was added to adjust the pH to 7.0 and the precipitate (barium sulfate) was removed by centrifugation.

Purification of this material was attempted using a DEAE-cellulose column in the sulfate form as described previously for the purification of (III). The column was washed with a linear gradient (300 ml.) of aqueous sulfuric acid (0-0.18 M) followed by 3 l. of sulfuric acid (0.18 M). However, the disulfate could not be eluted from the column.

SUMMARY

The most important conclusions for this work may be summarized as follows:

1. L-Ascorbic acid (I) is stable in concentrated sulfuric acid for at least 46 days at room temperature as shown by UV, c.m.r., and optical rotation.
2. The c.m.r. spectroscopy showed that L-ascorbic acid in concentrated sulfuric acid forms two products in the ratio of $\sim$ 9/1.
3. The principal product in a concentrated sulfuric acid solution of (I) was shown to be 6-sulfato-L-ascorbate (III).
4. The minor component is thought to be 5-sulfato-L-ascorbate (VIII).
5. A scheme to isolate and purify (III) was derived. The compound (III) was isolated as the barium salt in 48% yield.
6. Compound (III) was characterized by c.m.r. and UV.

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APPENDIX I

Table 1. Carbon-13 chemical shifts of L-ascorbic acid and selected derivatives.[a]

Compound	pH	C-1	C-2	C-3	C-4	C-5	C-6
L-Ascorbate (I)	7.1	179.71	115.70	177.87	80.96	72.26	65.33
4-d-L-Ascorbic acid	2.0	175.71	120.39	158.12	78.44	71.52	64.78
L-Ascorbic Acid (I)[b]	-	183.12	121.10	167.86	82.64	76.84	70.81
4-d-L-Ascorbic Acid[b]	-	182.96	121.06	167.40	--	76.74	70.69
2-Sulfato-L-Ascorbate	10.2	178.08	113.01	182.34	81.31	72.28	65.12
6-Sulfato-L-Ascorbate	6.7	179.37	116.06	176.14	80.51	69.81	71.50
Compound (V)[c]	7.4	172.71	114.21	171.15	149.36	108.16	57.66

[a]Chemical shifts are expressed in p.p.m. relative to external sodium-2,2-dimethyl-2-silapentane-5-sulfonate (DSS) in water except where noted.

[b]Solvent 99% sulfuric acid, the pH was not measured.

[c]2,3,4,6-Tetrahydroxy-2,4-hexadienoic acid γ-lactone 2-sulfate (V)

Internal DSS was used as a reference.

Table 2. Stability of L-ascorbic acid in concentrated sulfuric acid, as measured by optical rotation.

Time (hrs.)	Observed rotations[a] in degrees for solutions[b] given	
	A	B
1	17.10°	18.75°
19	17.10°	18.20°
25	17.10°	18.20°
39.5	17.10°	18.20°
42	17.10°	18.20°

[a]Storage and observations were done at room temperature (∿ 25°).

[b]Solutions: A, (I) (3.0 g.) dissolved in 99% H_2SO_4 (20 ml.)

B, (I) (3.0 g.) dissolved in 100% H_2SO_4 (20 ml.) + 4% SO_3

Table 3. Reaction with sprays and chromatographic mobilities of L-ascorbic acid and selected derivatives in solbent system A.[a]

Compound	Ra value[b]	Silver nitrate (neutral)	Silver nitrate (KOH)	Color with $FeCl_3$
L-Ascorbate	1.0	+	+	Purple or white[d]
4-Deuterio-L-ascorbate	1.0	+	+	Purple or white[d]
Barium 2-sulfato-L-ascorbate	0.50	-	-	Red
Barium 6-sulfato-L-ascorbate	0.27	+	+	Purple or white[d]
2-Sulfato-2,3,4,6-tetrahydroxy-2-4-hexadienoate γ-lactone	0.55	-	+	Red
Barium 2-keto-L-gulonate	0.55	-	+	--
2,5-Disulfato-6-O-valeryl-L-ascorbate	0.20[c]	-	+	Red

[a]Solvent system A: ethyl acetate, acetic acid, and water (6:3:2, v/v/v).

[b]Ra value = $\frac{\text{distance migrated by derivative}}{\text{distance migrated by L-ascorbate}}$

[c]Rf value

[d]Color observed for L-ascorbic acid or its derivatives with reducing power depends on chromatography solvent. Most solvents show white due to reduction of ferric to ferrous ions.

Table 4. Column chromatography of the neutralized reaction mixture.[a]

Component	Effluent pH	Total effluent[b]	Percent of mixture[c]	
			UV	Iodine titration
I	6.0	351	0.3	0.5
II	5.0	570	4.0	5.0
III	1.7	1118	85.0	81.0
IV	1.7	1464	0.2	<0.5
		Total Recovery	89.5	87.0

[a]Reaction and procedure are given on page 20.

[b]Total effluent in ml. to the beginning of each component.

[c]Percentage determined by comparison of absorbance or iodine titer of a peak to the total absorbance or iodine titer of the mixture applied to the column.

APPENDIX II

Figure 1.

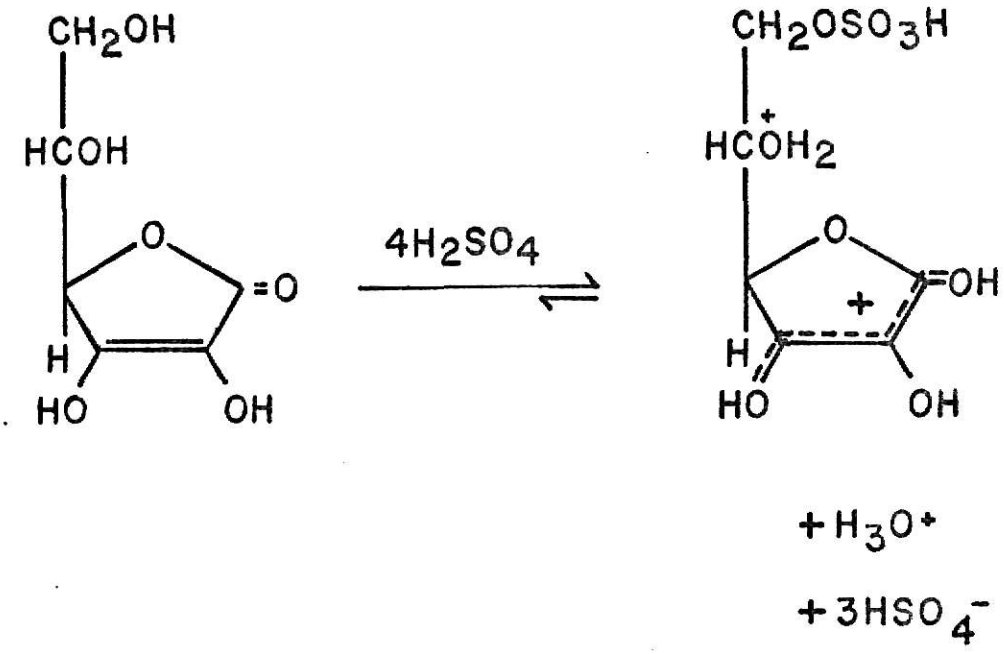
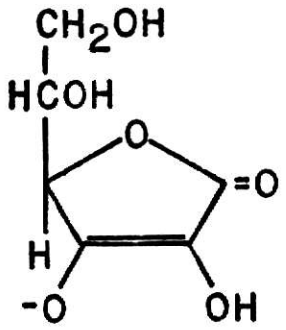
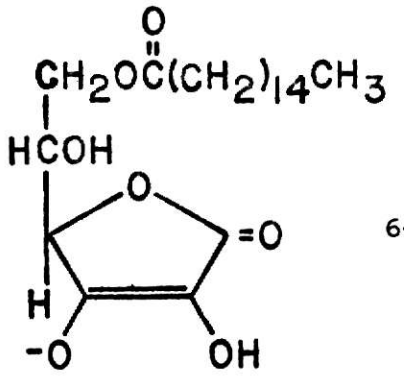


Figure 2.

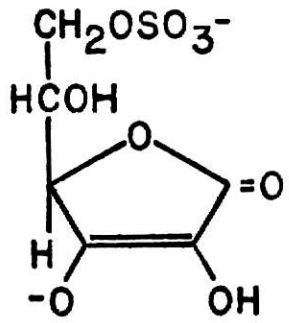
The structure of L-ascorbate and selected derivatives.



L-Ascorbate (I)

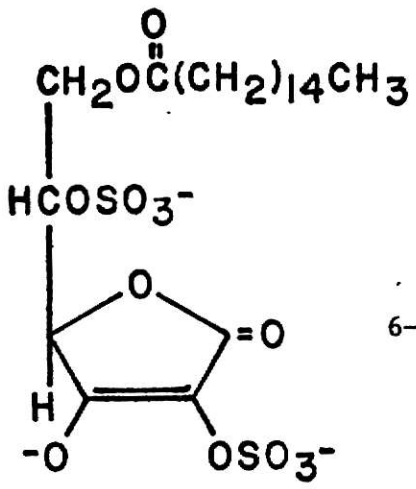


6-O-Palmitoyl-L-ascorbate (II)

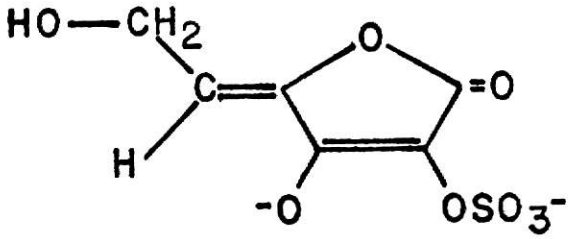


6-Sulfato-L-ascorbate (III)

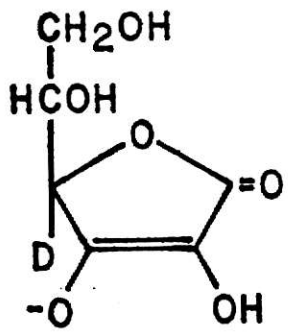
Figure 2., continued



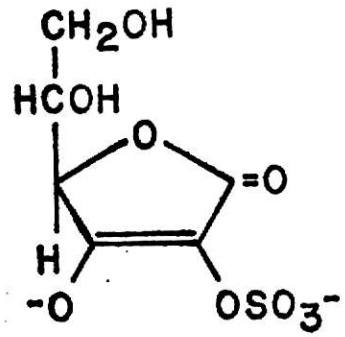
6-O-Palmitoyl-2,5-disulfato-L-ascorbate (IV)



2-Sulfato-2,3,4,6-tetrahydroxy-2, 4-hexadienoate, γ-lactone (V)



4-Deuterio-L-ascorbate (VI)



2-Sulfato-L-ascorbate (VII)

Figure 3. Column chromatography of the neutralized reaction mixture (strip-chart recording of the UV absorbance with time).

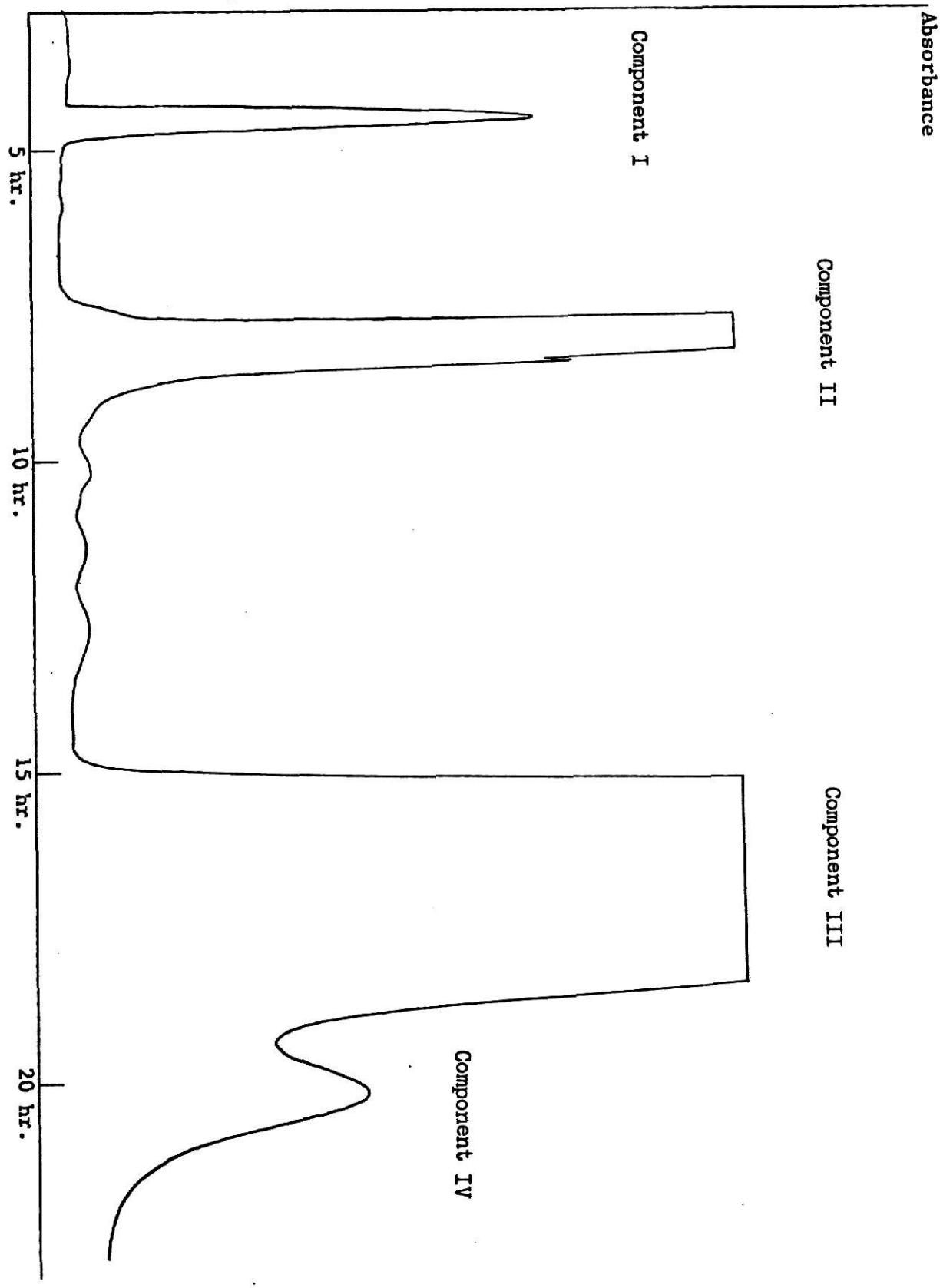


Figure 4.

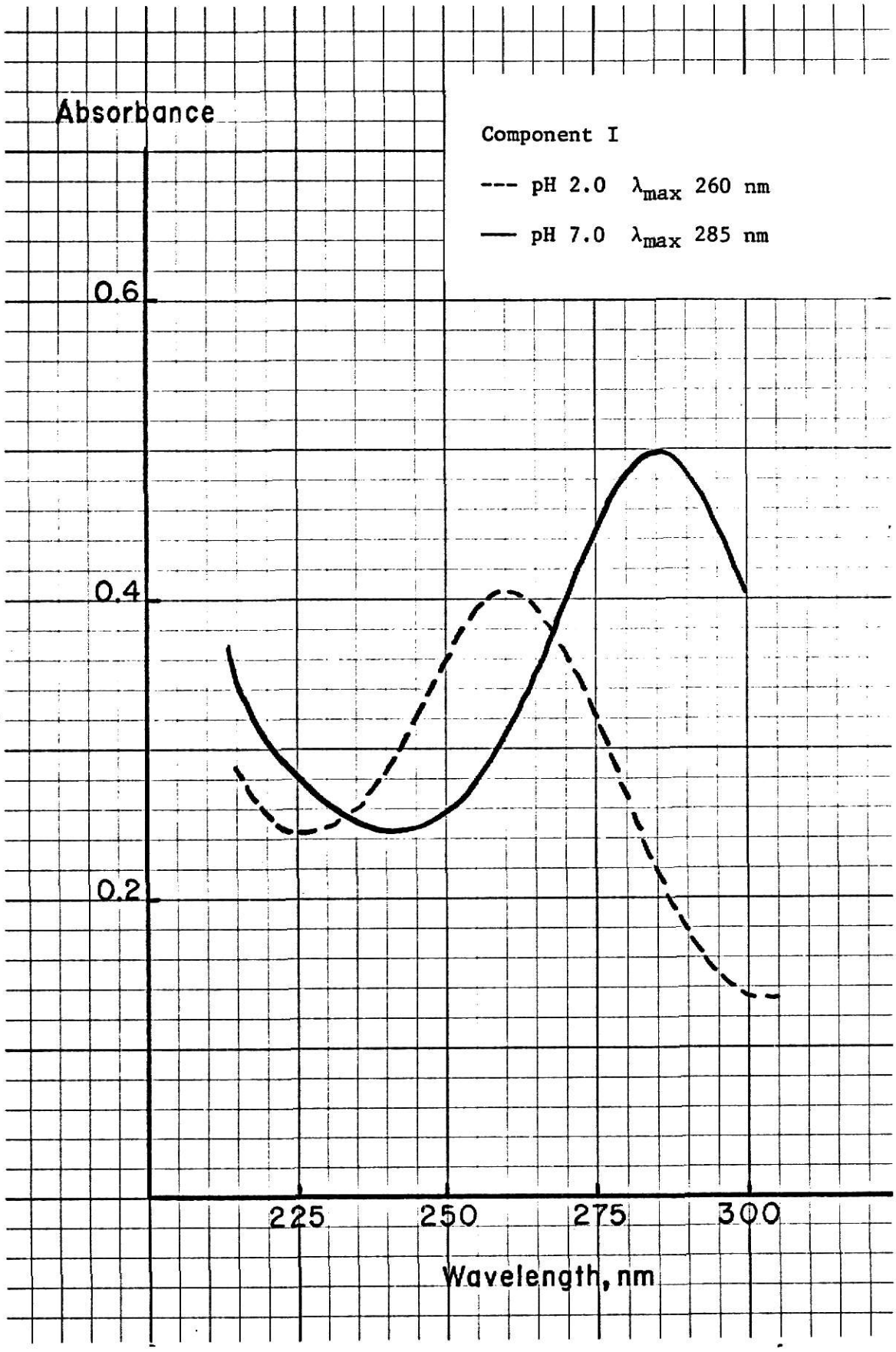

Absorbance
Component I
--- pH 2.0 λmax 260 nm
— pH 7.0 λmax 285 nm
0.6
0.4
0.2
225
250
275
300
Wavelength, nm

Figure 5.

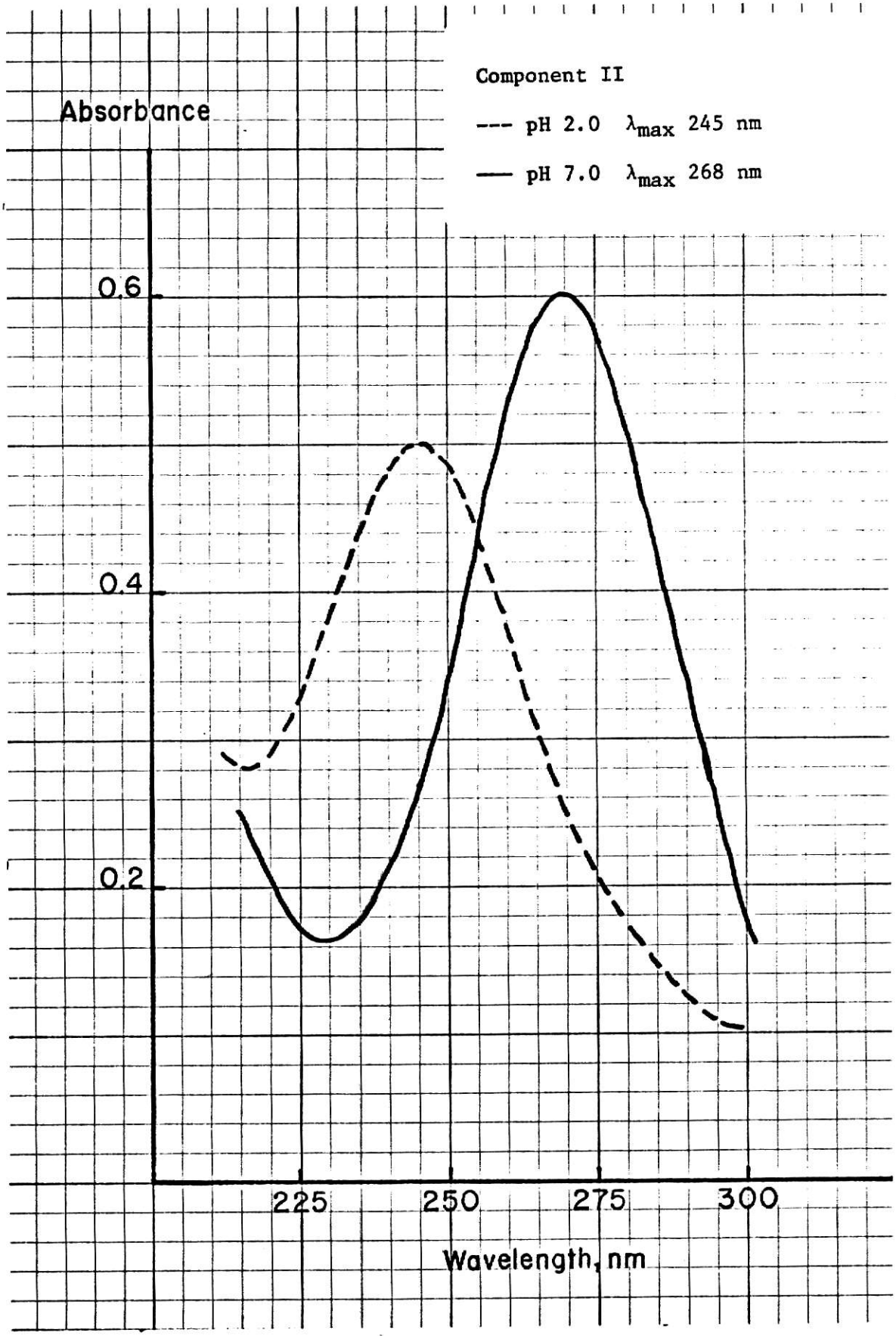

Component II
--- pH 2.0 λmax 245 nm
—— pH 7.0 λmax 268 nm
Absorbance
0.6
0.4
0.2
225
250
275
300
Wavelength, nm

Figure 6.

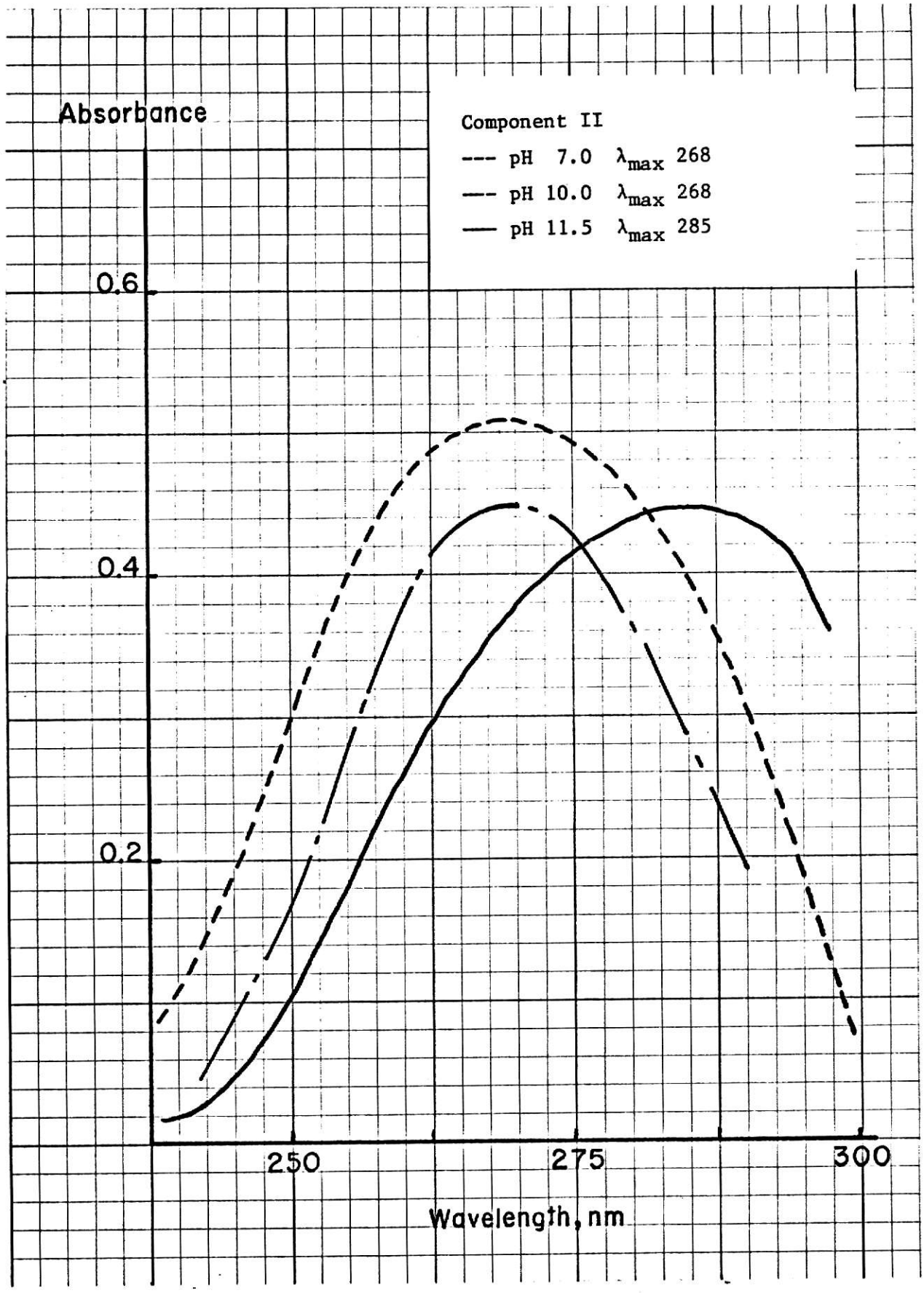

Absorbance
Component II
--- pH 7.0 λmax 268
— pH 10.0 λmax 268
—— pH 11.5 λmax 285
0.6
0.4
0.2
250
275
300
Wavelength, nm

Figure 7.

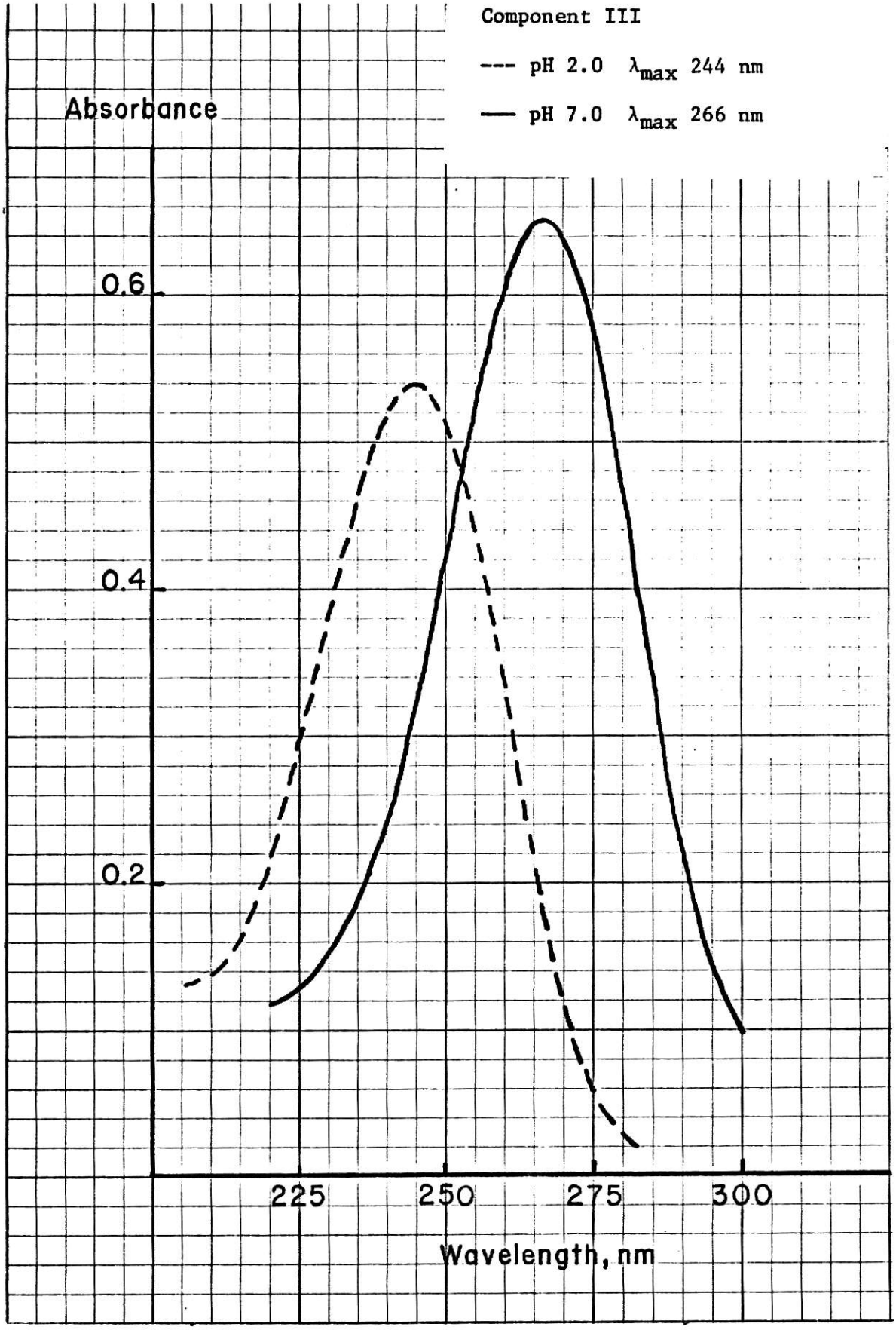

Component III
--- pH 2.0 λmax 244 nm
— pH 7.0 λmax 266 nm
Absorbance
0.6
0.4
0.2
225
250
275
300
Wavelength, nm

Figure 8.

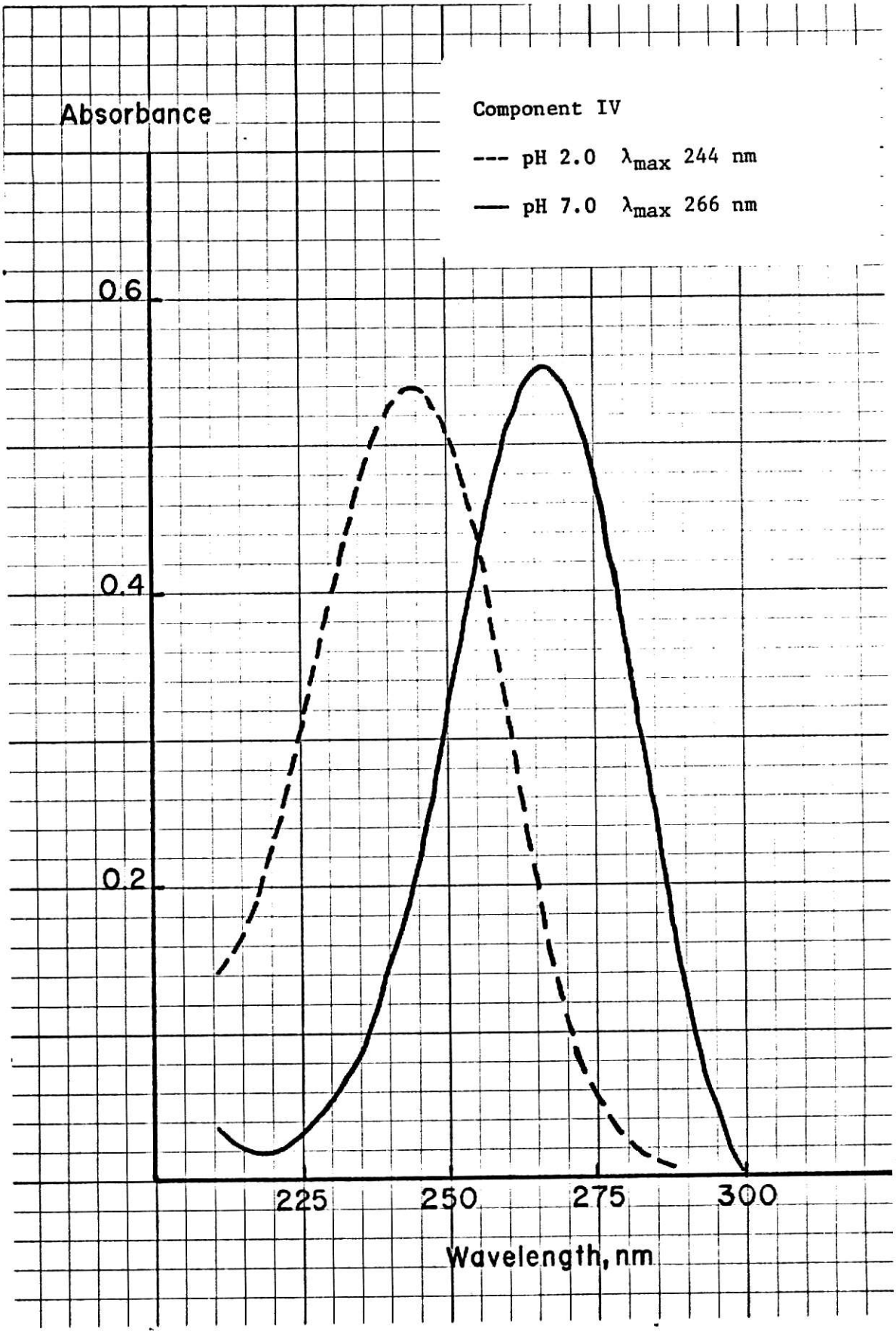
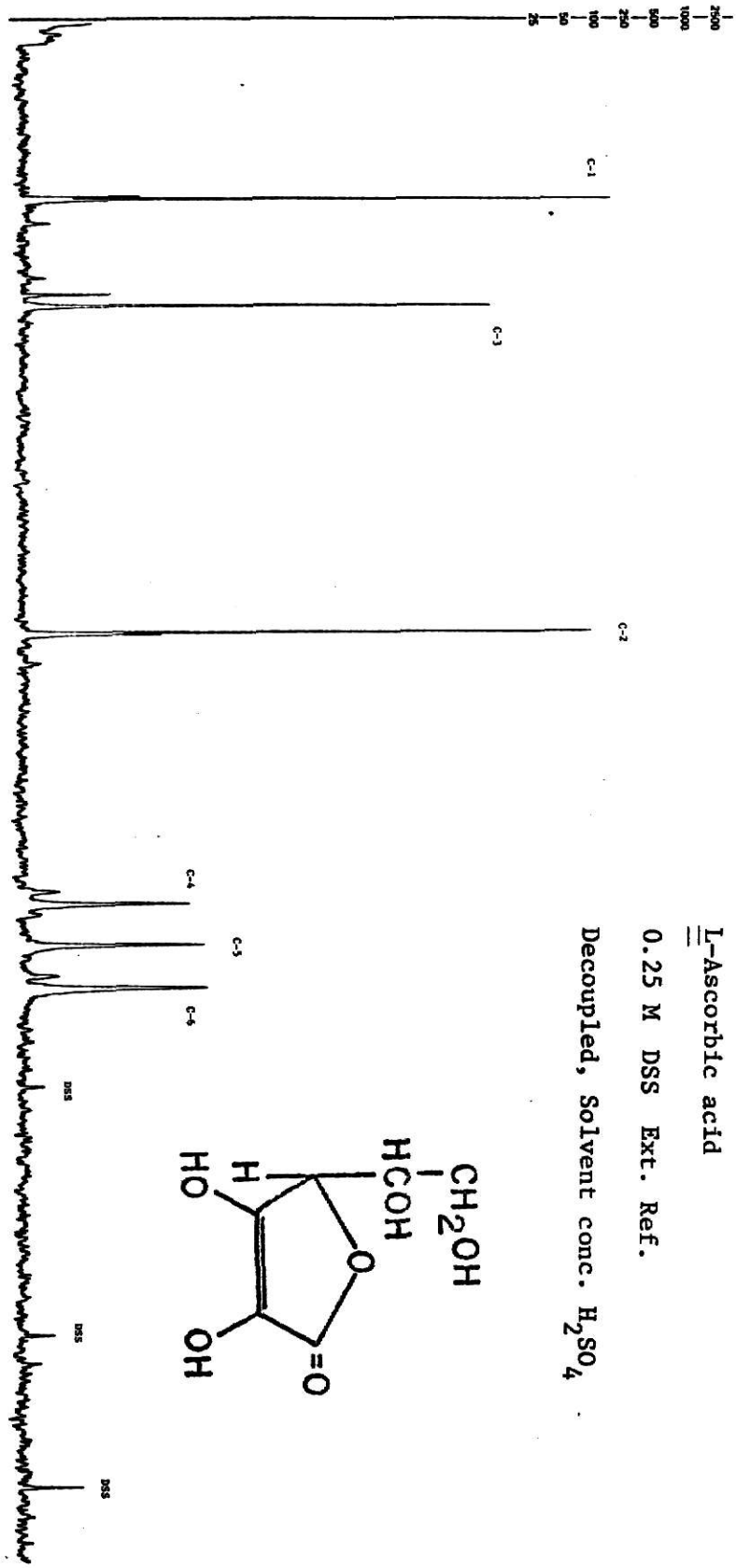

Absorbance
Component IV
--- pH 2.0 λmax 244 nm
— pH 7.0 λmax 266 nm
0.6
0.4
0.2
225
250
275
300
Wavelength, nm

Figure 9.



L-Ascorbic acid

0.25 M DSS Ext. Ref.

Decoupled, Solvent conc. H_2SO_4

Figure 10.

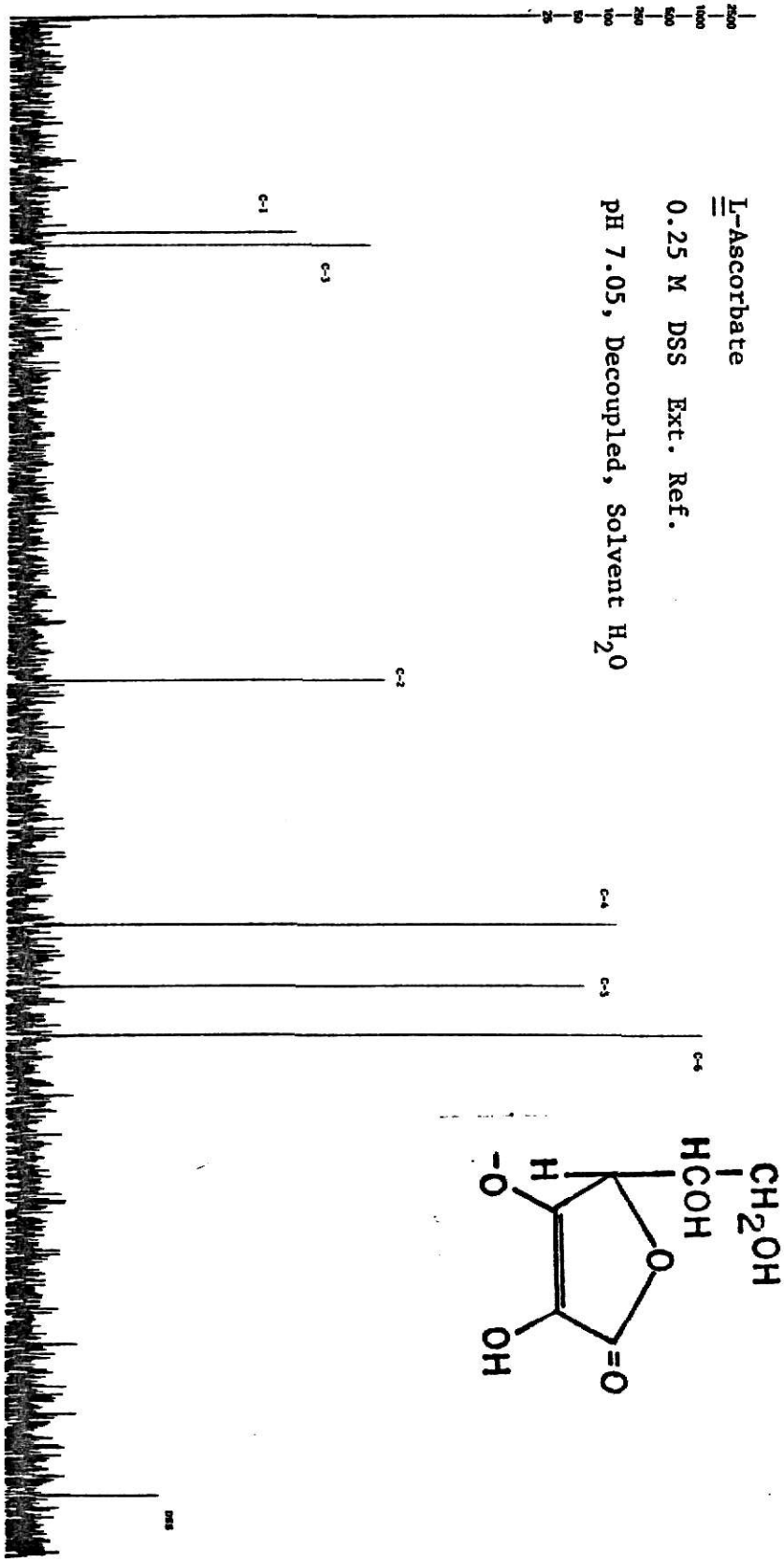

L-Ascorbate
0.25 M DSS Ext. Ref.
pH 7.05, Decoupled, Solvent H_2O
C-1
C-3
C-2
C-4
C-5
C-6
DSS
CH_2OH
HCOH
H
O
O
OH
=O

Figure 11.

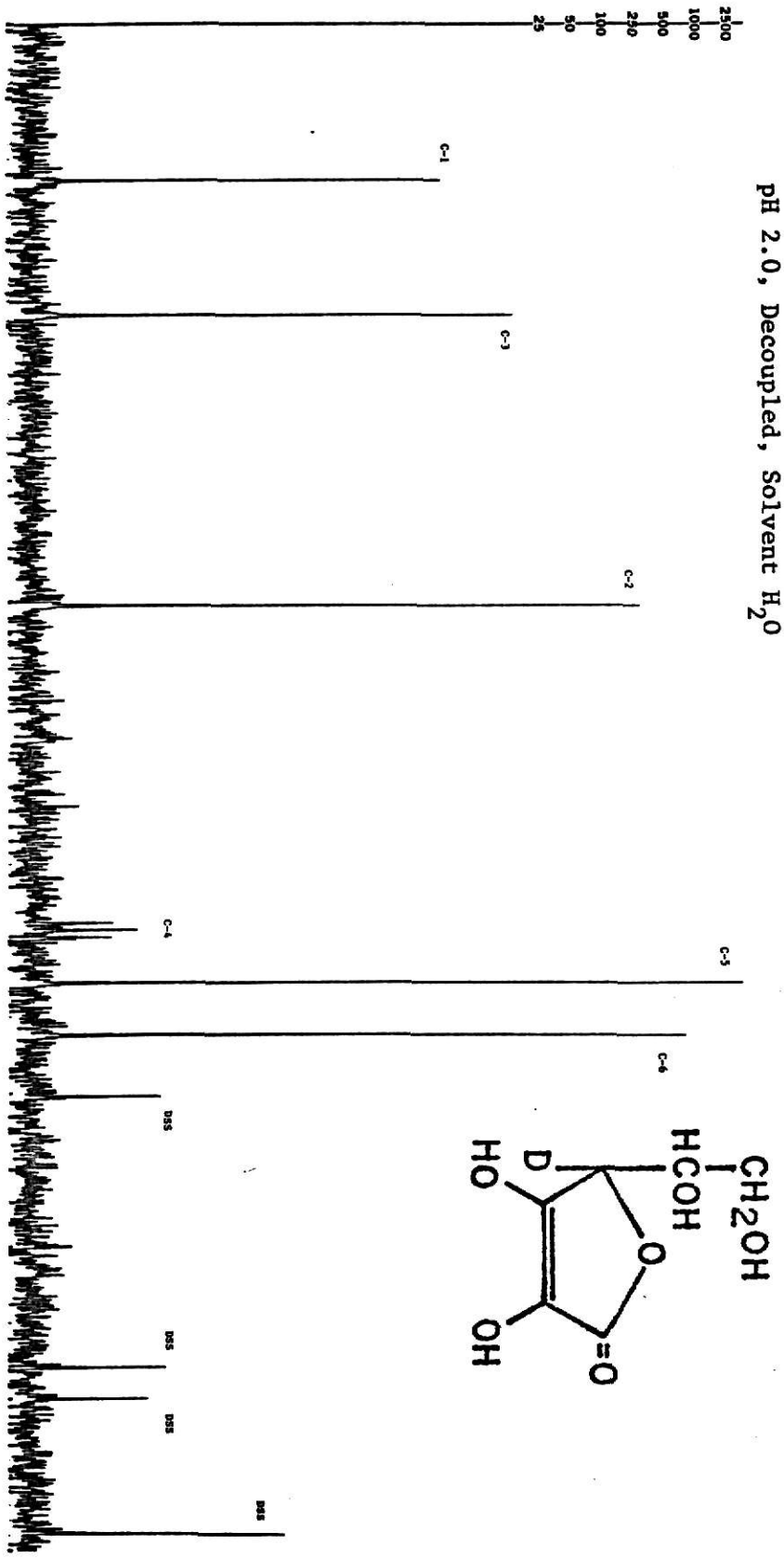
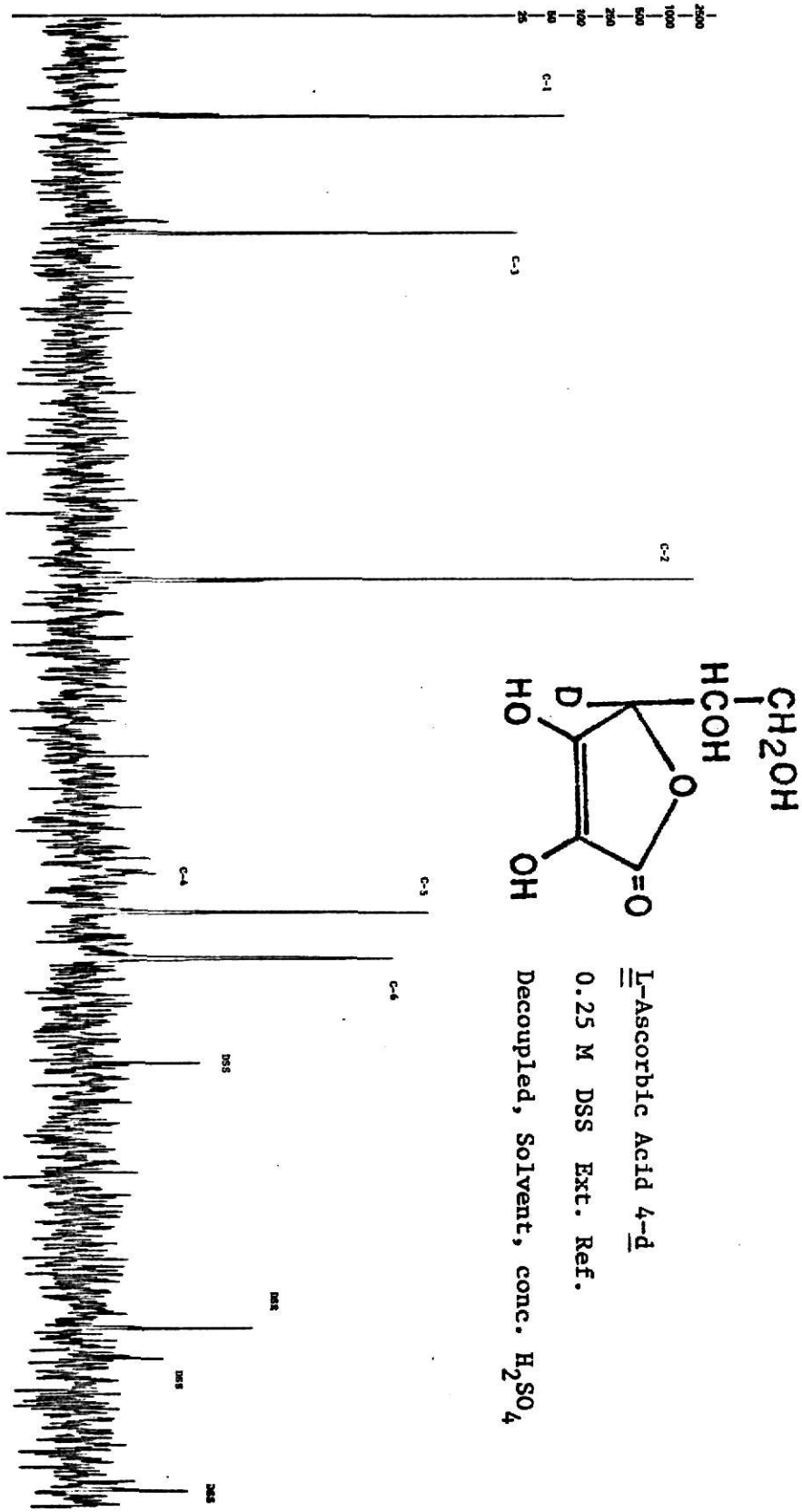

L-Ascorbic acid 4-d
0.25 M DSS Ext. Ref.
pH 2.0, Decoupled, Solvent H_2O
2500
1000
500
250
100
50
25
C-1
C-3
C-2
C-4
C-5
C-6
DSS
DSS
DSS
DSS
HO
D
HCOH
CH_2OH
O
OH
=O

Figure 12.



L-Ascorbic Acid 4-d

0.25 M DSS Ext. Ref.

Decoupled, Solvent, conc. H_2SO_4

Figure 13.

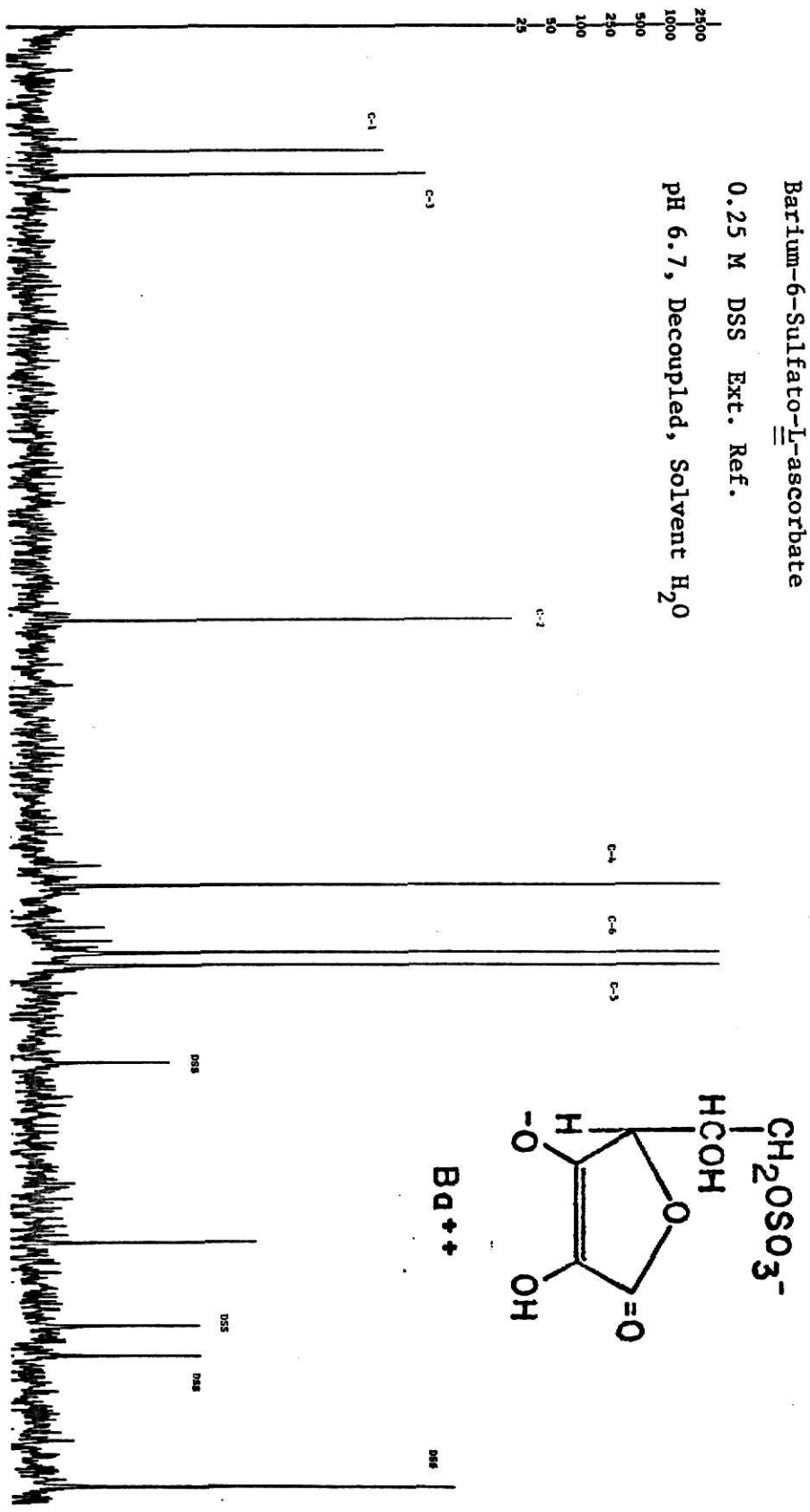

Barium-6-Sulfato-L-ascorbate
0.25 M DSS Ext. Ref.
pH 6.7, Decoupled, Solvent H_2O
2500
1000
500
250
100
50
25
C-1
C-2
C-3
C-4
C-5
C-6
DSS
DSS
DSS
$CH_2OSO_3^-$
HCOH
H
-O
O
OH
=O
Ba++

Figure 14.

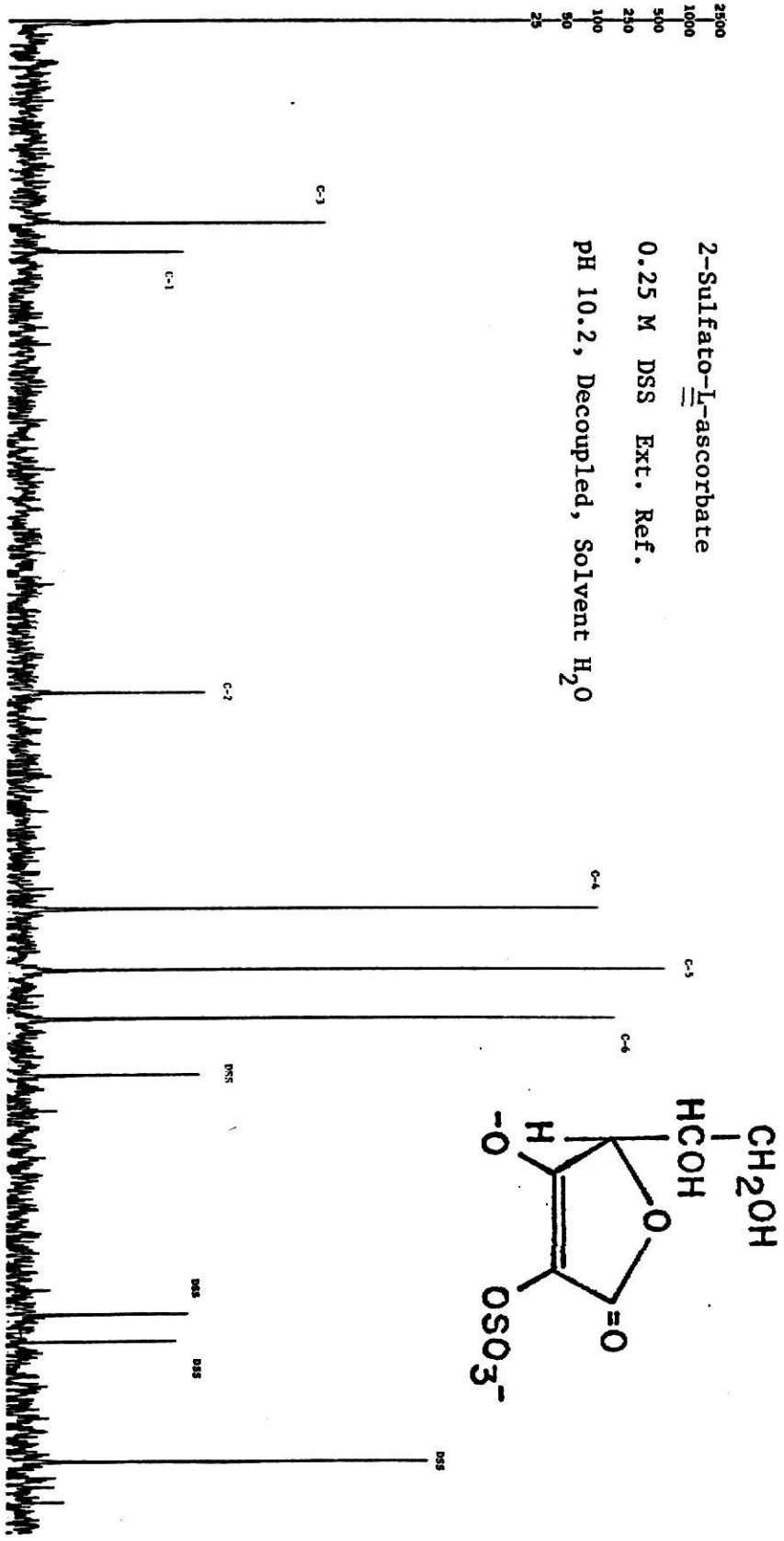
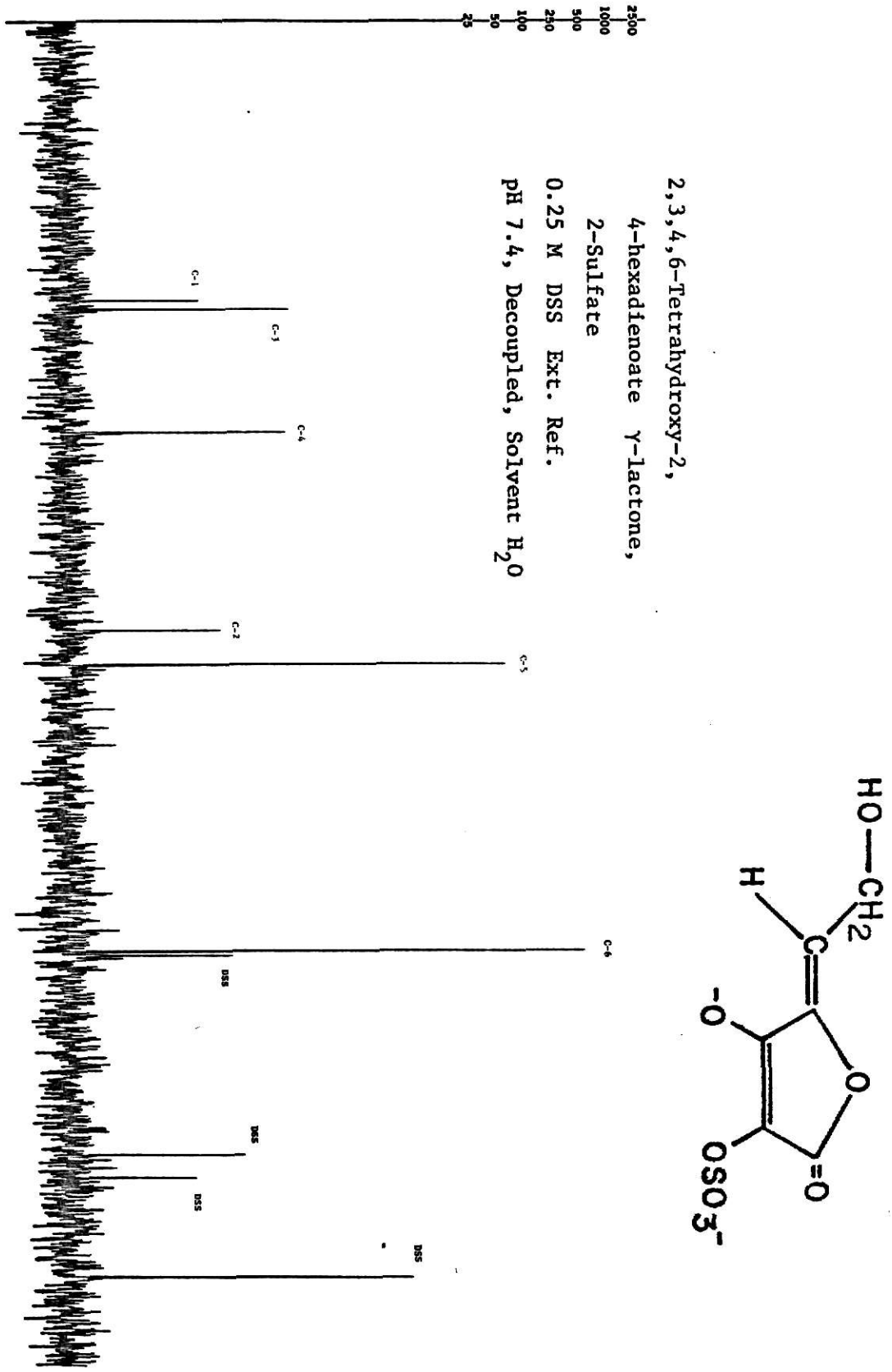
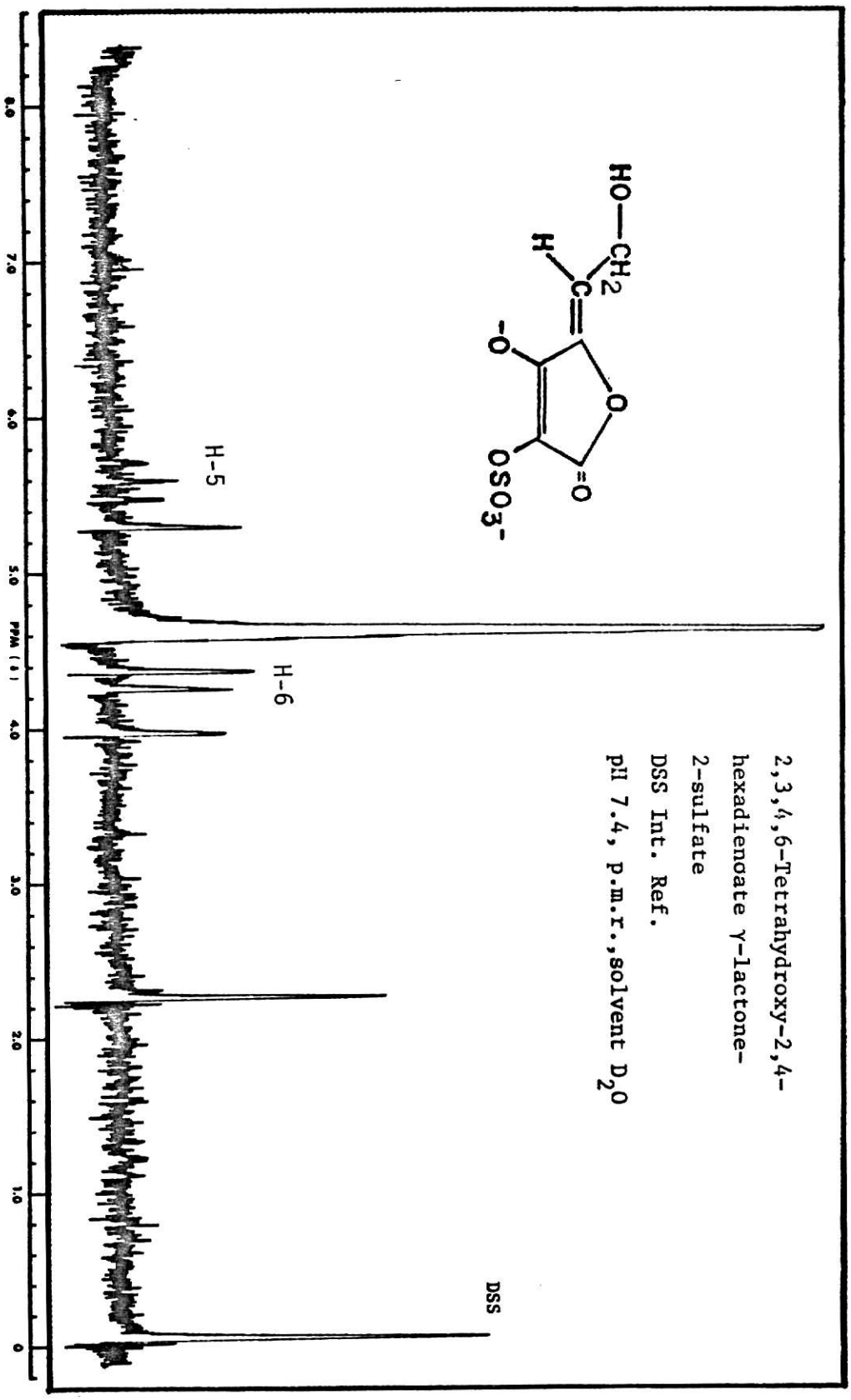

2500
1000
500
250
100
50
25
C-3
C-1
C-2
C-4
C-5
C-6
DSS
DSS
DSS
DSS
2-Sulfato-L-ascorbate
0.25 M DSS Ext. Ref.
pH 10.2, Decoupled, Solvent H2O
CH2OH
HCOH
H
-O
O
=O
OSO3-

Figure 15.



2,3,4,6-Tetrahydroxy-2,
4-hexadienoate γ-lactone,
2-Sulfate
0.25 M DSS Ext. Ref.
pH 7.4, Decoupled, Solvent H_2O

Figure 16.


2,3,4,6-Tetrahydroxy-2,4-
hexadienoate γ-lactone-
2-sulfate
DSS Int. Ref.
pH 7.4, p.m.r.,solvent D2O
HO—CH2
H
C
-O
O
=O
OSO3-
H-5
H-6
DSS
8.0
7.0
6.0
5.0
PPM (δ)
4.0
3.0
2.0
1.0
0