The Occurrence of Ascorbic Acid Sulfate in the Brine Shrimp, *Artemia salina**

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ABSTRACT: Undeveloped cysts of the brine shrimp, *Artemia salina*, contain a sulfur derivative of ascorbic acid, the properties of which are consistent with those expected of ascorbic acid sulfate.

Aqueous solutions of this substance are stable under a variety of conditions at room temperature. The compound is nonreducing, exhibits a characteristic ultraviolet absorption spectrum, and is converted by mild acid hydrolysis into a substance whose properties are identical with those of ascorbic acid.

Although the biosynthesis of organic sulfates has been shown to involve 3′-phosphoadenosine 5′-sulfate as a sulfating agent (Lipmann, 1958), other unknown cofactors have been implicated as sulfate carriers (Dodgson, 1959). Because of a number of indirect relationships between ascorbic acid deficiency and sulfate incorporation into biologically important compounds (Robertson, 1961), it has been postulated that ascorbic acid may be involved in biological sulfation. Recently, Ford and Ruoff (1965) synthesized 5,6-*O*-isopropylidene-L-ascorbic acid 3-sulfate and suggested that ascorbic acid may function biologically as a sulfating agent. Mumma (1968) prepared ascorbic acid 3-sulfate from this derivative and demonstrated that in the presence of a number of oxidizers, including air, the compound is an excellent sulfating agent. This report describes the isolation and purification of a compound from brine shrimp eggs whose chemical and physical properties indicate that it is a naturally occurring ascorbic acid sulfate.

Materials and Methods

Preparation of Acid-Soluble Extracts. Dry cysts (Sanders Co., Ogden, Utah) were ground in liquid nitrogen with a motorized mortar and pestle in 50-g batches for 10 min each. A total of 500 g of dry cysts was used for each preparation. The powdered cysts were suspended in 2 l. of 0.5 N HCl at 4° for 5 min with a Waring Blendor. The suspension was centrifuged at 14,000*g* for 60 min at 4° and the supernatant was filtered through Whatman No. 1 filter paper. The filtrate was diluted to 4 l. with water, the pH was adjusted to 2.0 with NaOH, and the final NaCl concentration was adjusted to 0.1 M by the addition of water.

Purification of Ascorbic Acid Sulfate. The acid-soluble extract was applied to a Dowex 1-X2 (Cl^-), 200–400 mesh (Bio-Rad) column (4 × 35 cm) which had been equilibrated with 0.01 N HCl containing 0.1 M NaCl. After application of the sample, the column was washed with 0.01 N HCl containing 0.1 M NaCl until no additional ultraviolet-absorbing material was eluted (approximately 8 l.). The column was then washed with water until the effluent was neutral. The remaining ultraviolet-absorbing compounds were eluted with 0.5 M NaCl and were found to consist of a mixture of ascorbic acid sulfate and P^1,P^3-diguanosine 5′-triphosphate (Warner and Finamore, 1965). This fraction was adjusted to 0.1 N with HCl and applied to a charcoal column (1.2 × 25 cm, Barnebey-Cheney active carbon SA) previously washed with 25 ml each of (1) water–toluene–isopropyl alcohol–concentrated NH_4OH (46:1:50:3, v/v), (2) absolute ethanol, (3) water, and (4) 1 N HCl (W. E. Cohn, 1968), unpublished data). After the sample was applied it was washed with 100 ml of water and eluted with 2 l. of ethanol–concentrated NH_4OH–water (2:1:2, v/v). The ultraviolet-absorbing fractions were collected and flash evaporated to near dryness at 30°. The separation of ascorbic acid sulfate from P^1,P^3-diguanosine 5′-triphosphate was accomplished by DEAE-cellulose column chromatography. DEAE-cellulose (DE 11, Whatman) was prepared according to Peterson and Sober (1956) and converted from the chloride form into the bicarbonate form by washing with 1 M NH_4HCO_3 adjusted to pH 8.6 with NH_4OH until free of chloride. The column (2.5 × 125 cm) was poured, equilibrated with 0.001 M NH_4HCO_3 (pH 8.6), and the sample was applied. Elution was performed with a linear gradient of NH_4HCO_3 (pH 8.6), from 0.01 to 0.3 M (2 l. of each). The first ultraviolet-absorbing compound eluted was collected and flash evaporated four or five times at 30° or until no NH_4HCO_3 was visible after dryness was reached. The residue, which had the appearance of a transparent film, was dissolved in water and stored at 4°.

Analytical Methods. Quantitative data were obtained by using the ultraviolet absorption molar extinction coefficients published for ascorbic acid and determined experimentally for ascorbic acid sulfate. Qualitative data were obtained by using the anthrone reaction described by Morris (1948) and a modification of the orcinol method (Brown, 1946). Total phosphorus was measured by the method of Griswold *et al.* (1951). Direct Nesslerization by the method of Koch and McMeekin (1924) was used to measure total nitrogen. Infrared spectrophotometry was done with a Beckman IR 12 spectrophotometer and performed by M. Murray of the Oak Ridge National Laboratory. Materials were examined as KBr pellets. Sulfur was determined by reduction of the sulfate

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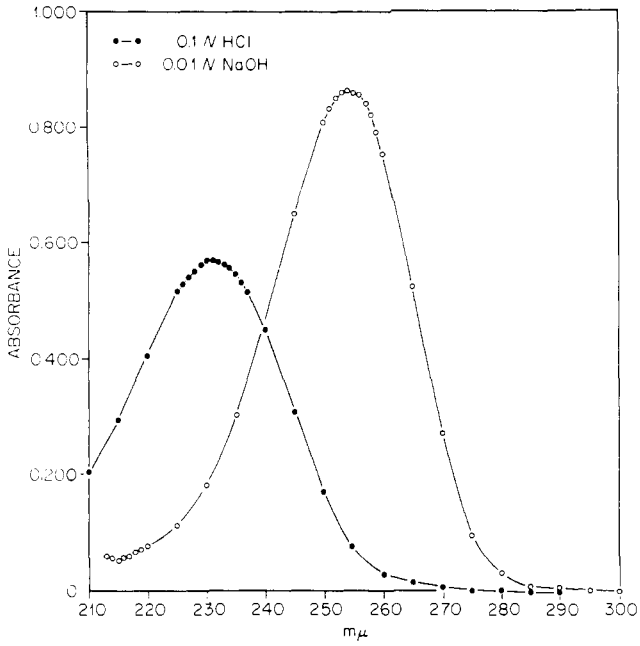

FIGURE 1: Ultraviolet absorption spectra of ascorbic acid sulfate.

to sulfide. The H_2S produced was then precipitated as CdS and washed, and the Cd was measured polarographically. Elemental analysis was performed by Galbraith Laboratories, Inc., Knoxville, Tenn.

Results and Discussion

Properties of Ascorbic Acid Sulfate. After purification of the compound by column chromatography on DEAE-cellulose, it behaved as a single ultraviolet-absorbing component when chromatographed on Whatman No. 1 paper in three different solvents and when chromatographed on DEAE paper eluted with two different concentrations of NH_4HCO_3 (Table I). Purity of the compound can also be demonstrated by column chromatography on Dowex 1-X2 formate, where a single ultraviolet-absorbing peak is eluted that corresponds

TABLE I: Paper Chromatographic Properties of Ascorbic Acid Sulfate.

System[a]	R_F
DEAE (0.1 M)	0.29
DEAE (0.25 M)	0.37
BAW	0.40
Pabst III	0.80
BPW	0.12

[a] Composition of solvent systems: DEAE, 0.1 M; DEAE, 0.25 M = Whatman DE 20 paper eluted with 0.1 or 0.25 M NH_4HCO_3, descending. BAW = 1-butanol–acetic acid–water (5:2:3, v/v), descending. Pabst III = 0.1 M potassium phosphate buffer (pH 6.8)–ammonium sulfate–propanol (100:60:2, v/v), ascending. BPW = 1-butanol–pyridine–water (6:4:3, v/v), descending.

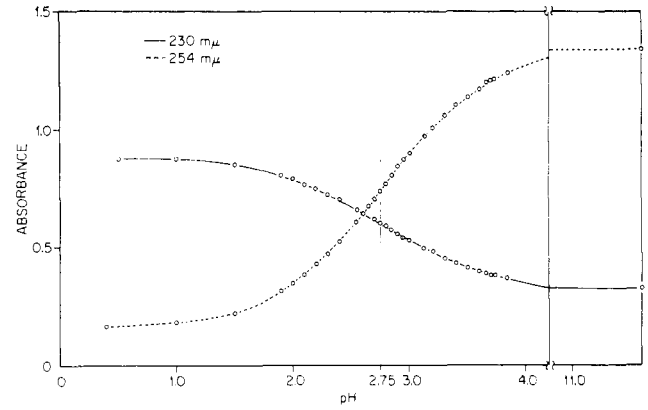

FIGURE 2: Relative ultraviolet absorbance at absorption maxima as a function of pH.

to all of the anthrone- and orcinol-positive material applied. The ultraviolet absorption spectrum of this material is shown in Figure 1, and a comparison of some of its properties with those of ascorbic acid is given in Table II. The compound is

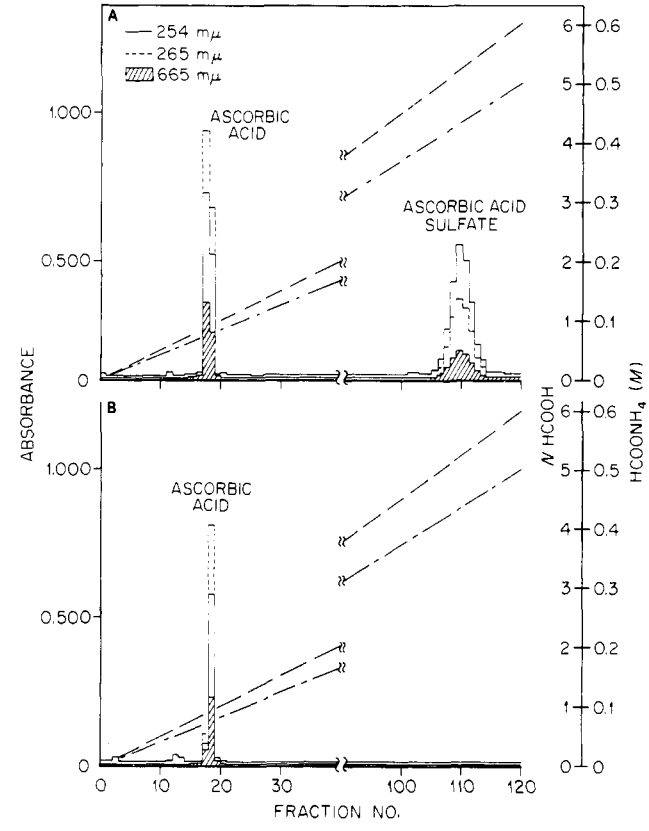

FIGURE 3: Column chromatography of ascorbic acid, ascorbic acid sulfate, and acid-hydrolyzed ascorbic acid sulfate. Chromatography was performed with a column (1 × 20 cm) of Dowex 1-X2 formate eluted with a linear gradient of formic acid and ammonium formate as indicated (1 l. each). The samples applied were (A) a mixture of ascorbic acid (10 μmoles) and ascorbic acid sulfate (3.25 μmoles) and (B) the acid hydrolysis product of ascorbic acid sulfate (3.25 μmoles heated at 100° in 1 N HCl for 5 min). The product was equivalent to 2.72 μmoles of ascorbic acid or an 84% recovery. The elution was followed by measurement of ultraviolet absorbancy at 254 mμ (absorption maximum of ascorbic acid sulfate) and 265 mμ (absorption maximum of ascorbic acid) of aliquots adjusted to pH 8.6 and by the orcinol reaction.

TABLE II: Properties of Ascorbic Acid Sulfate and Ascorbic Acid.

	Ascorbic Acid Sulfate		Ascorbic Acid	
	pH 1	pH 8.6	pH 2	pH 6.4
Ultraviolet absorption (mμ)				
λ_{max}	230	254	245	265
λ_{min}		220–221		
Molar extinction coefficient (λ_{max})[a]	22,500	35,000	12,240	16,550
Specific rotation $[\alpha]_D$ (deg)		+98.5		+24
Reduces $AgNO_3$		No		Yes

[a] The molar extinction coefficient for ascorbic acid sulfate is based on its sulfur composition. That for ascorbic acid is calculated from the data of Lawendel (1957).

stable for several days in aqueous solution at room temperature over a range of pH values from 1 to 12, and its ammonium salt is stable when dry at room temperature. When converted into the hydrogen form by passage through a Dowex 50 (H^+) column, the compound is unstable, forming a black product when dried over P_2O_5 at room temperature. Oxidation at room temperature with NaOCl or Br_2 results in an immediate loss of its ultraviolet-absorbing characteristics. Furfural is produced after prolonged heating in strong acid and gives a positive reaction to the orcinol and anthrone tests. Treatment with strong acid and heat produces a dark product ("browning reaction") identical in appearance with that produced by similar treatment of ascorbic acid. Figure 2 illustrates the effect of pH on the absorption spectrum of this compound. The shift in absorption maxima which is half-maximal at pH 2.75 reflects the ionization of an acidic group.

Formation of Ascorbic Acid by Acid Hydrolysis. When the compound is heated at 100° in 1 N HCl for short periods of time (2–5 min), the ultraviolet absorption maximum shifts from 230 to 242 mμ at pH 1 and from 254 to 265 mμ at pH 8.6. However, under alkaline conditions, the ultraviolet absorption of the acid-hydrolyzed compound decreases rapidly. The new absorption spectrum is similar to that reported for ascorbic acid (Herbert *et al.*, 1933), with absorption maxima at 245 mμ at pH 2 and 265 mμ at pH 6.4. After this mild acid hydrolysis the compound becomes a strong reducing substance, and like ascorbic acid it is capable of reducing $AgNO_3$ to Ag under neutral conditions (Szent-Györgyi, 1928). The chromatographic properties of this new reducing compound are identical with those of ascorbic acid, as detected by ultraviolet absorption and $AgNO_3$ reduction on paper chromatograms in two solvent systems (1-butanol–pyridine–water, 6:4:3, v/v, and 1-butanol–ethanol–acetone–water 5:4:3:2, v/v). Furthermore, ascorbic acid and the product of mild acid hydrolysis of ascorbic acid sulfate show identical chromatographic properties when chromatographed on Dowex 1-X2 formate columns (Figure 3). The specific optical rotation, $[\alpha]_D^{24}$, of the ammonium salt of the original compound is +202.8°. After the salt is converted into free acid by passage of the compound through Dowex 50-X8 (H^+), the $[\alpha]_D^{23}$ is +98.5°. After the compound is heated in 1 N HCl for 5 min at 100°, the $[\alpha]_D^{24}$ is +24.7°, a value almost identical with that reported for ascorbic acid (Herbert *et al.*, 1933).

Infrared Spectrophotometry. The infrared absorption spectrum of the sodium salt of the compound is illustrated in Figure 4a. Distinct absorption bands not present in the absorption spectrum of ascorbic acid (Figure 4b) are apparent at 1260 and 790 cm^{-1}. Absorption at the frequencies of 1210–1260 and 770–810 cm^{-1} has been shown by Lloyd *et al.* (1961)

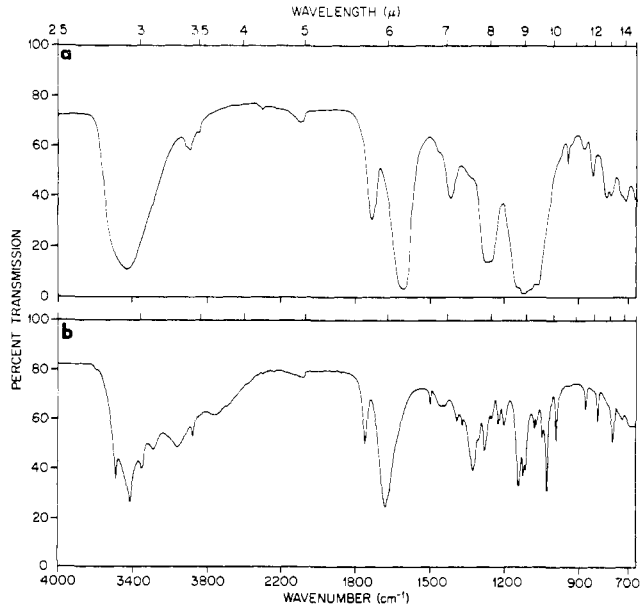

FIGURE 4: Infrared absorption spectra of (a) ascorbic acid sulfate (sodium salt) and (b) ascorbic acid.

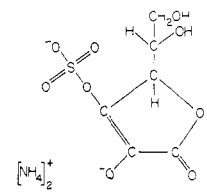

FIGURE 5: Proposed structure for ascorbic acid sulfate.

to be characteristic of acid sulfates of carbohydrates. According to Orr's (1954) original assignment, these bands correspond to the S=O and COS vibrations, respectively. The absorption spectrum of the hydrolysis product of ascorbic acid sulfate is complicated by the presence of other degradation products, but there are clearly no absorption bands at 1260 and 790 cm^{-1}, indicating the removal of the sulfate.

Conclusions

The properties of this compound are consistent with those expected of ascorbic acid sulfate. The nature of the substituted group is ascribed to a sulfate for three reasons. First, the acid lability of the compound is that expected of a sugar sulfate (Turvey, 1965; Rees, 1963). Second, the infrared absorption spectrum exhibits bands characteristic of acid sulfates of carbohydrates. Third, the elemental analysis of the compound (Table III) agrees with the calculated composition of ascorbic acid sulfate. The position of the sulfate has not been determined directly. Positions 2 and 3 are most likely because of the nonreducing properties of the compound, and because only substitutions of the vicinal enolic groups result in nonreducing derivatives of ascorbic acid (Hay *et al.*, 1967). It is presumed that the compound is the 3-sulfate, since the OH group of ascorbic acid at position 3 is considered to be the most acidic (Vestling and Rebstock, 1944) and since the pK observed spectrophotometrically at pH 2.75 is indicative of the ionization of an acidic group. Figure 5 illustrates the proposed structure of the compound.

TABLE III: Elemental Analysis of the Ammonium Salt of Ascorbic Acid Sulfate ($C_6H_{14}O_9SN_2 \cdot H_2O$).

	Observed (%)		
	Sample 1	Sample 2	Calcd (%)
C	23.49	23.54	23.38
H	5.23	5.18	5.23
O	51.85		51.90
S		10.12	10.40
N		8.91	9.09

To our knowledge this report represents the first demonstration of the natural occurrence of ascorbic acid sulfate, and it is tempting to speculate that the compound may be a means by which the organism stores ascorbic acid when encysted in the dry state. The observed stability of the purified compound would be consistent with this possibility. The substance may also function as a form of active sulfate, accumulated in the dry cysts to be used later during development. Thus, ascorbic acid sulfate could perform a dual function as a storage form of both ascorbic acid and sulfate. Studies on the change in concentration of the compound during development and tracer studies with ^{35}S may elucidate such functions.

Acknowledgments

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