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BICYCLIC RING FORMATION IS NOT NECESSARY FOR THE (AUTO)OXIDATION OF ASCORBIC ACID

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SUMMARY: The oxidation rates of ascorbic acid and several of its derivatives have been examined in order to delineate the role of bicyclic ring formation in the autooxidation of ascorbic acid. The compounds evaluated and their respective oxidation rates at pH 7.4 are in $uM \cdot min^{-1}$: ascorbate, 0.70; 5 methyl 3,4 dihydroxytetrone, 0.65; D-iso ascorbate, 0.73; and ascorbyl palmitate, 0.64. These data do not support the contention that bicyclic ring formation is required for the oxidation of ascorbic acid because both 5 methyl 3,4 dihydroxytetrone and ascorbyl palmitate, neither of which can form the bicyclic intermediate, have oxidation rates similar to that of ascorbate. Furthermore, evidence is presented which suggests that ionization of the oxygen on C_3 of ascorbic acid is an obligatory initial step in ascorbic acid autooxidation.

NMR studies have shown that closure of its side chain occurs when ascorbic acid oxidizes to its semidehydro form, the ascorbic acid free radical (HA) [1,2]. Ultimately, in a two one-step electron transfer reaction with HA as intermediate, ascorbic acid forms a hydrated bicyclic monomer, dehydroascorbic acid [3]. These observations suggested bicyclic ring formation as an obligatory step in the oxidation of ascorbic acid. Recent experiments of ours have shown that compounds which bind to ascorbic acid dramatically retard its rate of oxidation [4]. These ligands might be interacting with ascorbic acid through a mechanism which interferes with the formation of the bicyclic structure, thus affecting oxidation. We decided to test this hypothesis by determining the rate of oxidation of several ascorbic acid derivatives which have side chains that are not capable of the ring formation. Reported here are the oxidation rates of D,L 5-methyl-3,4-dihydroxytetrone, D-iso-ascorbic acid, ascorbic acid and ascorbyl palmitate in aqueous solution at physiological pH. The results show that formation of the ring structure is not an obligatory intermediate in the oxidation of ascorbic acid, since the oxidation rate was not influenced by structural or steric changes in the side chain.

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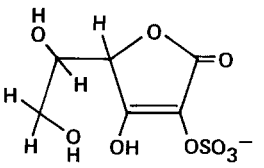
MATERIALS AND METHODS

Ascorbic acid and D iso-ascorbic acid were procured from Sigma Chemical Co., St. Louis, MO. Ascorbyl palmitate was obtained from Research Plus Inc., Denville, NJ. D,L 5-methyl-3,4-dihydroxytetrone was prepared from L-ascorbic acid according to the method of Tomita et al. [5]; its purity was confirmed by melting point determination (174.5-176.5°C), UV and IR spectrophotometry. Dilutions of freshly prepared stock solutions (1×10^{-3} M) were used for all assays. U.V. absorption spectra were obtained on all of the compounds at a concentration of 1×10^{-5} M. Each compound had the expected absorption peak at 265 nm. Autooxidation of these compounds (1.0×10^{-5} M) was determined by following the decrease in absorption at 265 nm [6]. Interference by oxidation products does not occur at this wavelength [6]. The effects of EDTA, bovine serum albumin and histidine on the rate of autooxidation of the ascorbate derivatives employed in this study was carried out in a manner identical to that used previously for ascorbic acid [4,7]. In essence, these compounds, which are known to strongly retard the oxidation of ascorbic acid, were added to the ascorbic acid derivative solutions at 10^{-5} M, and changes in the oxidation rate recorded [4,7]. Experiments with the superoxide radical, generated by the xanthine-xanthine oxidase system, were performed as described by Nishikimi [8]. All reactions were carried out at 25°C in a Gilford model 2600 microprocessor-controlled spectrophotometer coupled with a Hewlett Packard model 7225 A XY plotter. Absorbance was recorded automatically every 0.2 min for at least 30 minutes. The infrared spectrum of 5 methyl 3,4 dihydroxytetrone was obtained with a Pye-Unicam infrared spectrophotometer model 3-200.

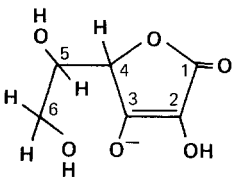
RESULTS AND DISCUSSION

Figure 1 shows the structures of the compounds employed in these studies. The L-ascorbate anion is depicted as reported by Sapper et al. [1]. The studies by this group showed, using NMR methodology, that during oxidation of the HA radical intermediate, loss of an electron from oxygen of C_3 results in bond formation between C_3 and the oxygen molecule on C_6 (Fig. 1). 5 methyl 3,4 dihydroxytetrone (Fig. 1) is not capable of forming a similar bicyclic structure owing to the short side chain and to the absence of a sterically-placed primary alcohol. Similarly, D-iso-ascorbate might not be capable of forming the same bicyclic intermediate because of steric hindrance due to the position of the OH on carbon 5 (Fig. 1). Also, formation of a similar bicyclic structure can not occur with ascorbyl palmitate, due to the esterification of the C_6 alcohol.

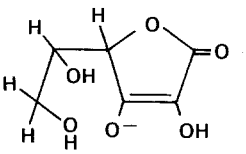
Figure 2 demonstrates that the oxidation rates of these compounds are all virtually identical; these reaction rates are in $uM \cdot min^{-1}$: ascorbate, .70; 5 methyl 3,4 dihydroxytetrone, .65; D-iso-ascorbate, .73; and ascorbyl palmitate, .64. These results clearly indicate several important points regarding the oxidation of ascorbate at physiological pH. First, the formation of a bicyclic structure is not required for its oxidation because 5 methyl 3,4 dihydroxytetrone, which clearly cannot form the suggested intermediate, has an oxidation rate similar to that of ascorbate. Second, the oxidation rate of ascorbate is not a function of the structure of its side chain as shown by the rate



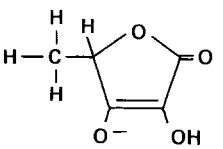
L–ASCORBIC ACID 2–SULFATE



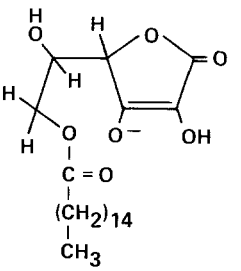
L–ASCORBATE ANION



D–ISOACORBATE ANION



D,L–5 METHYL 3,4 DIHYDROXYTETRONE



L–ASCORBYL PALMITATE

Figure 1. Structures of the ascorbate derivatives employed in the studies on oxidation rate. The configuration of the L-ascorbate anion is depicted as reported by Sapper et al. (1).

of ascorbyl palmitate autooxidation. We furthermore observed that compounds which retard the rate of autooxidation of the ascorbate also inhibit the oxidation of the ascorbate derivatives used in this study. Interestingly, generation of the superoxide radical by the xanthine-xanthine oxidase system accelerates the oxidation of all of these ascorbate derivatives as well as ascorbic acid to the same extent, i.e., all of these compounds have very similar antioxidant properties.

Our studies indicate that stabilization of semidehydro ascorbic acid by participation of the side chain alcohol is clearly a nonobligatory step in ascorbic acid oxidation. On the other hand, the replacement of the C_2 alcohol by sulfate yields a derivative that is stable to autooxidation at pH 7.4. In fact, the stability of 2-sulfate ascorbic acid at pH 7.4 rivals that of ascorbic acid below pH 4.2, the ionization constant for the C_3 alcohol.

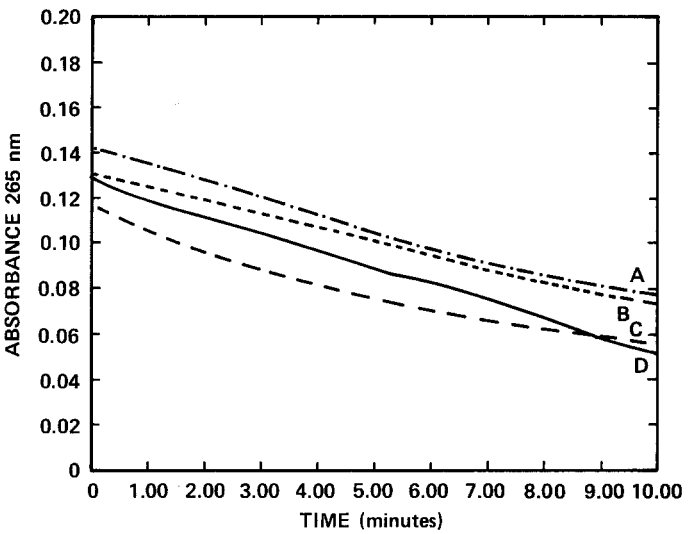


Figure 2. The oxidation rates of the ascorbate derivatives at 1.0 X 10^{-5}M.
A, L-ascorbate
B, 5-methyl 3,4 dihydroxytetrone
D, D-iso-ascorbate
All reactions carried out in 0.067M phosphate buffer, pH 7.4 at 25°C.

Since the ionization constant of the C_3 alcohol group of 2-sulfate ascorbic acid should be perturbed higher by the adjacent anionic sulfate moiety[1], important information about the autooxidation of ascorbic acid could be obtained from studies of the pH-dependence of 2-sulfate ascorbic acid titrations. These studies are currently underway.

We conclude from these data that the formation of the double ring structure which occurs on oxidation of the ascorbic acid free radical, does not play a role in the oxidation rate of ascorbic acid. We have speculated that ionization of the C_3 alcohol to the highly

[1]Calculations of the perturbation of the pK value of the C_3 alcohol group of ascorbic acid 2-sulfate, while crude, indicate that in fact this group may be unionized even at neutral pH. Thus, if we set the electrostatic potential for repulsion of two like point charges equal to the difference in free energy for dissociation of a 1:1 charge salt bridge, assigning K_1 to the unperturbated ascorbic acid ionization and K_2 to the ionization in 2-sulfate ascorbic acid, we obtain

$$RT \ln \frac{K1}{K_2} = \frac{q^2 N}{Dr}$$

where q is the electronic charge, N is Avogadro's number, D is the dielectric constant in the intervening medium, r is the distance between the C_3 alcohol and the anionic sulfate center (taken as 3A), and RT has its usual meaning. Taking the dielectric constant as 20 (the value of the effective dielectic constant for intramolecular charge-charge interactions of organic molecules in aqueous solution can be much less than 15 [Westheimer and Kirkwood, 1938, J. Chem. Phys. 6, 513]) we get a pK for the C_3 alcohol of about 8.3.

reactive alkoxide ion may be a necessary initial step in the mechanism of ascorbic acid oxidation and we are pursuing this possibility.

ACKNOWLEDGEMENT

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