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# ON A POSSIBLE MECHANISM OF ACTION OF ASCORBIC ACID: FORMATION OF IONIC BONDS WITH BIOLOGICAL MOLECULES

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## SUMMARY

Ascorbic acid is capable of forming an ionic bond with biological molecules at physiologic pH. The influence of such a bond on the reactivity of the molecular complex should be considerable because the bond does not directly affect the functional site of the ascorbate. It must bring about local alteration of the redox potential and accelerate charge transfer reactions. Facilitation of these properties may be one of the biological functions of ascorbic acid.

## INTRODUCTION

Many lyophilized biological tissues produce a characteristically shaped, asymmetrical ESR signal (a-signal) at about $g = 2.005$ (Fig. 1). Electron spin resonance (ESR) investigations of lyophilized blood of leukemic patients demonstrated this signal also in the washed erythrocytes of individuals with acute lymphatic leukemia. Therapeutic improvement of these patients was paralleled by a decrease in spin concentration and eventual disappearance of the a-signal (1). Laboratory investigations suggested that the a-signal was probably caused by a relatively high concentration of antioxidants in the leukemic blood. Specifically, ascorbic acid added to membrane components of erythrocytes of various sources affected the development and spin concentration of this complex signal (2). Subsequently, it was shown that interaction

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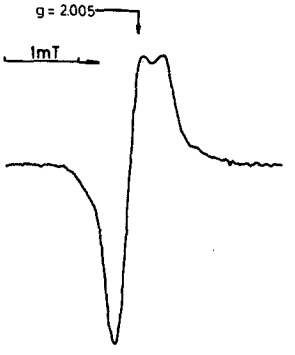


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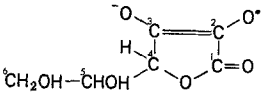

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Fig. 1: ESR signal typical of lyophilized biological tissue, in this instance plasma. The ESR detection conditions were: 0.2 mT modulation amplitude at 100 KHz and 5 mW microwave power at ~9.0 GHz.

Fig. 2: Molecular structure of ascorbic acid, here presented as the anion of its semidehydrous form; dot indicates the site of the unpaired electron.

of ascorbic acid with copper-containing proteins results in the a-signal (3). Power saturation studies carried out during this investigation revealed that the a-signal actually consists of two signals, $a^{I}$ and $a^{II}$, which originate from two different species. The $a^{I}$ peak at about g = 2.005 was believed to belong to the semi-dehydroascorbic acid radical and the $a^{II}$ peak, which is located slightly upfield, to the copper-protein. Since the type and atomic site of such an interaction of ascorbic acid with other molecules are still unknown, the complex formation between ascorbic acid and a variety of compounds of biological interest was investigated by means of ESR spectroscopy.

## MATERIALS AND METHODS

A series of biological compounds were permitted to react with ascorbic acid at different concentrations and pH before freeze-drying the aqueous solutions (Fig. 2). Similarly, synthetic organic and inorganic compounds were used in combination with ascorbic acid. Finally, ascorbic acid was allowed to interact with ion exchange resins at different pH. Typically, 10 ml of solutions containing up to 0.6 mM of ascorbic acid were passed through an anion exchange column (dry weight 500 mg) which was then rinsed with water before lyophilization and examination by ESR. The latter was carried out at room temperature with a Varian E9 spectrometer, usually with a modulation amplitude of 0.2 mT, a modulation frequency of 100 kHz and a microwave power of 5 mW at the x-band frequency. Most of the employed chemicals were of reagent grade and used without further purification. Particular attention was paid to

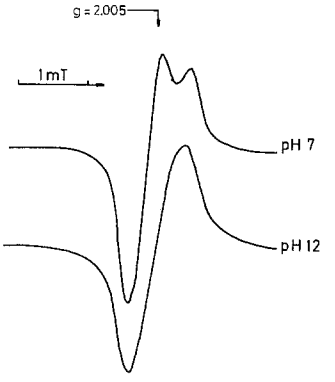


Fig. 3: ESR Spectra observed at room temperature in ascorbic acid-Dowex AG 1x8 complex. 0.3 mM ascorbic acid in 10 ml water at pH 7 (a) or pH 12 (b) was passed through the anion exchange column (dry weight 500 mg), the ascorbic acid-anion exchanger complex then washed with water and finally lyophilized. The ESR detection conditions were: 0.2 mT modulation amplitude at 100 kHz and 5 mW microwave power at ~9.0 GHz.

their contents of transition metals, especially Cu. The latter element was present at a concentration of 2 ppm in the ascorbic acid and the ion exchange resin, and reached levels of as much as 18 ppm in one of the batches of bovine serum albumin. These data are based on analyses by atomic absorption spectroscopy.

## RESULTS AND DISCUSSION

Pilot studies with a variety of compounds suggested that ascorbic acid may actually undergo a closer association with a partner molecule than hitherto suspected and that the interaction most likely is of the ionic type. In order to prove that such binding of the ascorbate anion to a partner molecule is a prerequisite for the appearance of the a-signal, an anion exchanger with well known properties (Dowex AG 1x8) was employed to immobilize the ascorbate anion. A solution of ascorbic acid at pH 6, at which the ascorbic acid is in its monovalent anion form, was passed through the anion exchange column and the unbound ascorbic acid was removed by rinsing with water. The resin-ascorbic acid complex was then examined by ESR. A very strong a-signal was obtained under these conditions (Fig. 3), which was directly proportional to the amount of bound ascorbic acid. Since the signal most likely originated from ascorbic

acid, it would have had to come from the semidehydroascorbic acid radical, the unpaired electron which is located at the oxygen atom attached to $C_2$ (4,5). This oxygen atom is also the site at which the second anion of ascorbic acid is formed at high pH. Therefore, if the bivalent anion of ascorbic acid is bound by both of its ionized groups to an anion exchanger, one can expect the radical not to appear. A solution of ascorbic acid at pH 12.5 was passed through the anion exchange colum and the unbound ascorbic acid was then removed. As expected, such a sample did not show the a-signal, thus proving that the peak at g = 2.005 indeed originates from ascorbic acid. Now only the second $a^{II}$-signal was visible (Fig. 3).

A systematic search was made for molecules giving rise to the a-signal when reacted with ascorbic acid. Certain proteins, such as serum albumin of various sources, as well as oxidized glutathione produce a strong a-signal. Interestingly, gelatin which is very poor in disulfide links exhibits only a relatively weak a-signal when mixed with ascorbic acid. This suggests, that disulfide bonds play a role in the production of the signal. In support of this assumption is the strong a-signal obtained with 4,4'-dithiopyridine (Aldrithiol-4), a synthetic organic compound of low molecular weight which contains a disulfide bond. This compound is a specific reagent for -SH groups with which it forms a disulfide link; in this process half of the molecule is released as the chromogen thiopyridone (6). One of the products of the reaction of 4,4'-dithiopyridine with ascorbic acid is also thiopyridone which was quantitated by optical spectroscopy. This proves that the S-S link of 4,4'-dithiopyridine is broken by ascorbic acid. However, more important, the basic 4,4'-dithiopyridine forms a bond with the anion of ascorbic acid, a process leading to the a-signal.

In summary, this study shows that the $a^{I}$-peak of the a-signal is produced only when the oxygen atom attached to the $C_3$ of the ascorbate anion is electrostatically bound to a partner molecule (via a nitrogen or possibly sulfur atom) and then, in a subsequent process, on the oxygen atom attached to the $C_2$, an unpaired electron appears. The intermolecular bond alters the relaxation time of the radical which results in the loss of the characteristic hyperfine splitting of the semidehydroascorbic acid signal. A prerequisite for the second process, the formation of the semi-

dehydroascorbate radical, is the presence of atmospheric oxygen. The second, the $a^{II}$-signal, with its peak at about g = 2.002, is almost symmetrical (Fig. 3); it is thought to originate from the partner molecule of ascorbic acid. This signal can be observed after lyophilization and subsequent oxygen exposure of many organic and inorganic compounds.

Although ascorbic aid was discovered half a century ago, its biological function has remained unknown (7). Its ability to form an ionic bond, at physiological pH, with body constituents is probably not an incidental property because of the changes in chemical behavior that must ensue. Such binding to a partner molecule should affect the steric molecular configuration and the electric charge of macromolecules or specialized regions on membranes. Furthermore, it should have a major influence on the reactivity of the molecular complex because the functional site of ascorbic acid, the highly active hydroxy group attached to the $C_2$, is not directly affected by the monovalent intermolecular bond at carbon atom 3. The resulting electronic mobility should have local effects on the redox potential and facilitate charge transfer reactions. Although a variety of molecules have been found to bind in vitro to the ascorbate anion, the number of "receptor" molecules in living tissues may be limited and they can be altered in disease. Indicative of the latter is the appearance of the a-signal in the erythrocytes of patients with a certain type of leukemia.

## ACKNOWLEDGEMENT

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