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Biophysics of Structure and Mechanism

Structure of Ascorbic Acid and its Biological Function

I. ESR Determination of the Ascorbyl Radical in Biological Samples and in Model Systems

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Abstract. ESR investigations on lyophilized systems have shown that the signal at $g = 2.005$ can be explained by an interaction between Na^+ or K^+ and the anionic ascorbyl radical. The unpaired electron is probably localized near the C(4) region and is produced by a cleavage of an H atom belonging to a water molecule bound tightly to C(4). Experiments on aqueous samples revealed that ascorbic acid in its radical configuration and in its highest concentration exists only at physiological pH and temperature. An additional splitting is obtained by the ring formation between C(3) and C(6)-OH. The coupling constants of the triplets produced by the CH_2-6 protons differ between ascorbic acid and isoascorbic acid. Thus, the ESR technique can be applied for an easy distinction between these two epimers.

Key words: Ascorbic acid – ESR – Na^+ – K^+

Introduction

The structure of ascorbic acid (ASC, vitamin C) has been determined soon after its crystallization (Szent-Györgyi 1978). Its complex function including its detailed redox mechanism and metabolism is, however, still unknown. The extreme sensitivity to physiological changes suggests its major biochemical role.

Despite the vast literature published up to now, an essential role for the ascorbate system on the molecular level has not been found except for its involvement in scorbut (Szent-Györgyi 1928), collagen production (Udenfried 1966), and its function in the control of mitotic activity (Edgar 1970). Accumulated evidence seems to suggest that it acts as a cofactor in some enzyme reactions (Myllylä et al. 1978; Ingebretsen et al. 1980), can cause lipid peroxidation (Haase and Dunkley 1969), and that its metabolism might be

modified in some types of cancer (Lohmann et al. 1982; Cameron and Pauling 1979).

Recently we could assign the electron spin resonance (ESR) signal located at $g = 2.005$ and present only in lyophilized blood or its constituents obtained from patients with acute lymphatic leukemia (ALL) to the ascorbyl (SDA) radical (Lohmann et al. 1979b). It was postulated that it might be the result of an interaction between vitamin C and probably Cu^{2+}-proteins (Lohmann et al. 1979a). Since the shape of the spectrum differs considerably from the doublet

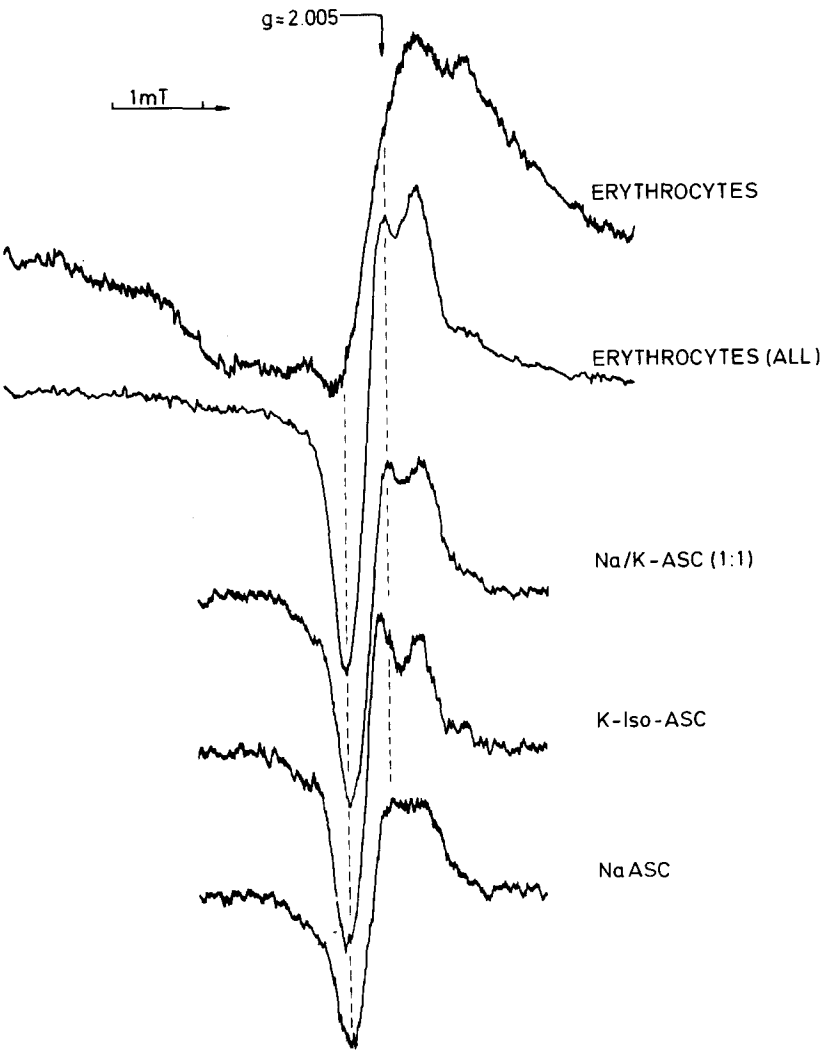

Fig. 1. ESR spectra of lyophilized erythrocytes obtained from healthy donors and from patients with an acute lymphatic leukemia (ALL). For comparison, ESR spectra of lyophilized samples containing 1 : 1 concentrations of K^+ : isoascorbate (K-Iso-ASC), Na^+ : ascorbate (Na-ASC), and Na^+ : K^+ : ASC (Na/K-ASC) are shown

usually observed with the SDA radical and is typical of π-system radicals with axial symmetry, it has been proposed that the ascorbyl radical is immobilized on a solid matrix such as a protein (Naktinis and Cerniauskiene 1974; Swartz 1982). These conclusions do not seem reasonable since the addition of ascorbate oxidase, a copper-containing protein, to e.g., erythrocytes obtained from patients with ALL results in the disappearance of the signal (Lohmann 1981).

The substance producing the SDA radical has, thus, not yet been identified. Recently we showed that the signal is produced only when the oxygen atom attached to C(3) of the ascorbate anion is electrostatically bound to a partner molecule (Bensch et al. 1981). There is some evidence that an alkaline cation might be involved. For this reason, a more detailed molecular investigation was conducted to determine the formation and the structure of the SDA radical. These findings might also give certain information about the biological function of the ascorbic acid system. The results obtained are described in a set of papers. In this report, the relevant ESR measurements are discussed.

Material and Methods

Erythrocytes and plasma of healthy donors and of patients with acute lymphatic leukemia (ALL; "ALL"-erythrocytes) were prepared according to a method described recently (Lohmann et al. 1979b).

Ascorbic acid (ASC; Merck, Darmstadt) and isoascorbic acid (Iso-ASC; Fluka, Buchs) were dissolved in either double distilled water or in NaOH or KOH respectively, to adjust to the pH value desired.

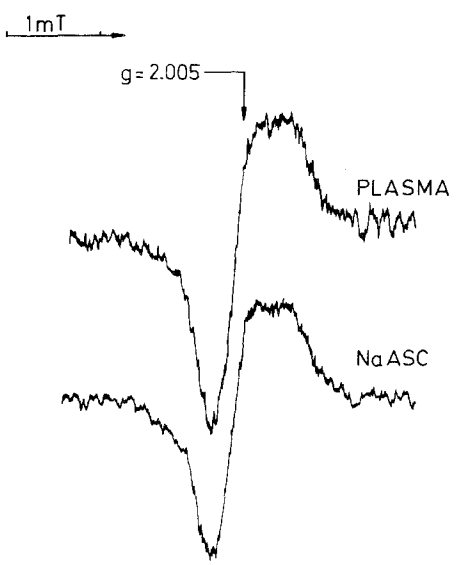

Fig. 2. ESR spectra of lyophilized plasma and Na-ASC (Na^+ : ASC $\triangleq$ 1 : 1)

The electron spin resonance (ESR) spectra of lyophilized samples were obtained with a Varian E-9, 100-kHz modulation X-band spectrometer. The modulation amplitude was ≤ 0.2 mT and the microwave power 5 mW. In the case of aqueous samples, an aqueous sample accessory, a 10 kHZ modulation with a modulation amplitude ≤ 1 μT, and a microwave power of 1 mW were used. All measurements were at room temperature. The relative spin concentration was obtained by taking the peak-to-peak height of the center line of the triplet observed ($m_I = 0$). The temperature was adjusted by a variable Varian temperature unit. For the temperature measurements, the samples were placed into a hematocrit tube right after preparation at room temperature ($t = 0$) and their spectra were measured immediately.

Results and Discussion

In Fig. 1, the ESR spectra of healthy and ALL erythrocytes (the two upper spectra) are shown. Since some evidence indicated the involvement of alkaline ions, the ESR spectra of lyophilized samples containing Na^+/K^+ ascorbate only were also registered. The ALL erythrocyte spectrum can be reproduced by ascorbate with appropriate Na^+/K^+ ratios. As can be seen, the K-isoascorbate

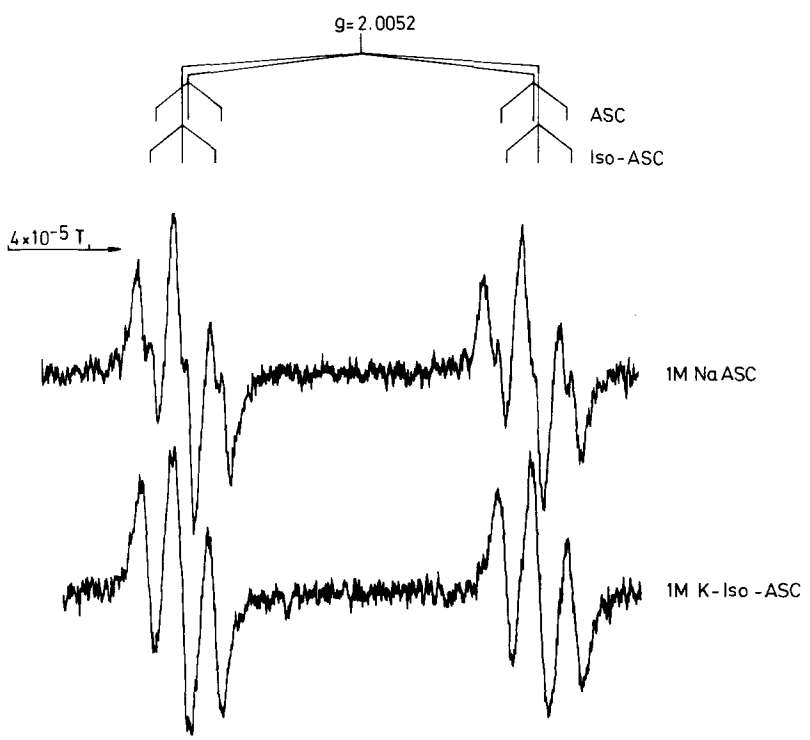

Fig. 3. ESR spectra of Na-ascorbate (Na-ASC) and K-isoascorbate (K-Iso-ASC) at pH 7.2

ESR spectrum differs in shape from the others just discussed. Only Na-ASC resembles exactly the plasma spectrum (Fig. 2).

To elucidate the formation and structure of the radical as well as the involvement of Na^+ and K^+ in this process, aqueous samples of Na^+ and K^+ with ASC or Iso-ASC were investigated at room temperature. It could be shown that the doublet obtained normally with lyophilized ascorbic acid (Lohmann et al. 1979) and which might be due to a cleavage of an H atom belonging to a water molecule bound tightly to C(4), exhibits an hf structure when ASC or Iso-ASC solutions are adjusted to pH 7 by either NaOH or KOH. At this pH value, the proton of C(3)-OH has also been cleaved allowing a ring formation between C(3) and C(6)-OH.

The hf structure seems to consist of two triplets belonging to the two different species ASC and Iso-ASC (Fig. 3). The spectra are superimposed; each containing a small portion of the other. They are produced by the interaction between the unpaired electron and the CH_2-6 protons.

The splitting of the doublet is slightly larger in the case of Iso-ASC. Therefore, the ESR technique can be applied for easy distinction between these two epimers of vitamin C.

Of great interest is the pH dependence of the spin concentration (Fig. 4). An optimum is obtained at around pH 7.2–7.4, which coincides with the

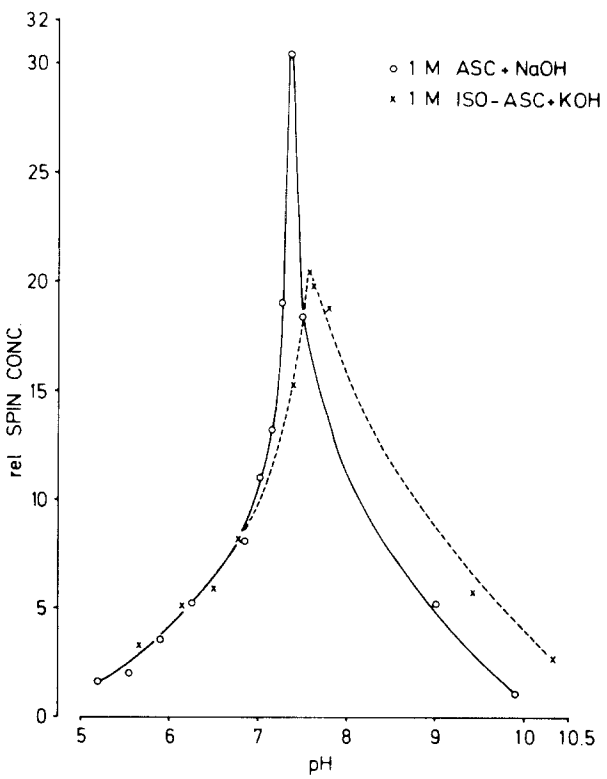

Fig. 4. pH dependence of the Na-ascorbyl (Na-ASC) and K-isoascorbyl (K-Iso-ASC) radicals. SD $\leq 5\%$

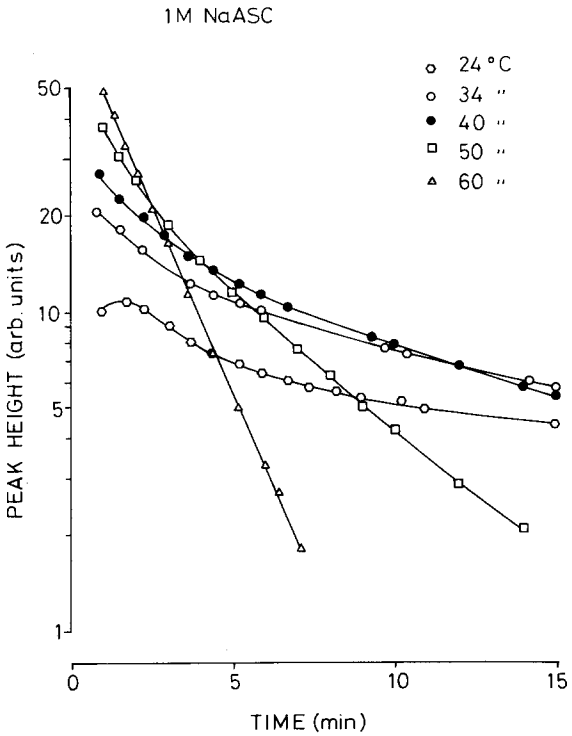

Fig. 5. Influence of temperature on the spin concentration of the Na-ASC radical (pH 7.2)

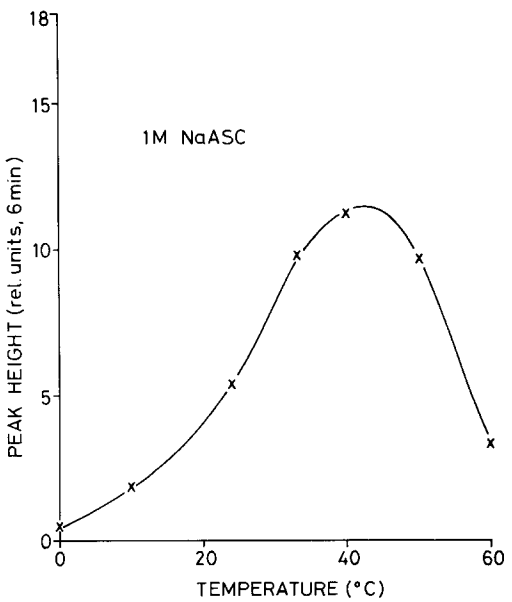

Fig. 6. Influence of temperature on the spin concentration of the Na-ASC radical (pH 7.2) taken 6 min after beginning the experiment. After this time, the samples are adjusted to the temperature. SD $\leq 5\%$

physiological pH value. At pH values < 4–5 (pK_1 = 4.17) and > 11 (pK_2 = 11.57), the signal disappears. That is, ASC is present in its radical configuration only at physiological pH value. Whether the difference in optimal intensity and pH, which exists between Na-ASC and K-Iso-ASC, has some biological relevance, is not known yet.

Some additional information with regard to the structure might be obtained by temperature studies. If a Na-ASC solution (pH 7.2) is exposed to different temperatures, the spin concentration increases first (perhaps due to the removal of bound water), followed by a rapid decrease, at least, at higher temperatures (Fig. 5). The samples were prepared at room temperature and their spectra recorded right after their placement into the cavity. At that time, of course, the temperature of the sample was not adjusted to the temperature given in the figure. Since the samples are rather small (hematocrit tubes containing 50 µl), they are equilibrated, however, after a few minutes. Then at about 6 min, the dependence of the spin concentration on the temperature was determined which reveals some interesting information. The highest spin concentration will be obtained at around 40° C, i.e., at physiological temperatures. At higher temperatures, the system breaks down rapidly, i.e., the radical disappears.

One possible reason for this effect might be the opening of the furanoid ring formed by the side chain. Experimental details and possible biological implications of this effect will be discussed in separate papers.

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