
CHAPTER 2

Electron spin resonance study on the formation of ascorbate free radical from ascorbate: the effect of dehydroascorbic acid and ferricyanide

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Summary

Ascorbate free radical is considered to be a substrate for a plasma membrane redox system in eukaryotic cells. Moreover, it might be involved in stimulation of cell proliferation. Ascorbate free radical can be generated by autoxidation of the ascorbate dianion, by transition metal-dependent oxidation of ascorbate or by an equilibrium reaction of ascorbate with dehydroascorbic acid. In this study, we investigated the formation of ascorbate free radical, at physiological pH, in mixtures of ascorbate and dehydroascorbic acid by electron spin resonance spectroscopy. It was found that at ascorbate concentrations lower than 2.5 mM, ascorbate free radical formation was not dependent on the presence of dehydroascorbic acid. Removal of metal ions by treatment with Chelex 100 showed that the rate of autoxidation under these conditions was less than 20% of the total oxidation. Therefore, it is concluded that at low ascorbate concentrations generation of ascorbate free radical mainly proceeds through metal-ion-dependent reactions. When ascorbate was present at concentrations higher than 2.5 mM, the presence of dehydroascorbic acid increased the ascorbate free radical signal intensity. This indicates that, under these conditions, ascorbate free radical is formed by a disproportionation reaction between ascorbate and dehydroascorbic acid, having a K_{equil} of $6 \cdot 10^{-17}$ M. Finally, it was found that the presence of excess ferricyanide completely abolished ascorbate free radical signals, and that the reaction between ascorbate and ferricyanide yields dehydroascorbic acid. We conclude that, for studies under physiological conditions, ascorbate free radical concentrations cannot be calculated from the disproportionation reaction, but should be determined experimentally.

Introduction

Ascorbate is considered to be one of the main anti-oxidants in biological systems (1-4). It can be oxidized by radicals and oxidants in two successive one-electron steps (5). The first one-electron oxidation gives ascorbate free radical, which can subsequently be oxidized to dehydroascorbic acid. This latter compound is unstable and is irreversibly degraded to potentially toxic compounds (6). In order to prevent accumulation of toxic ascorbate metabolites, cells are equipped with efficient ascorbate regenerating systems. One way to achieve this is by transporting extracellular DHA to the cell interior after which it can be reduced to ascorbate (7). Alternatively, it has been reported that a plasma membrane localized redox system may be involved in ascorbate regeneration (8, 9). This system would reduce extracellular AFR to ascorbate at the expense of intracellular reducing equivalents.

Interestingly, it has been claimed that this redox system is involved in regulation of growth of leukemic cells (10). Moreover, links to intracellular signal transduction pathways have been reported (11-13).

In order to study AFR-driven processes in cells, the generation of known amounts of AFR is a prerequisite. Experimentally, this has been achieved in two ways. One method makes use of the enzyme ascorbate oxidase which catalyzes the oxidation of ascorbate to AFR (14). The other method to generate AFR is by using mixtures of ascorbate and DHA, where AFR is formed in an equilibrium reaction (15, 16). Though the equilibrium results from both symproportionation and disproportionation reactions, it will be referred to as the disproportionation reaction. In mixtures of ascorbate and DHA, AFR concentrations can be calculated from ascorbate and DHA concentrations and the equilibrium constant. A number of studies on the effect of AFR on cells, and especially on plasma membrane redox systems, use the latter method to estimate AFR concentrations (10, 17-19). Recently, however, it was reported that addition of DHA to an ascorbate solution did not increase the AFR concentration, suggesting that AFR was not formed by the equilibrium reaction (20). The aim of this study was to determine whether the disproportionation reaction can indeed be used to estimate AFR concentrations. Since it has been assumed that potent oxidants, like ferricyanide, do not significantly influence the reaction between ascorbate and DHA (19), the influence of ferricyanide on AFR levels was also investigated. AFR formation was determined by electron spin resonance (ESR) spectroscopy and it is concluded that for studies under physiological conditions, the disproportionation reaction does not adequately describe AFR formation.

Materials and methods

Ascorbate and DHA were obtained from Aldrich, Chelex 100 from Bio-Rad and TEMPO (2,2,6,6-tetramethylpiperidine-N-oxyl) came from ICN Biochemicals. All solutions were prepared in ultrapure water that was prepared using a Millipore Milli-Q ultrapurification system. Experiments were performed at room temperature with air-saturated solutions in 0.2 M sodium phosphate buffer, pH 7.4. Stock solutions of ascorbate and DHA were always made up freshly in buffer in plastic tubes and kept on ice. When necessary, the pH was adjusted to 7.4. It was checked, by measuring the absorbance at 265 nm, that the ascorbate concentration of the stock solution remained constant during the course of the experiments. A solution of DHA could be kept for 60 min on ice without significant degradation. For chelex treatment, the 0.2 M sodium phosphate buffer, pH 7.4, was treated overnight with Chelex 100 resin by the batch method (21).

DHA concentrations were determined as described by Vera et al (22). Briefly, DHA samples were incubated with 5 mM dithiothreitol. This converts DHA into ascorbate, which was determined from its absorption at 265 nm, with an extinction coefficient of $14,500 \text{ M}^{-1} \text{ cm}^{-1}$ (21).

ESR spectra were obtained using a JEOL-RE2X spectrometer operating at 9.36 GHz with a 100-kHz modulation frequency, equipped with a TM_{110} cavity. The samples were transferred to the quartz flat cell by means of a rapid sampling device (23). In all experiments the complete spectrum of AFR was recorded within 3 min after preparing DHA solutions. The total area under the ESR absorption curve is proportional to the amount of paramagnetic species in the sample and this can be used to quantify AFR. The concentrations of AFR were determined by double integration of the ESR spectra with TEMPO as a standard. The TEMPO spectra were obtained with the same instrument settings as used for the AFR spectra, except for receiver gain. Saturation effects were accounted for and a microwave power of 40 mW was used (24). ESR spectrometer settings were: 1 Gauss modulation amplitude, 0.1 s time constant, 6 G/min scan rate.

Oxygen consumption was measured with a Clark-type electrode connected to a YSI Model 5300 Biological Monitor.

Ferricyanide concentrations were measured spectrophotometrically at 420 nm.

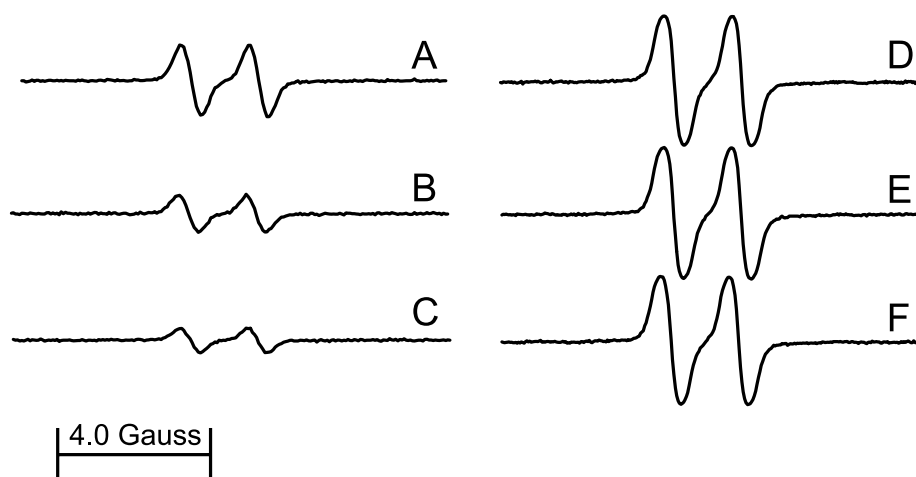
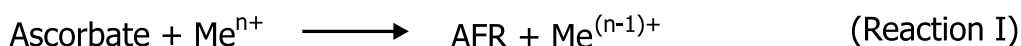


Figure 1: AFR generation from 6 mM ascorbate and 6 mM ascorbate plus 6 mM DHA. A) ESR signal obtained from a solution of 6 mM ascorbate in 0.2 M sodium phosphate, pH 7.4; B) as for A but with 50 μM EDTA; C) as for A but with 50 μM DTPA; D) 6 mM ascorbate plus 6 mM DHA in 0.2 M sodium phosphate, pH 7.4; E) as for D but with 50 μM EDTA; F) as for D but with 50 μM DTPA. Spectrometer settings were as described in Materials and Methods.

Results

Generation of ascorbate free radical

Ascorbate free radical can be detected by ESR spectroscopy as a doublet with hyperfine splitting $a^{\text{H4}} = 1.8 \text{ G}$ (25). This doublet can readily be observed in a solution of 6 mM ascorbate in phosphate buffer, showing that ascorbate free radical is formed in solution (Fig. 1A). Addition of the chelators EDTA or DTPA, to remove trace amounts of Cu^{2+} or Fe^{3+} ions respectively, decreased AFR signal intensity considerably (Figs. 1B-C). This indicates that a large part of the AFR formation from ascorbate was mediated by catalytic amounts of these metal ions, according to the reaction (21, 26, 27):



Addition of 6 mM DHA to a 6 mM ascorbate solution caused an increase in AFR signal intensity as compared to the signal obtained from ascorbate alone (compare Fig. 1D to A). In the presence of DHA the chelators DTPA and EDTA did not have any effect on AFR signal intensity (Fig. 1E, F). These data suggest that, at these concentrations, AFR formation is determined by a disproportionation reaction between DHA and ascorbate (15, 16):



However, when these experiments were performed with 1 mM ascorbate, the results were different (Fig. 2). The AFR doublet was again readily observed in a solution of ascorbate (Fig. 2A) and addition of EDTA and DTPA gave a similar reduction in AFR signal intensity as was found with 6 mM ascorbate (not shown). Chelex 100 treatment of the phosphate buffer, which removes both iron and copper ions, also considerably decreased signal intensity, confirming that AFR formation was mainly mediated by reaction I. The background signal that is observed after Chelex 100 treatment (Fig. 2B) is thought to arise from autoxidation of the ascorbate dianion (21):

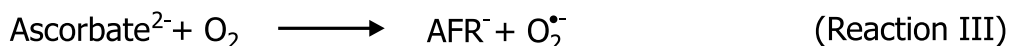


Figure 2C shows that addition of 1 mM DHA to 1 mM ascorbate did not increase signal intensity, compared to the situation where only ascorbate was present (Fig. 2A). This is in clear contrast with the results obtained with 6 mM DHA and ascorbate (Fig. 1A,D). In Chelex-100-treated buffer, however, addition of 1 mM DHA to 1 mM ascorbate did cause an increase in AFR signal intensity as compared

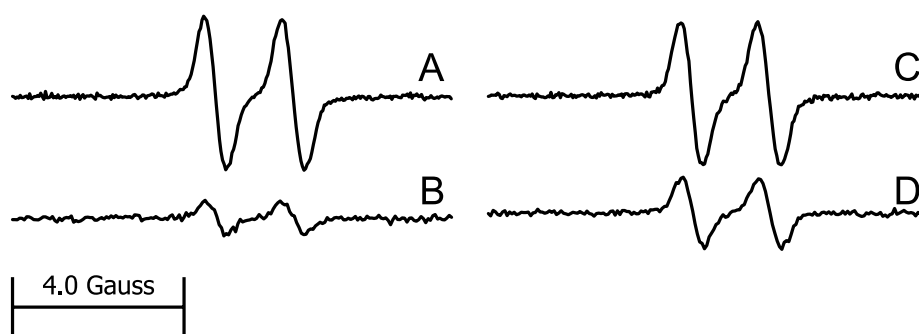


Figure 2: AFR generation from 1 mM ascorbate and 1 mM ascorbate plus 1 mM DHA. A) ESR signal obtained from a solution of 1 mM ascorbate in 0.2 M sodium phosphate, pH 7.4; B) as for A, but in buffer treated with Chelex 100; C) 1 mM ascorbate plus 1 mM DHA in 0.2 M sodium phosphate, pH 7.4; D) as for C, but in buffer treated with Chelex 100. Spectrometer settings were as described in Materials and Methods.

to ascorbate alone (compare Fig. 2D to B). In the presence of EDTA or DTPA the same signal was obtained as shown in Fig. 2D (data not shown).

These results suggest that, when metal ions are present, reaction I determines AFR formation at low ascorbate and DHA concentrations, whereas at higher ascorbate and DHA concentrations reaction II prevails (Figs. 1 and 2). Therefore, we decided to determine AFR generation at various concentrations of ascorbate and DHA, both in 'regular' buffer and in buffer treated with Chelex 100 (Fig. 3). This was compared to the amount of AFR formed in ascorbate solutions in regular buffer. It was found that, in regular buffer, at ascorbate concentrations lower than 2.5 mM the formation of AFR was independent of the presence of DHA, and was mainly driven by metal ions. Only at higher concentrations, DHA could augment AFR formation, showing that reaction II determined the AFR concentration.

In reaction I, reduced metal ions are reoxidized in an O_2 -dependent way (28). Thus, it can be expected that addition of ascorbate to a solution would induce O_2 consumption. Figure 4 shows that this is indeed the case. It should be noted that after 5 min of incubation only about 20 % of the oxygen was consumed. This shows that AFR measurements by ESR spectroscopy were not oxygen-limited, since these measurements never took more than 3 min. In the presence of DHA similar results were obtained (data not shown).

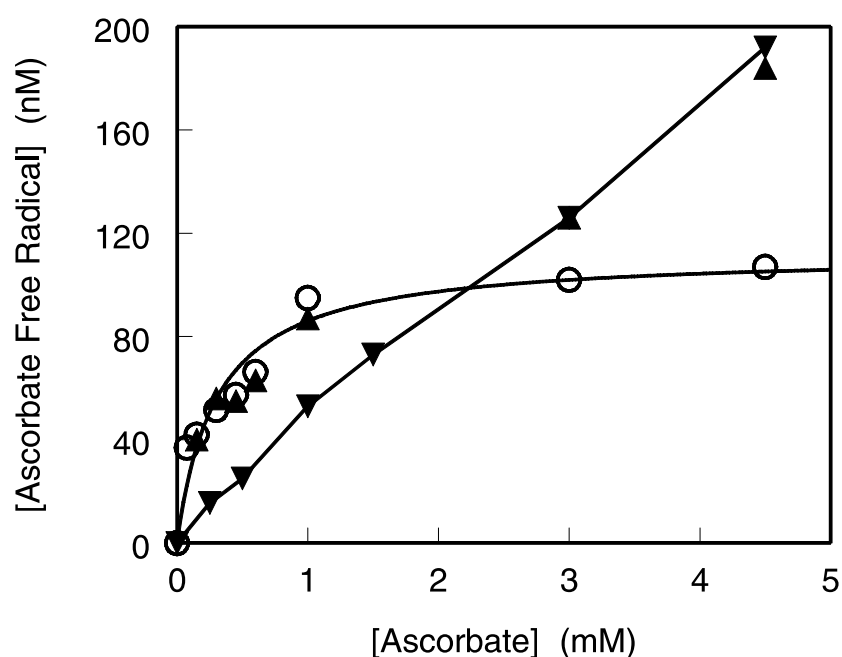


Figure 3: Dependence of the AFR generation on the ascorbate concentration. AFR generation was measured in 0.2 M sodium phosphate, pH 7.4, containing ascorbate (○) or equimolar concentrations of ascorbate and DHA (▲), or in buffer treated with Chelex 100, containing equimolar concentrations of ascorbate and DHA (▼). Spectrometer settings were as described in Materials and Methods.

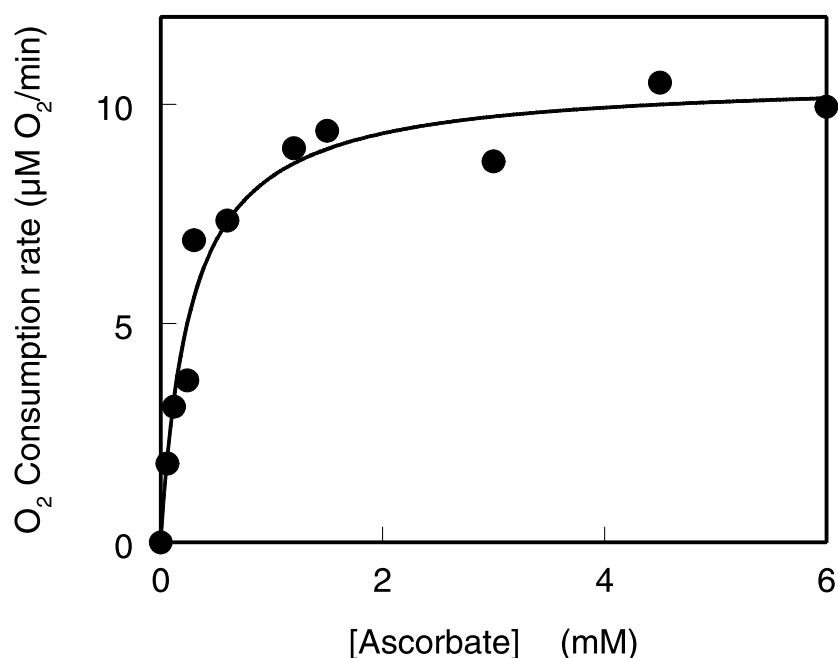


Figure 4: Dependence of the oxygen consumption rate on the ascorbate concentration. The initial rate of oxygen consumption was measured in 0.2 M sodium phosphate pH 7.4, containing various amounts of ascorbate.

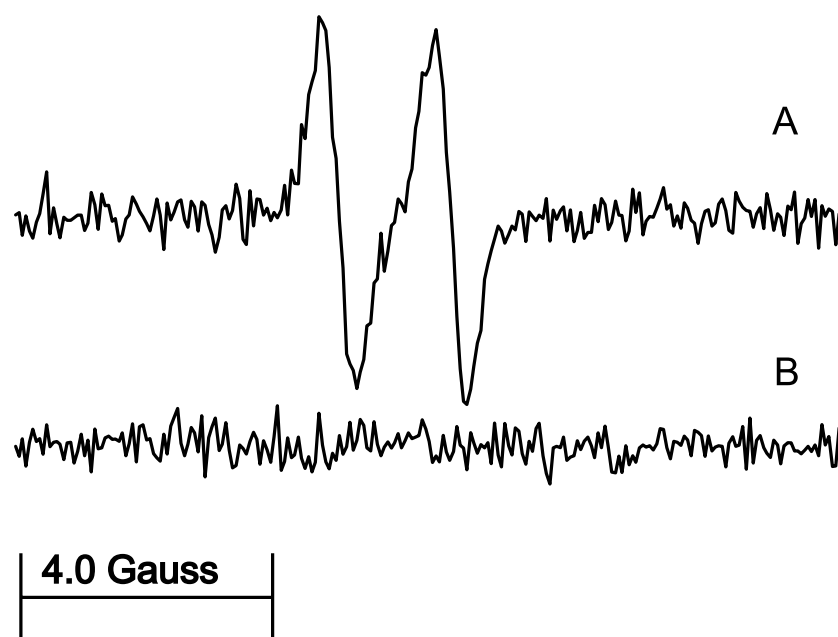


Figure 5: The influence of ferricyanide on the formation of AFR. A) 25 μM ascorbate in 20 mM Tris-HCl, 150 mM NaCl, pH 7.4; B) as for A but with 1 mM ferricyanide. ESR settings were as in figure 1.

Equilibrium constant

The disproportionation reaction is an equilibrium reaction for which the equilibrium constant can be described as (29):

$$K_{\text{equil}} = \frac{[\text{AFR}^-]^2 [\text{H}^+] (K_a + [\text{H}^+])}{[\text{Ascorbate}]_{\text{total}} [\text{DHA}] K_a} \quad (\text{Equation 1})$$

with $[\text{ascorbate}]_{\text{total}} = [\text{ascorbate}^-] + [\text{H-ascorbate}]$

and $K_a = [\text{ascorbate}^-][\text{H}^+]/[\text{H-ascorbate}]$.

At a particular pH, K_{equil} can be determined by measurement of the AFR concentration by ESR spectroscopy in a solution of known concentrations of ascorbate and DHA. DHA is unstable at pH 7.4 and at room temperature, and decays with a half-life of about 12 min (data not shown). Therefore, AFR measurements were performed within 3 min after mixing ascorbate and DHA. After correction for decay of DHA, it was found that, at pH 7.4, in a mixture with an initial concentration of 30 mM ascorbate and 30 mM DHA, K_{equil} amounted to $6 \cdot 10^{-17}$ M.

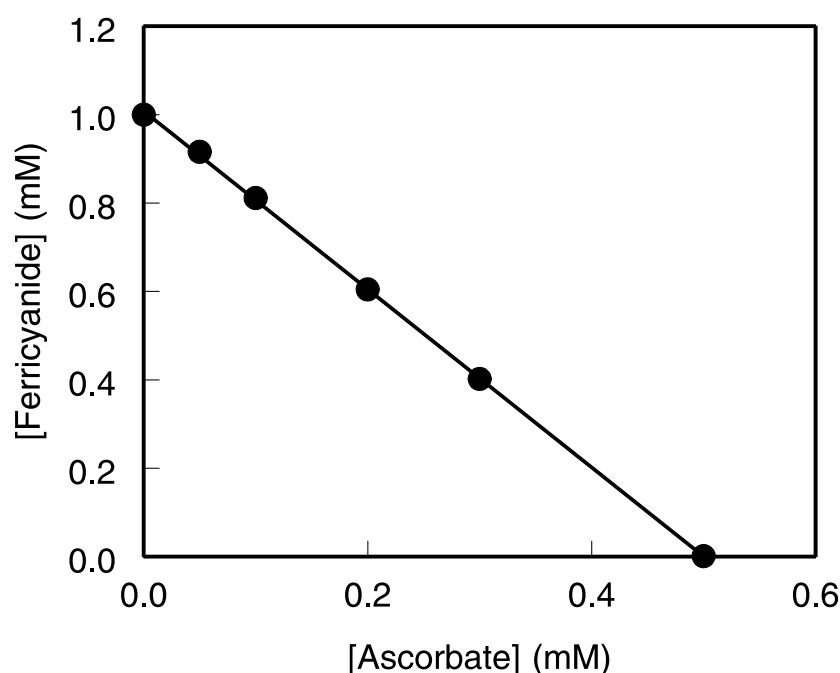


Figure 6: The influence of ascorbate on the ferricyanide concentration. Ferricyanide (1 mM) was mixed with various amounts of ascorbate in 20 mM Tris-HCl, 150 mM NaCl, pH 7.4. Ferricyanide concentration was determined from its absorbance at 420 nm.

Effect of ferricyanide

In a number of studies on plasma membrane redox systems, ascorbate or ascorbate plus DHA were incubated simultaneously with ferricyanide (19, 30). Though ferricyanide is a potent oxidant, it was assumed that it did not significantly affect the AFR concentration (19). To determine the AFR concentration under these experimental settings, ESR studies were performed. Figure 5 shows that incubation of 25 mM ascorbate with 1 mM ferricyanide completely abolished the AFR signal, indicating that ascorbate was completely converted to dehydroascorbic acid. This was corroborated by the experiments presented in figure 6, which show that upon addition of ascorbate to 1 mM ferricyanide, the concentration of ferricyanide decreased with a stoichiometry of two mol ferricyanide per mol of ascorbate added.

Discussion

Ascorbate free radical can be formed from ascorbate in solution by various mechanisms (reactions I-III). Autoxidation of the ascorbate dianion (reaction III) results in the formation of AFR, and is dependent on pH and ascorbate concentration (24). The experiments performed in this study were all done at physiological pH 7.4, where autoxidation is low (Fig. 2B). A second mechanism (reaction I) involves the

transition-metal-ion-catalyzed oxidation of ascorbate (21) Under normal laboratory conditions buffers are always contaminated by trace amounts of iron and copper (21). A third mechanism (reaction II) involves an equilibrium reaction between ascorbate and DHA yielding two AFR molecules (16). Finally, AFR can also be generated enzymatically with ascorbate oxidase.

Our results show that at ascorbate concentrations lower than 2.5 mM the metal-ion-dependent reaction prevails (Fig. 3). First, removal of metal ions by Chelex 100 treatment decreased the AFR signal by at least 80%, showing that the rate of autoxidation is less than 20% of the total rate under these conditions (Fig. 2). Second, addition of DHA did not affect AFR signal intensities in 'regular' buffer (Figs. 2 and 3). Only when metal ions were removed, by addition of EDTA or DTPA (data not shown) or by treatment with Chelex 100 (Fig. 2D), the addition of DHA increased AFR signal intensity. This shows that, when the concentration of metal ions was low, the disproportionation reaction determined the AFR concentration. In regular buffer the disproportionation reaction will also take place, but at ascorbate concentrations lower than 2.5 mM the AFR concentration is determined by metal-ion-dependent oxidation of ascorbate. Thus, it is concluded that, in ascorbate-DHA mixtures, the actual AFR level strongly depends on the reaction conditions, and especially on the presence of metal ions and on the concentrations of ascorbate and DHA.

It has been reported that the metal ions involved in this reaction are Fe^{3+} and Cu^{2+} ions (21). During ascorbate oxidation these ions are reduced to Fe^{2+} and Cu^{1+} and must be re-oxidized in order to remain catalytically active. This oxidation involves molecular oxygen and probably generates hydroxyl radicals (28). The results presented in figure 4 reveal that ascorbate stimulates O_2 consumption under conditions where AFR is formed. Moreover, it shows that O_2 consumption does not have a strong dependence on the ascorbate concentration. This is consistent with the view that the catalyst of the reaction is present in trace concentrations, and therefore that regeneration of the metal ions is rate limiting. It should be noted that oxygen consumption is not affected by the addition of DHA (data not shown). At concentrations above 2.5 mM, the final AFR concentration is determined by the equilibrium reaction, but autoxidation and metal ion dependent reactions still take place at the same level as without DHA. Thus, oxygen is consumed.

At ascorbate concentrations higher than 2.5 mM the disproportionation reaction determines the AFR concentration, as equimolar mixtures of DHA plus ascorbate gave higher AFR signal intensities than ascorbate alone (Figs. 1 and 3). The fact that under these conditions neither EDTA, DTPA nor Chelex 100 treatment influenced

the AFR concentration shows that the disproportionation reaction is not mediated by metal ions and strengthens the view that this mechanism is different from the metal-ion-dependent reaction.

Equilibrium constants for the disproportionation reaction have been determined by Von Foerster in the pH range 4-6.4, according to equation 2 (16):

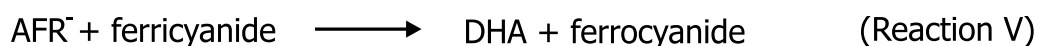
$$K_{\text{equil}}^{\text{Foerster}} = \frac{[\text{AFR}]^2}{[\text{Ascorbate}][\text{DHA}]} \quad (\text{Equation 2})$$

Extrapolation of the data to pH 7.4 yielded a constant of 10^{-8} (15). A more general way of describing the equilibrium constant is the one presented in equation 1, which relates to equation 2 as:

$$K_{\text{equil}} = K_{\text{equil}}^{\text{Foerster}} \frac{[\text{H}^+](K_a + [\text{H}^+])}{K_a} \quad (\text{Equation 3})$$

Thus, the equilibrium constant of 10^{-8} , determined by Lumper et al, would, in equation 3, give a K_{equil} of $4 \cdot 10^{-16}$ M at pH 7.4, which is much higher than the value we determined in this study, i.e., $6 \cdot 10^{-17}$ M. The reason for this difference may be due to the settings of the ESR spectrometer. We utilized settings as determined by Buettner and Jurkiewicz, which give optimal signal-to-noise ratios (24). Secondly, we used a different calibration procedure, using the water soluble standard TEMPO, whereas Foerster et al used the solid standard diphenylpicryl-hydrazyl (16). Since AFR signals were generated in solution, a water-soluble standard should be used, rather than a solid one. Finally, our measurements were performed at pH 7.4, whereas the previously used constant of 10^{-8} was obtained through extrapolation from data at more acidic pH values.

Ferricyanide can react with ascorbate and AFR, according to the reactions:



It has been suggested that reaction V proceeds more slowly than reaction IV, and that in mixtures of ascorbate and ferricyanide AFR would still be present (19). On the other hand, it has been claimed that AFR is more reactive towards ferricyanide than ascorbate (5), suggesting that once AFR is formed it would rapidly react to DHA. Our results confirm the latter view, as no AFR could be detected in mixtures of ascorbate/AFR plus ferricyanide (Fig. 5). Moreover, ascorbate caused a decrease of the ferricyanide concentration within seconds, with a stoichiometry of two mol

ferricyanide reduced per mol ascorbate added (Fig. 6). This shows that in mixtures where ferricyanide is present in excess, as used for some plasma membrane reductase measurements, no AFR is present.

AFR is referred to as a substrate for a plasma membrane redox system, and could play a role in cell proliferation. In several studies on these topics, AFR concentrations have been calculated using the disproportionation reaction (10, 17-19). Thus, it has been assumed that AFR concentrations would be higher in solutions containing ascorbate plus dehydroascorbic acid than in solutions of only ascorbate. The results presented in this chapter show that one has to be cautious with these conclusions. Especially at low ascorbate concentrations, the AFR concentration is not determined by the disproportionation reaction, but rather by the metal-ion-catalyzed oxidation of ascorbate. With ascorbate concentrations in the micromolar range huge differences occur between calculated AFR concentrations, based on reaction II, and the experimentally measured concentrations. This conclusion is not only valid for sodium-phosphate buffers but also for more physiological media, like RPMI supplemented with fetal bovine serum. In the latter medium we observed that, at low ascorbate concentrations, the AFR concentration was much higher than calculated on basis of the disproportionation reaction (data not shown), which indicates that the presence of proteins does not prevent metal-driven AFR formation. This once again shows that AFR concentrations cannot simply be calculated, but should be determined using ESR spectroscopy, as it is strongly dependent on medium conditions.

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