Analysis of Free Radicals in Biological Systems
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Ascorbate radical: A valuable marker of oxidative stress

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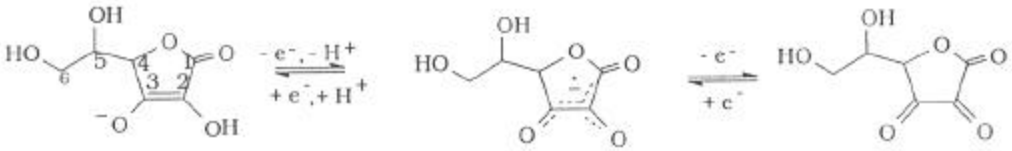
Summary. The ascorbate anion is an endogenous water-soluble antioxidant that is present in biological systems. The one-electron oxidation of ascorbate produces the ascorbate free radical that is easily detectable by electron paramagnetic resonance (EPR), even in room temperature aqueous solution. The ascorbate radical has a relatively long lifetime compared to other free radicals, such as hydroxyl, peroxyl, and carbon-centered lipid radicals. This longer lifetime in conjunction with its relatively narrow EPR linewidth makes it easily detectable by EPR. In this essay we describe the EPR detection of the ascorbate radical and its use as a marker of oxidative stress.

Ascorbate, the terminal small-molecule antioxidant

Introduction

Ascorbate (Asc H^-) is ubiquitous, yet there is still much to be learned about its chemistry, biochemistry, and biology. Ascorbate is an excellent reducing agent [1–5]. It readily undergoes two consecutive, yet reversible, one-electron oxidation processes to form the ascorbate radical ($Asc^{\bullet -}$) as an intermediate. Loss of a second electron yields dehydroascorbic (DHA) [1].



$AscH^-$ $Asc^{\bullet -}$ DHA

Because $Asc^{\bullet -}$ has its unpaired electron in a highly delocalized π-system, it is a relatively unreactive free radical. These properties make ascorbate a superior biological, donor antioxidant [3–17].

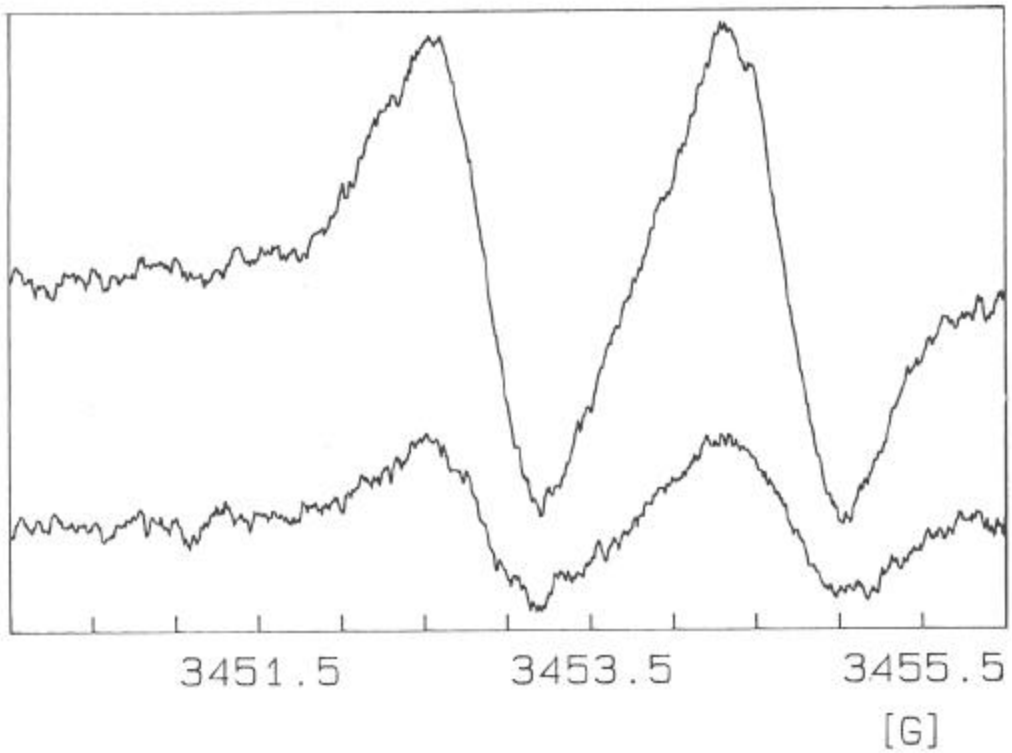


Fig. 1. The ascorbate radical doublet EPR signal in bovine lens increases upon exposure to UV light. An approximate 1 cm^2 section of lens was placed into the well of an EPR tissue cell, then positioned in an EPR TM_{110} cavity, and subsequently exposed to UV light. Lower: ambient $Asc^{\cdot -}$; Upper: $Asc^{\cdot -}$ during exposure to UV light. See the section on lens below for details.

The ascorbate free radical is a strong acid having a pK_a of -0.86 [18]. Thus, it will exist as a monoanion, $Asc^{\cdot -}$, over the entire biological pH range.[1]

When biological fluids or tissues are examined by electron paramagnetic resonance spectroscopy (EPR), $Asc^{\cdot -}$ will most likely be observed. See Figure 1 below. This is consistent with ascorbate's role as the terminal small-molecule antioxidant [3, 4].

Ascorbate thermodynamics and kinetics

As can be seen in Table 1, ascorbate is thermodynamically at the bottom of the pecking order of oxidizing free radicals. That is, all oxidizing free radials with greater reduction potentials, which includes, $HO^{\cdot}$, $RO^{\cdot}$, $LOO^{\cdot}$, $GS^{\cdot}$, the urate radical, and even the tocopheroxyl

[1]A note on nomenclature: $Asc^{\cdot -}$ is usually referred to in brief as the ascorbate free radical. The ending "ate" being used because it is a charged species. The short name ascorbyl radical would be used for $AscH^{\cdot}$, the neutral protonated form of $Asc^{\cdot -}$. The ending "yl" being used for this neutral species.

Table 1. One-electron reduction potentials at pH 7.0 for selected radical couples

Redox couple	$E^{0'}$/mV
$HO^{\bullet}, H^{+}/H_2O$	+2310
$RO^{\bullet}, H^{+}/ROH$ (aliphatic alkoxyl radical)	+1600
$ROO^{\bullet}, H^{+}/ROOH$ (alkyl peroxyl radical)	+1000
$GS^{\bullet}/GS^{-}$ (glutathione)	+920
$PUFA^{\bullet}, H^{+}/PUFA\text{-}H$ (bis-allylic-H)	+600
$HU^{\bullet -}, H^{+}/UH_2^{-}$ (Urate)	+590
$TO^{\bullet}, H^{+}/TOH$ (Tocopherol)	+480
$H_2O_2, H^{+}/H_2O, HO^{\bullet}$	+320
$Ascorbate^{\bullet -}, H^{+}$/Ascorbate monoanion	+282
Fe(III)EDTA/Fe(II)EDTA	+120
$O_2/O_2^{\bullet -}$	−330
Paraquat/$Paraquat^{\bullet +}$	−448
Fe(III)DFO/FE(II)DFO (Desferal)	−450
RSSR/$RSSR^{\bullet -}$ (GSH)	−1500
H_2O/e_{aq}^{-}	−2870

This table is adapted from references [3, 12, 19].

Table 2. Rate constants for the reaction of the equilibrium mixture of $AscH_2/AscH^{-}/Asc^{2-}$ at pH 7.4 unless noted otherwise

Radical	$k_{obs}/M^{-1}s^{-1}$	(pH 7.4)	Ref.[a]
$HO^{\bullet}$	1.1×10^{10}		[20]
$RO^{\bullet}$ (*tert*-butyl alkoxyl radical)	1.6×10^{9}		[21]
$ROO^{\bullet}$ (alkyl peroxyl radial, e.g., $CH_3OO^{\bullet}$)	$1\text{–}2 \times 10^{6}$		[22]
$Cl_3COO^{\bullet}$	1.8×10^{8}		[23]
$GS^{\bullet}$ (glutathiyl radical)	6×10^{8}	(5.6)	[24, 25]
$PUFA^{\bullet}$	—[b]		
$UH^{\bullet -}$ (Urate radical)	1×10^{6}		[26]
$TO^{\bullet}$ (Tocopheroxyl radical)	2×10^{5}[c]		[3]
$Asc^{\bullet -}$ (dismutation)	2×10^{5}[d]		[27]
$CPZ^{\bullet +}$ (Chlorpromazine radical cation)	1.4×10^{9}	(5.9)	[28]
Fe(III)EDTA/Fe(II)EDTA	$\approx 10^{2}$[e]		
$O_2^{\bullet -}/HO_2^{\bullet}$	1×10^{5}[d]		[29, 30]
	2.7×10^{5}		[31]
Fe(III)DFO/Fe(II)DFO	Very slow		[32, 33]

[a]A complete summary of free radical solution kinetics can be found in [34].
[b]We were unable to find data that addresses this reaction directly.
[c]Estimated k_{obs} for $TO^{\bullet}$ when in a biological membrane.
[d]k is pH dependent, thus this is k_{obs} at pH 7.4.
[e]Estimated from data in [35, 36, 63].

radical ($TO^{\bullet}$), can be repaired by ascorbate. Therefore, we have:

$$AscH^{-} + X^{\bullet} \longrightarrow Asc^{\bullet -} + XH,$$

where $X^{\bullet}$ can be any of these oxidizing free radicals. From Table 2, we see that the kinetics of these electron (hydrogen atom) transfer reactions are rapid. Thus, both thermodynamically and kinetically, ascorbate can be considered to be an excellent antioxidant.

Although ascorbate itself forms a radical in this reaction, a potentially very dangerous oxidizing radical ($X^{\cdot}$) is replaced by the domesticated $Asc^{\cdot -}$. $Asc^{\cdot -}$ does not react by an addition reaction with O_2 to form dangerous peroxyl radicals. Ascorbate (probably Asc^{2-}, *vida infra*) and/or $Asc^{\cdot -}$ appear to produce very low levels of superoxide [37, 38]. But by removing $O_2^{\cdot -}$, superoxide dismutase provides protection from this possibility [39, 40]. Thus, the biological organism is protected from further free radical-mediated oxidations. In addition. $Asc^{\cdot -}$ as well as dehydroascorbic can be reduced back to ascorbate by enzyme systems. Thus, it is recycled. Ascorbate's ubiquitous presence in biological systems in conjunction with its role as an antioxidant suggests that the ascorbate free radical would also be present.

Equilibrium

The ascorbate free radical will be present in solutions due to both the autoxidation and the metal catalyzed oxidation of ascorbate. Forester et al. observed that $Asc^{\cdot -}$ can also arise from comproportionation of $AscH^-$ and DHA [41],

$$AscH^- + DHA \rightleftharpoons 2\ Asc^{\cdot -} + H^+$$

$$K = \frac{[Asc^{\cdot -}]^2}{[AscH^-]\,[DHA]}$$

Using EPR, they determined the equilibrium constant for this process and noted that it was pH dependent. The equilibrium constant K was found to vary from 5.6×10^{-12} at pH 4.0 to 5.1×10^{-9} at pH 6.4. Later, after the acid-base properties of ascorbic acid and ascorbate free radical were understood, it was then possible to develop an expression for K at any pH value [27].

$$K = \frac{[Asc^{\cdot -}]^2\,[H^+] + \{1 + [H^+]/10^{-pK_1}\}}{[DHA]\,[AscH_2]_{total}} = 2.0 \times 10^{-15}\ M^{-2}$$

where pK_1 is the first ionization constant of ascorbic acid and $[AscH_2]_{total}$ is the analytical concentration of $AscH_2$, i.e., $[AscH_2]_{total} = [AscH_2] + [AscH^-] + [Asc^{2-}]$. [27, 41].

Using $Asc^{\cdot -}$ as a marker of oxidative stress

Overview

The ascorbate free radical is naturally detectable by EPR at low steady-state levels in biological samples, such as leaves from crops [42], plasma [14, 43, 44], synovial fluid [45], skin [46, 47], and lens of the eye,

vida infra. As oxidative stress increases in a system, the steady-state $Asc^{\cdot -}$ concentration increases [4]. These findings are consistent with ascorbate's role as the terminal small-molecule antioxidant (see Tab. 1). It is proposed that ascorbate, i.e., the ascorbate free radical, which is naturally present in biological systems, can be used as a noninvasive indicator of oxidative stress [4, 48].

The ascorbate radical as a marker of oxidative flux has been shown to be useful in the study of free radical oxidations in many biological systems including mouse skin [46, 47, 49], hepatocytes [50], and ischemia reperfusion of hearts [51–53][2]. Human sera and rat plasma intoxicated with paraquat and diquat, known superoxide generators, have increased ascorbate radical levels [56]. In animal experiments, sepsis has also been shown to increase $Asc^{\cdot -}$, indicating the involvement of oxidative stress with this health problem [57]. Sasaki et al. have investigated in human serum the use of $Asc^{\cdot -}$ signal intensity in combination with measurements of $AscH^-$ and DHA as an indicator of oxidative stress in human health problems that range from aging to xenobiotic metabolism [58–62]. Taken together, these studies demonstrate that the asorbate radical level in biological systems may be useful for monitoring free radical oxidations *in vivo*, particularly when free radical production is low and other methods are insensitive.

Absorption spectra

Pure ascorbic acid solutions are colorless as neither the diacid nor the monoanion have significant absorbances in the visible region of the spectrum. However, each has an absorbance in the ultraviolet region.

(1) Ascorbic acid: The diacid has an approximately symmetrical Gaussian absorption spectrum with $\varepsilon_{244} = 10\,800\ M^{-1} \cdot cm^{-1}$ in aqueous solution [1].
(2) Ascorbate monoanion: Compared to the diacid, the peak of the absorption curve for the monoanion is red-shifted to 265 nm. A wide range of molar extinction coefficients have been reported, ranging from $7500–20\,400\ M^{-1} \cdot cm^{-1}$ [1]. We find that $\varepsilon_{265} = 14\,500\ M^{-1} \cdot cm^{-1}$ best reflects our experimental observations when doing experiments in near-neutral buffered aqueous solutions [63].
(3) Ascorbate radical: The ascorbate free radical has an approximately symmetrical Gaussian shaped absorption curve with $\varepsilon_{360} =$

[2]In a quite different approach Pietri et al. [54, 55] have used $Asc^{\cdot -}$ as a probe for plasma ascorbate concentrations. In their approach, a 1:1 mixture of plasma and dimethylsulfoxide is examined for $Asc^{\cdot -}$ by EPR. They claim that the $Asc^{\cdot -}$ is an index of the transient changes in plasma ascorbate status during ischemia/reperfusion. Whereas, in our studies the $Asc^{\cdot -}$ levels reflect the ongoing free radical flux in the system being examined [4, 47, 53].

3300 $M^{-1} \cdot cm^{-1}$ and a half-width at half-maximum of about 50 nm [27]. With this small extinction coefficient, $Asc^{\cdot -}$ will not be observable by standard UV-VIS spectroscopy in steady-state experiments.

(4) Dehydroascorbic: Dehydroascorbic (acid) has a weak absorption at 300 nm, $\varepsilon_{300} = 720\ M^{-1} \cdot cm^{-1}$ [1].

EPR Detection of the ascorbate free radical

The ascorbate free radical is usually detected by EPR as a doublet signal with $a^{H} = 1.8$ G, $\Delta H_{pp} \approx 0.6$ G and $g = 2.0052$, Figure 1. However, each line of the ascorbate doublet is actually a triplet of doublets, $a^{14} = 1.76$ G, $a^{16}(2) = 0.19$ G, and $a^{15} = 0.07$ G [64].

In most biological experiments where the $Asc^{\cdot -}$ EPR signal will be weak, a compromise is made in the choice of modulation amplitude. The usual choice is to sacrifice resolution of the hyperfine structure for improved sensitivity. We find that a modulation amplitude of ≈ 0.65 G maximizes the ascorbate free radical double peak-to-peak signal amplitude [65].

The EPR power saturation curve of $Asc^{\cdot -}$ in room temperature aqueous solutions shows that saturation effects begin at ≈ 16 mW and maximum signal height is achieved at 40 mW nominal power when using an aqueous flat cell and a TM cavity, see Figure 2. Thus, if quantitation of the $Asc^{\cdot -}$ levels is desired, appropriate corrections for saturation effects must be included in the calculations.

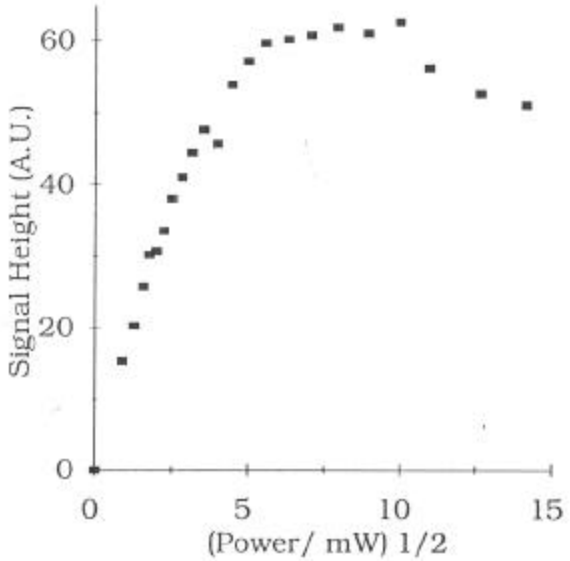


Fig. 2. EPR Power Saturation Curve for $Asc^{\cdot -}$. Signal heights are arbitrary units. $Asc^{\cdot -}$ was observed in a demetalled 50 mM pH 7.8 phosphate buffer containing 10 mM ascorbate. Bruker ESP-300 instrument settings were: 0.65 modulation amplitude; 10 G/167 s scan rate; 0.167 s time constant [65].

Applications

$Asc^{\cdot -}$ in solution

Stock ascorbate solutions

In our work with ascorbate in solution we have found that the quality of the stock solution determines the quality and reproductivity of the results. We prepare ascorbate stock solutions using only the diacid. It is prepared as a 0.100 M stock solution (10 mL) using high purity water. This solution is colorless, having a pH of ≈ 2. It is stored in a volumetric flask with a tight-fitting plastic stopper, thus oxygen is kept from the solution during long-term storage. As the solubility of oxygen in air-saturated water is ≈ 0.25 mM, the solution will become anaerobic with loss of $< 1\%$ of the original ascorbate. If the flask is indeed clean, we have found that the solution can be kept for several weeks without significant loss of ascorbate due to the low pH and lack of oxygen. The appearance of a yellow color is an indication of ascorbate deterioration. We avoid the use of sodium ascorbate as it invariably contains substantial quantities of oxidation products as evidenced by the yellow color of the solution. [63].

Autoxidation and metal catalyzed oxidations

Before beginning this discussion it must be understood that we use the term *autoxidation* to mean oxidation in the absence of metal catalysts [66]. The term oxidation is used more broadly and includes all oxidations, with or without catalysts.

Ascorbate is readily oxidized. However, the rate of this oxidation is dependent upon pH and the presence of catalytic metals [32, 33, 35, 36, 63, 67–70]. The diacid is very slow to oxidize. Consequently, at low pH, i.e., less than 2 or 3, ascorbic acid solutions are quite stable, assuming catalytic transition metal ions are not introduced into the solutions. However, as the pH is raised above pK_1 (4.2), $AscH^-$ becomes dominant and the stability of the ascorbate solution decreases. This loss of stability is usually the result of the presence of adventitious catalytic metals (on the order of 1 μM) in the buffers and salts that are typically employed in studies at near neutral pH [63]. For example, we have found that in room temperature aerated, aqueous solutions at pH 7.0 (50 mM phosphate buffer) 10–30% of 125 μM ascorbate is lost in just 15 min. This large variation is the result of different sources and grades of phosphate used in the buffer preparation. However, if care is taken to remove these trace levels of transition metals, this rate of loss can be lowered to as little as 0.05%/15 min [63], thus demonstrating the extreme importance of metals in controlling ascorbate stability. At pH 7.0 we have set an upper limit for the observed rate constant for the

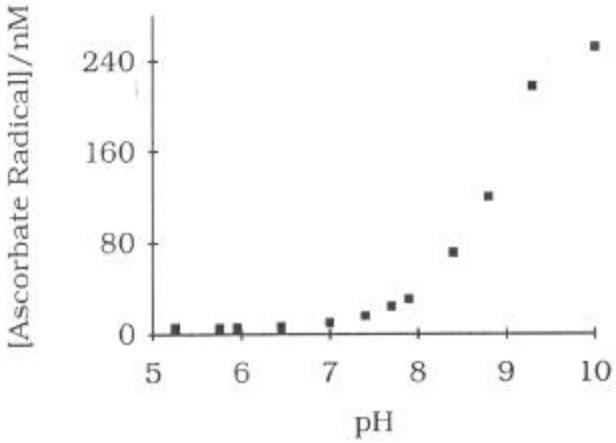


Fig. 3. Background [Asc$^{\cdot -}$] *vs.* pH: Each solution was made with 50 mM demetalled phosphate buffer that contained 50 μM desferoxamine mesylate, for at least 12 hours. To these solutions 500 μM ascorbate was added and the EPR spectra were collected. The points represent the Asc$^{\cdot -}$ concentration observed in the second of three EPR scans, where the values had a standard deviation of less than 1 nonomolar (adapted from [4]). These data demonstrate the importance of pH control. At pH values greater than ≈ 8 $[Asc^{\cdot -}]_{ss}$ is not a good indicator of oxidative stress, but at near neutral pH it is excellent.

oxidation of ascorbate to be 6×10^{-7} s^{-1} under our experimental conditions [63]. However, even in carefully demetalled solutions as the pH is varied the rate of oxidation increases, Figure 3 [4].

We attribute this increase in rate at higher pH values to the increasing concentrations of the ascorbate dianion. Williams and Yandell have made an estimate based on the Marcus theory of electron transfer that the ascorbate dianion would undergo true autoxidation at a significant rate [38].

$$k \approx 10^2 \text{ M}^{-1} \text{ s}^{-1}$$

$$Asc^{2-} + O_2 \longrightarrow Asc^{\cdot -} + O_2^{\cdot -}$$

Our experimental results are consistent with these estimates [4, 33, 63]. Marcus theory would predict that the rate of the true autoxidation of AscH$^-$ would be much slower.

Thus, at pH $\approx$ 7.4 the rate of autoxidation of an ascorbate solution is determined predominantly by Asc^{2-}.

Typical buffers employed in biochemial and biological research have on the order of 1 μM iron and <1 μM copper [63]. But because copper is $\approx$80 times more efficient as a catalyst for ascorbate oxidation than iron, it is the adventitious copper that is the biggest culprit in catalyzing ascorbate oxidation [63].

We have developed two assays that take advantage of this chemistry:

Iron analysis at the nM level

Fe-EDTA is an excellent catalyst of ascorbate oxidation, while Cu-EDTA is a very poor catalyst. We have found that with careful

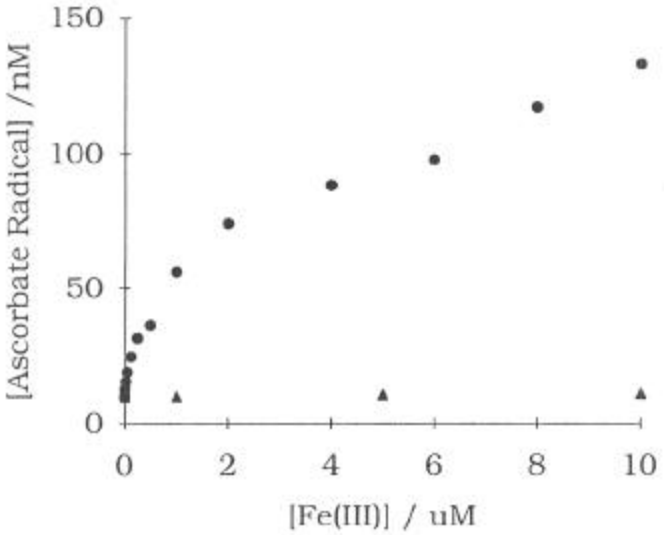


Fig. 4. These data were gathered using EPR spectroscopy to quantitate the steady state level of $Asc^{\bullet -}$. The curves were obtained in 50 mM demetalled phosphate buffer, pH 7.40 with 250 μM EDTA (●) or 50 μM Desferal (▲) with 125 μM ascorbate present (adapted from [33]).

attention to detail to ensure that all glassware, pipettes and pipette tips are scrupulously clean, we can estimate iron levels in phosphate buffer to a lower limit of $\approx$100 nM using UV-Vis spectroscopy [63]. However, using EPR spectroscopy this limit can be as low as $\approx$5 nM [33], Figures 4 and 5. For the EPR method of analysis we add EDTA to the solution to be assayed. This converts the iron to a "standard" catalytic form. We

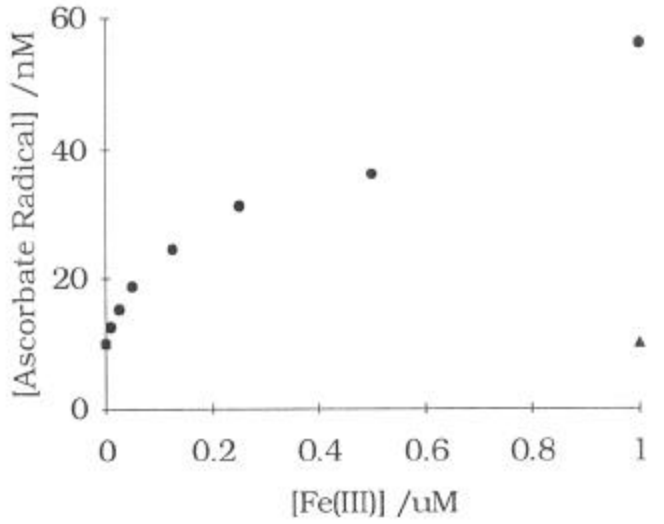


Fig. 5. This figure is an expansion of the 0–1 μM [Fe(III)] region of Figure 4. The experimental conditions are the same as in Figure 4. The curves were obtained in 50 mM demetalled phosphate buffer, pH 7.40 with 250 μM EDTA (●) or 50 μM Desferal (▲) with 125 μM ascorbate present (adapted from [33]).

then introduce ascorbate and determine by EPR the steady-state concentration (i.e., signal height) of $Asc^{\bullet -}$. From a standard curve we can then estimate the iron concentration from $\approx 5\,nM \rightarrow \approx 10\,\mu M$. To achieve estimates at the lowest end of this range, extreme care must be taken with each step and the EDTA must be pure; recrystallized at least three times using methods that will produce the best result. The standard curve must be obtained using the same buffer/salt system and exact pH. This buffer/salt must be demetalled using a chelating resin such as Chelex 100 [63]. This method is useful if there is interference from standard colormetric assays of iron, or if only "loosely bound" iron is to be estimated [71].

For the UV visible method, the experiment is similar except the rate of loss of ascorbate is followed at 265 nm. This rate is plotted vs. Fe(III)EDTA concentration for the standard curve, from which unknown concentrations of iron are estimated.

Removal of Trace Metals

We have also found that ascorbate is an excellent tool to ascertain the effectiveness of adventitious catalytic metal removal from near-neutral buffer systems. In this method we follow the loss of ascorbate due to oxidation by monitoring its absorbance at 265 nm. In our standard test we add $\approx 3.5\,\mu L$ of 0.100 M ascorbic acid solution to 3.00 mL of the buffer in a standard 1 cm quartz cuvette. This results in an initial absorbance of 1.8. The loss of ascorbate is followed for 15 min. A loss of more than $\approx 0.5\%$ in this time indicates significant metal contamination. (If using a diode array spectrometer, interrogate the solution only a few times as the UV radiation near 200 nm will itself initiate ascorbate photooxidation.) [63, 72].

Plasma

The free radical initiator AAPH (2,2′-*azo-bis*(2-amidinopropane) dihydrochloride) undergoes thermal decomposition at a constant rate (at a fixed temperature) producing carbon-centered sigma radicals that react with O_2 at nearly diffusion-controlled rates yielding peroxyl radicals [73]. Thus, AAPH, in an oxygen-containing system, produces a constant flux of oxidizing free radicals that can oxidize ascorbate or produce spin adducts with the spin trap DMPO (5,5-dimethylpyrroline-1-oxide). When using AAPH as a source of oxidizing radicals in plasma a linear increase in $[Asc^{\bullet -}]_{ss}$ is seen with increasing concentrations of AAPH (Fig. 6). This plasma sample contained 58 μM ascorbate, a value typical of physiological conditions. Thus, in plasma $[Asc^{\bullet -}]_{ss}$ is indeed an excellent indicator of oxidative stress. [4].

Cells

Iron and ascorbate are well-known as a prooxidant combination that will initiate lipid peroxidation [9, 74–79]. Lipid-derived radicals from

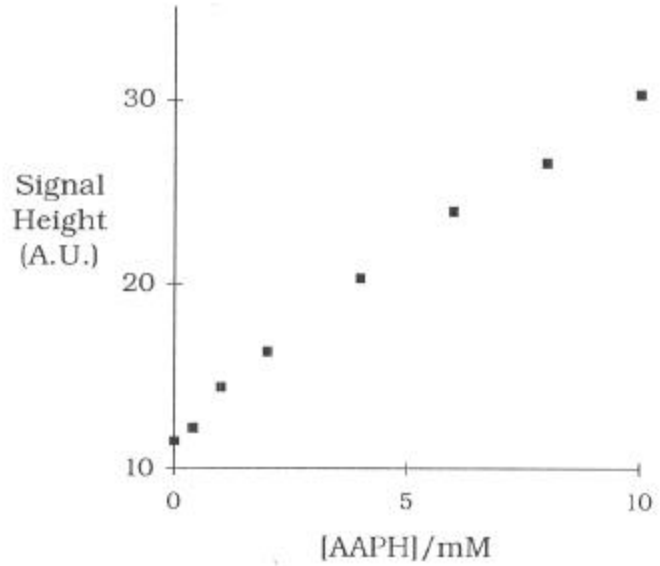


Fig. 6. Azo Initiator-Produced $Asc^{\bullet -}$ Radicals in Plasma: $Asc^{\bullet -}$ EPR signal height (arbitrary units) *versus* AAPH concentration. The plasma contained 58 μM ascorbate and varying amounts of AAPH [4].

cells have been detected using EPR spin trapping techniques when cells are exposed to iron and ascorbate [78, 79]. The introduction of edelfosine, an ether lipid drug being investigated for use in cancer treatment, to an L1210 murine leukemia cell suspension with 20 μM iron and 100 μM ascorbate present, results in a burst of $Asc^{\bullet -}$ production within 1–2 min after the addition. This burst of $Asc^{\bullet -}$ production corresponds with an increase in the rate of cellular lipid peroxidation as observed by EPR spin trapping, consistent with $[Asc^{\bullet -}]_{ss}$ being a real time reflection of the oxidation flux in the system [78].

$Asc^{\bullet -}$ in tissues

To examine tissues by EPR, e.g., skin, lens or samples whose viscosity precludes the use of an aqueous EPR flat sample cell, tissue cells (sometimes called cavity cells) such as produced by Wilmad Glass Co. (Buena, New Jersey) are available. These cells generally have a 0.5 mm depth sample cavity well and two supporting rods. In our experience, those cells with two stems are prone to breakage. Thus, we use a one stem tissue cell, i.e., no lower positioning rod. This reduces the incidence of breakage and facilitates tuning of the sample in the EPR cavity. A cover slip fits over the sample cavity well. Phosphor-bronze clips are provided to hold the cover over the well. However, we find that to prevent potential scraping of the inside of the EPR cavity with these clips that Parafilm ties can be used to provide an even more secure fit.

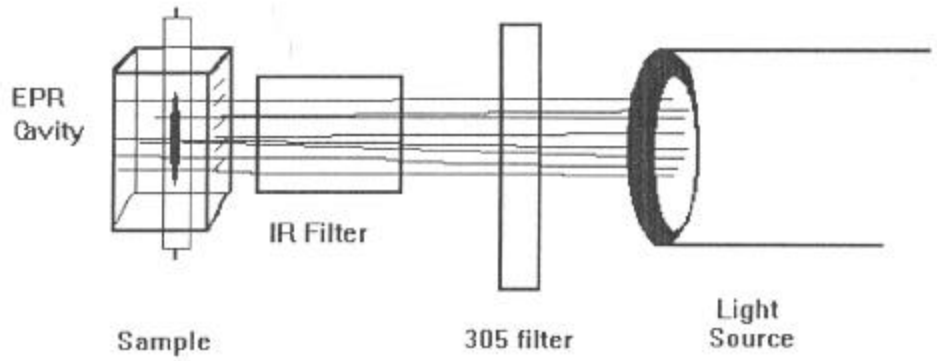


Fig. 7. EPR and light source setup for EPR tissue cell experiments. The 305 filter cuts off those wavelengths below ≈300 nm. The IR filter is a water-filled large diameter (50 mm) cylindrical, quartz UV-visible cell having a 50 mm path length. Our light source is an Oriel Photomax system with a 150 W Xe lamp. This system requires no special air handling due to ozone production. For our skin and lens experiments the lamp was operating at 3 mW/cm^2.

We find Parafilm to be an excellent tool in EPR experiments as it yields no significant EPR signals. These ties are made by cutting ≈2 mm wide strips of Parafilm and then wrapping them tightly around the cell and cover plate at the indentions that are provided for the clips. A diagram of the experimental setting is given in Figure 7.

Skin

Whole skin harvested from SKH-1 hairless male mice (Charles River Laboratories, Portage, Michigan) is cut into EPR usable pieces (≅1.0 cm^2, epidermis and dermis), placed in a Wilmad Glass Co. (Buena, New Jersey) one stem tissue cell, and positioned in the EPR cavity. EPR spectra are obtained at room temperature. The EPR spectrometer settings for the ascorbate radical experiments are: microwave power, 40 milliwatts; modulation amplitude, 0.66 G; time constant 0.3 s; scan rate 8 G/41.9 s; receiver gain, 2×10^6. The epidermal surface of the skin is exposed to UV light while in the EPR cavity. The light source is a Photomax 150 W xenon arc lamp (Oriel Corporation, Stratford, Connecticut) operating at 32 W; wavelengths below 300 nm are filtered out using a Schott WG 305 filter (Duryea, Pennsylvania). Infrared radiation from the light is removed by a 5 cm water filter. The filtered light fluence rate, including the visible wavelengths, as measured using a Yellow Springs Instrument (Yellow Springs, Ohio) model 65A radiometer with a 6551 probe, was 3 mW/cm^2, assuming the cavity grid transmits 75% of the incident light.

Lens

There is considerable evidence that UV-induced epithelial damage can be related to lens opacity and subsequent cataract formation [80]. The involvement of free radicals in cataract formation has been suggested [81, 82]. Ascorbic acid is clearly of importance as an antioxidant in the

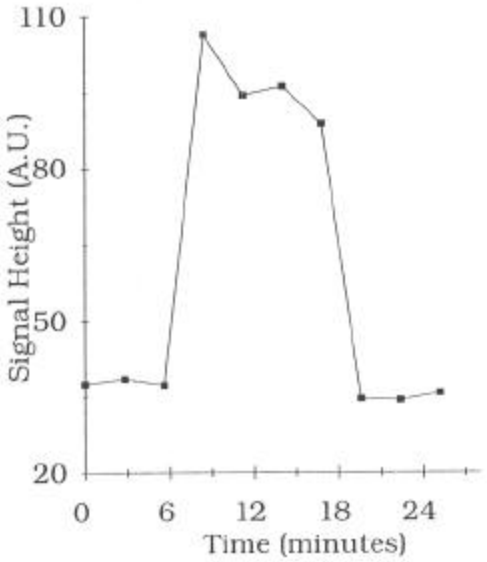


Fig. 8. $Asc^{\cdot -}$ in bovine lens subjected to UV light-induced oxidative stress. The $Asc^{\cdot -}$ signal height is shown in arbitrary units. The lens was exposed to the UV light after collection of the third data point. The light was turned off after collection of the seventh data point. The light source and EPR experimental setup is depicted in Figure 7.

lens of the eye, present at steady-state concentrations of 1–2 mM in the human lens and adjacent aqueous and vitreous humors. To examine $Asc^{\cdot -}$ levels in bovine lens tissue, lens tissue is placed in an EPR tissue cell and irradiated as described above in the skin experiments. A low steady-state level of the ascorbate free radical is detectable by EPR in the lens of the bovine eye. During UV photooxidative stress the levels of ascorbate free radical significantly increase (Fig. 8). When the light is turned off, the ascorbate free radical signal returns to baseline levels.

Whole bovine lens was cut into EPR usable pieces ($\approx 1.0\ cm^2$), placed in a Wilmad Glass Co. (Buena, New Jersey) one stem tissue cell, and positioned in a TM_{110} EPR cavity. EPR spectra were obtained at room temperature using a Bruker ESP 300 spectrometer operating at 9.74 GHz with 100 kHz modulation frequency. The EPR spectrometer settings for the ascorbate radical experiments were: microwave power, 40 milliwatts; modulation amplitude, 0.63 G; time constant, 1.3 s; scan rate, 6 G/167.7 s; receiver gain, 2×10^6. While in the EPR cavity, the lens was exposed to UV light after the third consecutive scan, and turned off after the seventh scan.

$Asc^{\cdot -}$ in vivo

Rat in vivo/ex vivo $Asc^{\cdot -}$

Mori et al. [83, 84] have observed $Asc^{\cdot -}$ in the circulatory blood of living rats with EPR. In these experiments a 1 mm tube was used to

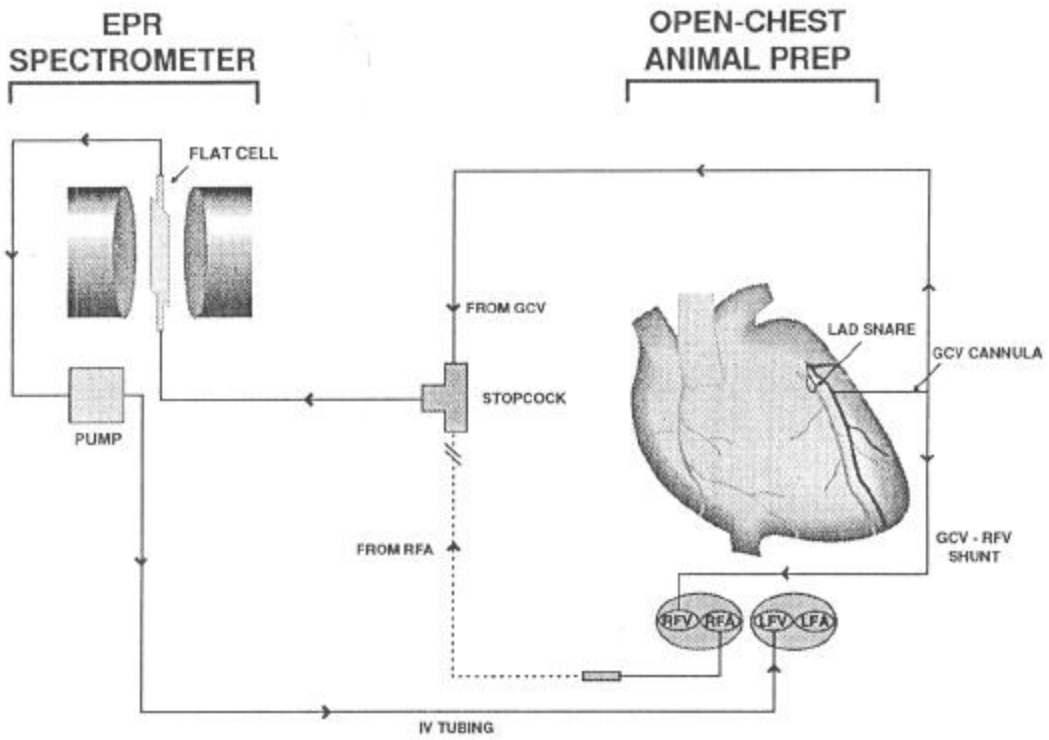


Fig. 9. Schematic of EPR setup for monitoring of $Asc^{\bullet -}$ in whole blood from the canine myocardium *ex vivo*. Note that the blood is returned to the animal. The lower end of the flat cell (Wilmad WG-813) is connected to the coronary venous cannula using thin (0.5 mm outer diameter) Teflon tubing of ≈1 meter in length. The end of the Teflon tubing is placed completely into the lower stem of the flat cell so the blood emerges directly into the bottom of the flat portion of the cell. The pump is an IV infusion pump; we draw from the top of the flat cell and push into the LFV. The flow rate of the pump is set to 600 mL/hour. (RFA, right femoral artery; RFV, right femoral vein; LFA, left femoral vein; LAD, left anterior descending coronary artery; GCV, great cardiac vein; and IV, intravenous.) The arrows indicate the direction of blood flow.

make a shunt from a femoral artery of the rat to an EPR cell positioned in an EPR cavity; the blood was returned to the rat by a continuation of the shunt from the EPR cell back to a femoral vein of the animal. In these experiments, the investigators demonstrated that introduction of iron, as ferric citrate, to the rat results in an increase in the circulating $[Asc^{\bullet -}]_{ss}$. This increase in $[Asc^{\bullet -}]$ correlates with other parameter of oxidative stress.

Canine in vivo/ex vivo *myocardial ischemia/reperfusion studies*

Free radical mediated oxidative stress is now thought to be a significant source of tissue damage during myocardial ischemia/reperfusion episodes. We have developed a means to monitor by EPR whole blood *ex vivo* from an open-chest canine method of myocardial ischemia/reperfusion [53]. Using this method we can monitor for the presence of $Asc^{\bullet -}$ in myocardial blood within ≈4–5 s from leaving the heart. By following the intensity of the $Asc^{\bullet -}$ signal versus time we can determine

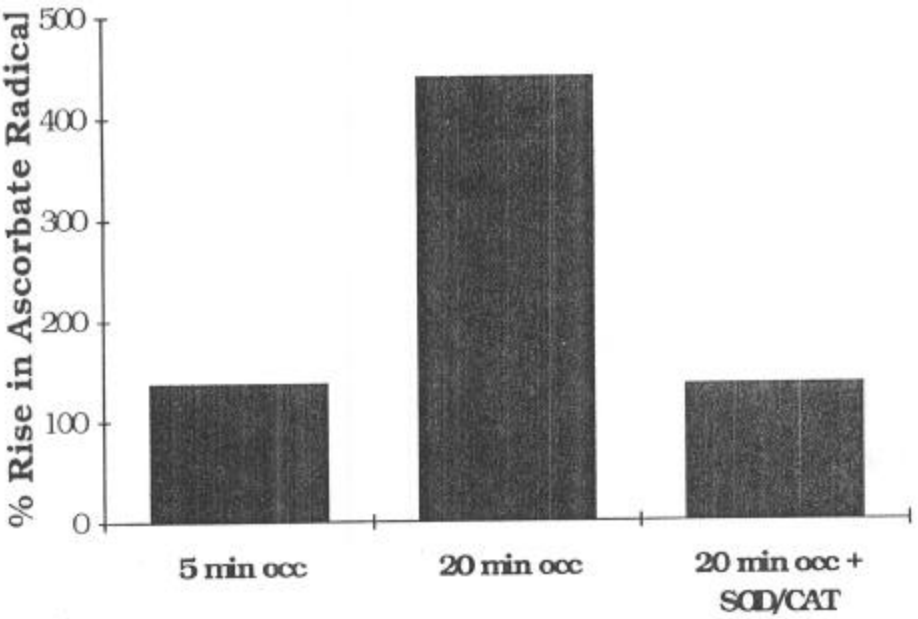


Fig. 10. These data demonstrate the increase in [Asc$^{\cdot -}$] during ischemia/reperfusion episodes. The percent rise is the change in the area under the curves of plots of [Asc$^{\cdot -}$] vs. time; the areas were determined using the Simpson integration method; the limits being from the beginning of reperfusion through the thirty minute time point [53]. Both the 5 min occlusion ($+134 \pm 73\%$) and the 20 min occlusion + SOD/CAT experiment ($+136 \pm 94\%$) were statistically different $p < 0.005$ than the 20 min occlusion ($+440 \pm 236\%$) experiments.

the changes in oxidative stress within the myocardium with various interventions during ischemia/reperfusion episodes [53].

These studies have used an open-chest canine model (≈ 20 kg) model of ischemia. General anesthesia is achieved with fentanyl droperidol. Briefly, a midsternal thoracotomy is performed and the heart exposed. A cannula is manipulated into the coronary sinus. Blood is withdrawn from the coronary sinus and passed through the EPR spectrometer, which is positioned next to the animal; we have refined this system so that the blood is scanned by EPR within $\approx 4-5$ s of withdrawal from the coronary sinus, Figure 9. At the beginning of the experiment we administer 1 gram of vitamin C as an intravenous bolus, followed by an intravenous infusion (usually 3.8–15.2 mg/min) in order to attain a steady-state arterial concentration of ascorbate free radical. Arterial blood is also initially passed through the EPR spectrometer to demonstrate the steady-state arterial level, which is usually ≈ 14 nM. The venous level is usually ≈ 8 nM. The blood is periodically rescanned to further demonstrate that the arterial level has not changed (if it has changed, the IV infusion of ascorbate is adjusted as necessary). In spite of this, if the arterial level of AFR is shown to vary more than 15% during a study, that study is discarded. During the experiment the coronary venous blood is continuously scanned to determine ascorbate

free radical signal intensity: the amplitude of the ascorbate radical signal is linearly proportional to the concentration of the radical, thus permitting real-time quantitative AFR determination, a demonstrated index of oxygen free radical generation.

In this method we have observed that 20 min of regional ischemia will increase the integrated $Asc^{\bullet -}$ signal intensity by over 400% upon reperfusion, Figure 10. A 5-min occlusion produced substantially less $Asc^{\bullet -}$; superoxide dismutase (SOD) and catalase (CAT) are able to blunt the 20-min reperfusion oxidative stress, bringing it to near the 5-min occlusion results.

The disadvantage of this method is that we lose information on the exact radicals being produced. However, the big advantage is that we are able to get a relative estimate on the total free oxidative flux, in real time. Because many types of radicals are produced during the oxidative cascade no one primary radical can be a reliable marker of the total radical flux. However, ascorbate, being at the bottom of the pecking order for oxidizing free radicals, can serve as a marker of the total free radical oxidative flux in a carefully controlled system.

Conclusion

Ascorbate is well known for its reducing properties. As such, it is an excellent antioxidant; it is thermodynamically at the bottom of the pecking order for oxidizing free radicals [3], thus we view it as the terminal small-molecule antioxidant [4]. Ascorbate protects cells from oxidative stress by scavenging free radicals and recycling other antioxidants, such as vitamin E. We have described here how using EPR, the ascorbate free radical can be used as a maker of oxidative stress.

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References

1. Lewin, S. (1976) *Vitamin C: Its Molecular Biology and Medical Potential*. Academic Press, New York.
2. Davies, M.B., Austin, J. and Partridge, D.A. (1991) *Vitamin C: Its Chemistry and Biochemistry*. Royal Society of Chemistry, Cambridge.
3. Buettner, G.R. (1993) The pecking order of free radicals and antioxidants: Lipid peroxidation, α-tocopherol, and ascorbate. *Arch. Biochem. Biophys.* 300: 535–543.
4. Buettner, G.R. and Jurkiewicz, B.A. (1993) Ascorbate free radical as a marker of oxidative stress: An EPR study. *Free Radic. Biol. Med.* 14: 49–55.
5. Creutz, C. (1981) The complexities of ascorbate as a reducing agent. *Inorg. Chem.* 20: 4449–4452.
6. Frei, B., England, L. and Ames, B.N. (1989) Ascorbate is an outstanding antioxidant in human blood plasma. *Proc. Natl. Acad. Sci. USA* 86: 6377–6381.

7. Halliwell, B. (1990) How to characterize a biological antioxidant. *Free Rad. Res. Comms.* 9: 1–32.
8. McCay, P.B. (1985) Vitamin E: Interactions with free radicals and ascorbate. *Ann. Rev. Nutr.* 5: 323–340.
9. Rees, S. and Slater, T.F. (1987) Ascorbic acid and lipid peroxidation: The cross-over effect. *Acta Biochim. Biophys. Hung.* 22: 241–249.
10. Niki, E. (1991) Vitamin C as an antioxidant. *World Rev. Nutr. Diet.* 64: 1–30.
11. Krinsky, N.I. (1992) Mechanism of action of biological antioxidants. *Proc. Soc. Exp. Biol. Med.* 200: 248–254.
12. Koppenol, W.H. and Butler, J. (1985) Energetics of interconversion reactions of oxy radicals. *Adv. Free Radical Biol.* 1: 91–131.
13. Kalyanaraman, B., Darley-Usmar, V.M., Wood, J., Joseph, J. and Parthasarathy, S. (1992) Synergistic interaction between the probucol phenoxyl radical and ascorbic acid in inhibiting the oxidation of low density lipoprotein. *J. Biol. Chem.* 267: 6789–6795.
14. Sharma, M.K. and Buettner, G.R. (1993) Interaction of vitamin C and vitamin E during free radical stress in plasma: An ESR study. *Free Radic. Biol. Med.* 14: 649–653.
15. Rose, R.C. and Bode, A.M. (1993) Biology of free radical scavengers: An evaluation of ascorbate. *FASEB J.* 7: 1135–1142.
16. Retsky, K.L., Freeman, M.W. and Frei, B. (1993) Ascorbic acid oxidation product(s) protect human low density lipoprotein against atherogenic modification. *J. Biol. Chem.* 268: 1304–1309.
17. Navas, P., Villalba, J.M. and Cordoba, F. (1994) Ascorbate function at the plasma membrane. *Biochim. Biophys. Acta* 1197: 1–13.
18. Davis, H.F., McManus, H.J. and Fessenden, R.W. (1986) An ESR study of free-radical protonation equilibria in strongly acid media. *J. Phys. Chem.* 90: 6400–6404.
19. Wardman, P. (1989) Reduction potentials of one-electron couples involving free radicals in aqueous solution. *J. Phys. Chem. Ref. Data.* 18: 1637–1755.
20. Buxton, G.V., Greenstock, C.L., Helman, W.P. and Ross, A.B. (1988) Critical review of rate constants for reactions of hydrated electrons, hydrogen atoms and hydroxyl radicals ($^{\cdot}OH/^{\cdot}O^{-}$) in aqueous solution. *J. Phys. Chem. Ref. Data* 17: 513–886.
21. Erben-Russ, M., Michel, C., Bors, W. and Saran, M. (1987) Absolute rate constants of alkoxyl radical reactions in aqueous solution. *J. Phys. Chem.* 91: 2362–2365.
22. Neta, P., Huie, R.E. and Ross, A.B. (1990) Rate constants for reactions of peroxyl radicals in fluid solutions. *J. Phys. Chem. Ref. Data* 19: 413–513.
23. Packer, J.E., Willson, R.L., Bahnemann, P. and Asmus, K.D. (1980) Electron transfer reactions of halogenated aliphatic peroxyl radicals: measurement of absolute rate constant by pulse radiolysis. *J. Chem. Soc. Perkin Trans. II.* 2: 296–299.
24. Tamba, M. and O'Neill, P. (1991) Redox reactions of thiol free radicals with the antioxidants ascorbate and chlorpromazine: Role in radioprotection. *J. Chem. Soc. Perkin Trans. II* 1681–1685.
25. Forni, L.G., Monig, J., Mora-Arellano, V.O. and Willson, R.L. (1983) Thiyl free radicals: Direct observations of electron transfer reactions with phenothiazines and ascorbate. *J. Chem. Soc. Perkin Trans. II* 961–965.
26. Simic, M.G. and Jovanovic, S.V. (1989) Antioxidation mechanisms of uric acid. *J. Am. Chem. Soc.* 111: 5778–5782.
27. Bielski, B.H.J. (1982) Chemistry of ascorbic acid radicals. *In*: Seib, P.A. and Tolbert, B.M. (eds): *Ascorbic Acid: Chemistry, Metabolism, and Uses.* Washington DC, American Chemical Society, pp 81–100.
28. Pelizzetti, E., Meisel, D., Mulac, W.A. and Neta, P. (1979) On the electron transfer from ascorbic acid to various phenothiazine radicals. *J. Am. Chem. Soc.* 101: 6954–6959.
29. Bielski, B.H.J., Cabelli, D.E. and Arudi, R.L. (1985) Reactivity of HO_2/O_2^- radicals in aqueous solution. *J. Phys. Chem. Ref. Data* 14: 1041–1100.
30. Cabelli, D.E. and Bielski, B.H.J. (1983) Kinetics and mechanism for the oxidation of ascorbic acid/ascorbate by HO_2/O_2^- radicals. A pulse radiolysis and stopped-flow photolysis study. *J. Phys. Chem.* 87: 1809–1812.
31. Nishikimi, M. (1975) Oxidation of ascorbic acid with superoxide anion generated by the xanthine-xanthine oxidase system. *Biochem. Biophys. Res. Comms.* 63: 463–468.
32. Buettner, G.R. (1986) Ascorbate autoxidation in the presence of iron and copper chelates. *Free Rad. Res. Comms.* 1: 349–353.

33. Buettner, G.R. (1990) Ascorbate oxidation: UV absorbance of ascorbate and ESR spectroscopy of the ascorbyl radical as assays for iron. *Free Rad. Res. Comms.* 10: 5–9.
34. Ross, A.B., Mallard, W.G., Hleman, W.P., Buxton, G.V., Huie, R.E. and Neta, P. (1994) NDRL-NIST Solution Kinetics Database: -Ver. 2.0. Gaithersburg: NIST.
35. Kahn, M.M.T. and Martell, A.E. (1967) Metal ion and metal chelate catalyzed oxidation of ascorbic acid by molecular oxygen. I. Cupric and ferric ion catalyzed oxidation. *J. Am. Chem. Soc.* 89: 4176–4185.
36. Khan, M.M.T. and Martell, A.E. (1967) Metal ion and metal chelate catalyzed oxidation of ascorbic acid by molecular oxygen. II. Cupric and ferric chelate catalyzed oxidation. *J. Am. Chem. Soc.* 89: 7104–7111.
37. Scarpa, M., Stevanto, R., Viglino, P. and Rigo, A. (1983) Superoxide ion as active intermediate in the autoxidation of ascorbate by molecular oxygen. *J. Biol. Chem.* 258: 6695–6697.
38. Williams, N.H. and Yandell, J.K. (1982) Outer-sphere electron-transfer reaction of ascorbate anions. *Aust. J. Chem.* 35: 1133–1144.
39. Winterbourn, C.C. (1993) Superoxide as an intracellular radical sink. *Free Radic. Biol. Med.* 14: 85–90.
40. Koppenol, W.H. (1993) A thermodynamic appraisal of the radical sink hypothesis. *Free Radic. Biol. Med.* 14: 91–94.
41. Foerster, G., Weis, W. and Staudinger, H. (1965) Messung der Elektronenspinresonanz an Semidehydroascorbinsäure. *Annalen der Chemie* 690: 166–169.
42. Stegmann, H.B., Schuler, P., Westphal, S. and Wagner, E. (1993) Oxidative stress of crops monitored by EPR. *Z. Naturforsch. C.* 48: 766–772.
43. Minetti, M., Forte, T., Soriani, M., Quaresima, V., Menditoo, A. and Ferrari, M. (1992) Iron-induced ascorbate oxidation in plasma as monitored by ascorbate free radial formation. *Biochem. J.* 282: 459–465.
44. Miller, D.M. and Aust, S.D. (1989) Studies of ascorbate-dependent, iron catalyzed lipid peroxidation. *Arch. Biochem. Biophys.* 271: 113–119.
45. Buettner, G.R. and Chamulitrat, W. (1990) The catalytic activity of iron in synovial fluid as monitored by the ascorbate free radical. *Free Radic. Biol. Med.* 8: 55–56.
46. Buettner, G.R., Motten, A.G., Hall, R.D. and Chignell, C.F. (1987) ESR detection of endogenous ascorbate free radical in mouse skin: Enhancement of radical production during UV irradiation following topical application of chlorpromazine. *Photochem. Photobiol.* 46: 161–164.
47. Jurkiewicz, B.A. and Buettner, G.R. (1994) Ultraviolet light-induced free radical formation in skin: An electron paramagnetic resonance study. *Photochem. Photobiol.* 59: 1–4.
48. Roginsky, V.A. and Stegmann, H.B. (1994) Ascorbyl radical as natural indicator of oxidative stress: Quantitative regularities. *Free Radic. Biol. Med.* 17: 93–103.
49. Timmins, G.S. and Davies, M.J. (1993) Free radical formation in murine skin treated with tumour promoting organic peroxides. *Carcinogensis* 14: 1499–1503.
50. Tomasi, A., Albano, E., Bini, A., Iannone, A.C. and Vannini, V. (1989) Ascorbyl radical is detected in rat isolated hepatocytes suspensions undergoing oxidative stress: and early index of oxidative damage in cells. *Adv. in the Biosciences* 76: 325–334.
51. Arroyo, C.M., Kramer, J.H., Dickens, B.F. and Weglicki, W.B. (1987) Identification of free radicals in myrocardial ischemia/reperfusion by spin trapping with nitrone DMPO. *FEBS Lett.* 221: 101–104.
52. Nohl, H., Stolze, K., Napetschnig, S. and Ishikawa, T. (1991) Is oxidative stress primarily involved in reperfusion injury of the ischemic heart? *Free Radic. Biol. Med.* 11: 581–588.
53. Sharma, M.K., Buettner, G.R., Spencer, K.T. and Kerber, R.E. (1994) Ascorbyl free radical as a real-time marker of free radical generation in briefly ischemic and reperfused hearts. *Circulation Res.* 74: 650–658.
54. Pietri, S., Culcasi, M., Stella, L. and Cozzone, P.J. (1990) Ascorbyl free radical as a reliable indicator of free-radical-mediated myocardial ischemic and post-ischemic injury. *Eur. J. Biochem.* 193: 845–854.
55. Pietri, S., Seguin, J.R., D'Arbigny, P. and Culcasi, M. (1994) Ascorbyl free radical: A noninvasive marker of oxidative stress in human open-heart surgery. *Free Radic. Biol. Med.* 16: 523–528.
56. Minakata, K., Suzuki, O., Saito, S. and Harada, N. (1993) Ascorbate radical levels in human sera and rat plasma intoxicated with paraquat and diaquat. *Arch. Toxicol.* 67: 126–130.

57. Stark, J.M., Jackson, S.K., Rowlands, C.C. and Evans, J.C. (1988) Increases in ascorbate free radical concentration after endotoxin in mice. *In*: C. Rice-Evans and B. Halliwell (eds): *Free Radicals: Methodology and Concepts*. Richelieu, London, pp 201–209.
58. Sasaki, R., Kurokawa, T. and Tero-Kubota, S. (1982) Nature of serum ascorbate radical and its quantitative estimation. *Tohoku J. Exp. Med.* 136: 113–119.
59. Sasaki, R., Kurokawa, T. and Tero-Kubota, S. (1983) Ascorbate radical and ascorbic acid level in human serum and age. *J. Gerontology* 1: 26–30.
60. Sasaki, R., Kobayasi, T., Kurokawa, T., Shibuya, D. and Tero-Kubota, S. (1984) Significance of the equilibrium constant between serum ascorbate radical and ascorbic acids in man. *Tohoku J. Exp. Med.* 144: 203–210.
61. Sasaki, R., Kurokawa, T. and Shibuya, D. (1985) Factors influencing ascorbate free radical formation. *Biochem. Intern.* 10: 155–163.
62. Ohara, T., Sasaki, R., Shibuya, D., Asaki, S. and Toyota, T. (1992) Effect of omeprazole on ascorbate free radical formation. *Tohoku J. Exp. Med.* 167: 185–188.
63. Buettner, G.R. (1988) In the absence of catalytic metals ascorbate does not autoxidize at pH 7: ascorbate as a test for catalytic metals. *J. Biochem. Biophys. Meth.* 16: 27–40.
64. Laroff, G.P., Fessenden, R.W. and Schuler, R.H. (1972) The electron spin resonance spectra of radical intermediates in the oxidation of ascorbic acid and related substances. *J. Am. Chem. Soc.* 94: 9062–9073.
65. Buettner, G.R. and Kiminyo, K.P. (1992) Optimal EPR detection of weak nitroxide spin adduct and ascorbyl free radical signals. *J. Biochem. Biophys. Meth.* 24: 147–151.
66. Miller, D.M., Buettner, G.R. and Aust, S.D. (1990) Transition metals as catalysts of "autoxidation" reactions. *Free Radic. Biol. Med.* 8: 95–108.
67. Guzman Barron, E.S., DeMeio, R.H. and Klemperer, F. (1936) Studies of biological oxidations. Copper and hemochromogens as catalysts for the oxidation of ascorbic acid. The mechanism of the oxidation. *J. Biol. Chem.* 112: 625–640.
68. Borsook, H., Davenport, H.W., Jeffreys, C.E.P. and Warner, R.C. (1937) The oxidation of ascorbic acid and its reduction *in vitro* and *in vivo*. *J. Biol. Chem.* 117: 237–279.
69. Weissberger, A., LuValle, J.E. and Thomas, D.S. (1943) Oxidation processes. XVI. The autoxidation of ascorbic acid. *J. Am. Chem. Soc.* 65: 1934–1939.
70. Halliwell, B. and Foyer, C.H. (1976) Ascorbic acid, metal ions and the superoxide radical. *Biochem. J.* 155: 697–700.
71. Britigan, B.E., Pou, S., Rosen, G.M., Lilleg, D.M. and Buettner, G.R. (1990) Hydroxyl radical is not a product of the reaction of xanthine oxidase and xanthine. *J. Biol. Chem.* 265: 17533–17538.
72. Buettner, G.R. (1990) Use of ascorbate as test for catalytic metals in simple buffers. *Meth. Enzymol.* 186: 125–127.
73. Niki, E. (1990) Free radical initiators as source of water- or lipid-soluble peroxyl radicals. *Methods Enzymol.* 186: 100–108.
74. Willis, E.D. (1969) Lipid peroxide formation in microsomes, general considerations. *Biochem. J.* 113: 315–324.
75. Willis, E.D. (1969) Lipid peroxide formation in microsomes, the role of non-haem iron. *Biochem. J.* 113: 325–332.
76. Willis, E.D. (1966) Mechanisms of lipid peroxide formation in animal tissues. *Biochem. J.* 99: 667–675.
77. Baysal, E., Sullivan, S.G. and Stern, A. (1989) Prooxidant and antioxidant effects of ascorbate on tBuOOH-induced erythrocyte membrane damage. *Int. J. Biochem.* 21: 1109–1113.
78. Wagner, B.A., Buettner, G.R. and Burns, C.P. (1993) Increased generation of lipid-derived and ascorbate free radicals by L1210 cells exposed to the ether lipid edelfosine. *Cancer Res.* 53: 711–713.
79. Wagner, B.A., Buettner, G.R. and Burns, C.P. (1993) Free radical-mediated lipid peroxidation in cells: Oxidizability is a function of cell lipid bis-allylic hydrogen content. *J. Biol. Chem.* 33: 4449–4453.
80. Taylor, H.R., West, S.K., Rosenthal, F.S., Munoz, B., Newland, H., Abbey, H. and Emmett, E.A. (1988) Effect of ultraviolet radiation on cataract formation. *New Eng. J. Med.* 319: 1429–33.

81. Weiter, J.J. and Finch, E.D. (1975) Paramagnetic species in cataractous human lenses. *Nature* 254: 536–537.
82. Murakami, J., Okazaki, M. and Shiga, T. (1989) Near UV-induced free radicals in ocular lens, studies by ESR and spin trapping. *Photochem. Photobiol.* 49: 465–473.
83. Mori, A., Wang, X. and Liu, J. (1994) Electron spin resonance assay of ascorbate free radicals *in vivo*. *Methods Enzymology* 233: 149–154.
84. Wang, X., Liu, J., Yokoi, I., Kohno, M. and Mori, A. (1992) Direct detection of circulating free radicals in the rat using electron spin resonance spectrometry. *Free Radic. Biol. Med.* 12: 121–126.

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