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\(^{\cdot}\)) as an intermediate. Loss of a second electron yields dehydroascorbic (DHA) [1].

![Chemical structures of AscH\(^+\), Asc\(^{\cdot}\), and DHA](image)

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

When biological fluids or tissues are examined by electron paramagnetic resonance spectroscopy (EPR), $\text{Asc}^-$ 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 radicals with greater reduction potentials, which includes, HO', RO', LOO', GS', the urate radical, and even the tocopheroxyl

¹A note on nomenclature: $\text{Asc}^-$ 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 $\text{AscH}^+$, the neutral protonated form of $\text{Asc}^-$. The ending “yl” being used for this neutral species.
Table 1. One-electron reduction potentials at pH 7.0 for selected radical couples

<table>
<thead>
<tr>
<th>Redox couple</th>
<th>$E^\circ$/mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>HO', H'+/H₂O</td>
<td>+2310</td>
</tr>
<tr>
<td>RO', H'/ROH (aliphatic alkoxy radical)</td>
<td>+1600</td>
</tr>
<tr>
<td>ROO', H'/ROOH (alkyl peroxy radical)</td>
<td>+1000</td>
</tr>
<tr>
<td>GS'/GS^- (glutathione)</td>
<td>+920</td>
</tr>
<tr>
<td>PUFA', H'/PUFA-H (bis-allylic-H)</td>
<td>+600</td>
</tr>
<tr>
<td>HU'^- , H'/UH_2 (Urate)</td>
<td>+590</td>
</tr>
<tr>
<td>TO', H'/TOH (Tocopherol)</td>
<td>+480</td>
</tr>
<tr>
<td>H₂O₂, H'/H₂O, HO'</td>
<td>+320</td>
</tr>
<tr>
<td>Ascorbate'^- , H'/Ascorbate monoanion</td>
<td>+282</td>
</tr>
<tr>
<td>Fe(III)/EDTA/Fe(II)/EDTA</td>
<td>+120</td>
</tr>
<tr>
<td>O₂/O^-</td>
<td>-330</td>
</tr>
<tr>
<td>Paraquat/Paraquat'^-</td>
<td>-448</td>
</tr>
<tr>
<td>Fe(III)/DFO/Fe(II)/DFO (Desferal)</td>
<td>-450</td>
</tr>
<tr>
<td>RSSR/RSSR'^- (GSH)</td>
<td>-1500</td>
</tr>
<tr>
<td>H₂O/e⁻</td>
<td>-2870</td>
</tr>
</tbody>
</table>

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

Table 2. Rate constants for the reaction of the equilibrium mixture of AscH₂/Asc^-/Asc²^- at pH 7.4 unless noted otherwise

| Radical                                      | $k_{obs}$/M⁻¹ s⁻¹ | (pH 7.4) | Ref.^
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>HO' ( tert-butyl alkoxy radical)</td>
<td>1.1 × 10¹⁰</td>
<td></td>
</tr>
<tr>
<td>RO' (CH₃COO')</td>
<td>1.6 × 10⁹</td>
<td></td>
</tr>
<tr>
<td>ROO' (alkyl peroxy radical, e.g., CH₃OO')</td>
<td>1–2 × 10⁶</td>
<td></td>
</tr>
<tr>
<td>Cl₄COO'</td>
<td>1.8 × 10⁸</td>
<td></td>
</tr>
<tr>
<td>GS' (glutathial radical)</td>
<td>6 × 10⁸</td>
<td>(5.6)</td>
</tr>
<tr>
<td>PUFA'</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>UH'^- (Urate radical)</td>
<td>1 × 10⁶</td>
<td></td>
</tr>
<tr>
<td>TO' (Tocopherol radical)</td>
<td>2 × 10⁵</td>
<td></td>
</tr>
<tr>
<td>Asc'^- (dissmutation)</td>
<td>2 × 10⁵</td>
<td></td>
</tr>
<tr>
<td>CPZ'^+ (Chlorpromazine radical cation)</td>
<td>1.4 × 10⁹</td>
<td>(5.9)</td>
</tr>
<tr>
<td>Fe(III)/EDTA/Fe(II)/EDTA</td>
<td>≈10²⁺</td>
<td></td>
</tr>
<tr>
<td>O₂^- /HO₂</td>
<td>1 × 10⁵</td>
<td></td>
</tr>
<tr>
<td>Fe(II)/DFO/Fe(II)/DFO</td>
<td>Very slow</td>
<td></td>
</tr>
</tbody>
</table>

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

The radical (TO'), can be repaired by ascorbate. Therefore, we have:

$$\text{Asc}^- + X' \rightarrow \text{Asc}^\cdot + XH,$$

where $X'$ 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') is replaced by the domesticated Asc⁻⁻. Asc⁻⁻ does not react by an addition reaction with O₂ to form dangerous peroxyl radicals. Ascorbate (probably Asc²⁻, \textit{vida infra}) and/or Asc⁻⁻ appear to produce very low levels of superoxide [37, 38]. But by removing O₂⁻, superoxide dismutase provides protection from this possibility [39, 40]. Thus, the biological organism is protected from further free radical-mediated oxidations. In addition, Asc⁻⁻ 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.

\textit{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⁻⁻ can also arise from comproportionation of AscH⁻ and DHA [41],

\[ \text{AscH}^- + \text{DHA} \rightleftharpoons 2 \text{Asc}^- + \text{H}^+ \]

\[ K = \frac{[\text{Asc}^-]^2}{[\text{AscH}^-][\text{DHA}]} \]

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 $5.6 \times 10^{-12}$ at pH 4.0 to $5.1 \times 10^{-9}$ 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].

\[ K = \frac{[\text{Asc}^-]^2[H^+]+\{1+[H^+]/10^{-pK_1}\}}{[\text{DHA}][\text{AscH}_2]_{\text{total}}}, \]

where $pK_1$ is the first ionization constant of ascorbic acid and $[\text{AscH}_2]_{\text{total}}$ is the analytical concentration of AscH₂, i.e., $[\text{AscH}_2]_{\text{total}} = [\text{AscH}_2] + [\text{AscH}^-] + [\text{Asc}^2-]$. [27, 41].

\textit{Using Asc⁻⁻ as a marker of oxidative stress}

\textit{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$^-$ 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]. 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$^-$, indicating the involvement of oxidative stress with this health problem [57]. Sasaki et al. have investigated in human serum the use of Asc$^-$ 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 ascorbate 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 $\varepsilon_{360} =$

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*In a quite different approach Pietri et al. [54, 55] have used Asc$^-$ as a probe for plasma ascorbate concentrations. In their approach, a 1:1 mixture of plasma and dimethylsulfoxide is examined for Asc$^-$ by EPR. They claim that the Asc$^-$ is an index of the transient changes in plasma ascorbate status during ischemia/reperfusion. Whereas, in our studies the Asc$^-$ levels reflect the ongoing free radical flux in the system being examined [4, 47, 53].*
3300 M$^{-1}$·cm$^{-1}$ and a half-width at half-maximum of about 50 nm [27]. With this small extinction coefficient, Asc$^-$ will not be observable by standard UV-VIS spectroscopy in steady-state experiments.

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

**EPR Detection of the ascorbate free radical**

The ascorbate free radical is usually detected by EPR as a doublet signal with $a_{11} = 1.8$ 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, $a_{11} = 1.76$ G, $a_{11}(2) = 0.19$ G, and $a_{11} = 0.07$ G [64].

In most biological experiments where the Asc$^-$ 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 $\approx 0.65$ G maximizes the ascorbate free radical doublet peak-to-peak signal amplitude [65].

The EPR power saturation curve of Asc$^-$ in room temperature aqueous solutions shows that saturation effects begin at $\approx 16$ 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$^-$ levels is desired, appropriate corrections for saturation effects must be included in the calculations.

![Fig. 2. EPR Power Saturation Curve for Asc$^-$](image)

Signal Height (A.U.) vs. \([\text{Power/ mW}]^{1/2}\)
Applications

Asc\textsuperscript{−} 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 reproducibility 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 \( \approx 2 \). 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 \( \approx 0.25 \text{ mM} \), 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\textsubscript{a} (4.2), Asc\textsuperscript{−} 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 \( \mu\text{M} \)) 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 \( \mu\text{M} \) 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
oxidation of ascorbate to be $6 \times 10^{-7}$ s$^{-1}$ under our experimental conditions [63]. However, even in carefully demetalated 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].

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

$$\text{Asc}^{2-} + \text{O}_2 \rightarrow \text{Asc}^{\cdot+} + \text{O}_2^{\cdot+}$$

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 biochemical and biological research have on the order of 1 $\mu$M iron and $<1$ $\mu$M 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 \( \text{Asc}^- \). The curves were obtained in 50 mM demetalled phosphate buffer, pH 7.40 with 250 \( \mu \text{M} \) EDTA (●) or 50 \( \mu \text{M} \) Desferal (▲) with 125 \( \mu \text{M} \) 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 \( \approx 100 \text{ nM} \) using UV-Vis spectroscopy [63]. However, using EPR spectroscopy this limit can be as low as \( \approx 5 \text{ nM} \) [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 \( \mu \text{M} \) [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 \( \mu \text{M} \) EDTA (●) or 50 \( \mu \text{M} \) Desferal (▲) with 125 \( \mu \text{M} \) ascorbate present (adapted from [33]).
then introduce ascorbate and determine by EPR the steady-state concentration (i.e., signal height) of \( \text{Asc}^- \). From a standard curve we can then estimate the iron concentration from \( \approx 5 \text{ nM} \rightarrow \approx 10 \mu \text{M} \). 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 colorimetric 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 \( \approx 3.5 \mu \text{L} \) 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-aminopropane) dihydrochloride) undergoes thermal decomposition at a constant rate (at a fixed temperature) producing carbon-centered sigma radicals that react with \( \text{O}_2 \) 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 \([\text{Asc}^-]_{ss}\) is seen with increasing concentrations of AAPH (Fig. 6). This plasma sample contained 58 \( \mu \text{M} \) ascorbate, a value typical of physiological conditions. Thus, in plasma \([\text{Asc}^-]_{ss}\) 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
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⁻⁻⁻ production within 1–2 min after the addition. This burst of Asc⁻⁻⁻ production corresponds with an increase in the rate of cellular lipid peroxidation as observed by EPR spin trapping, consistent with [Asc⁻⁻⁻],s being a real time reflection of the oxidation flux in the system [78].

 Asc⁻⁻⁻ 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.
We find Parafilm to be an excellent tool in EPR experiments as it yields no significant EPR signals. These ties are made by cutting \( \approx 2 \text{ mm} \) wide strips of Parafilm and then wrapping them tightly around the cell and cover plate at the indentations 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 (\( \approx 1.0 \text{ cm}^2 \), 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, \( 2 \times 10^6 \). 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
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Fig. 8. Asc'− in bovine lens subjected to UV light-induced oxidative stress. The Asc'− 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'− 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 (≈1.0 cm²), placed in a Wilmas Glass Co. (Buena, New Jersey) one stem tissue cell, and positioned in a TM₁₁₀ 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 × 10⁶. 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'− in vivo

Rat in vivo/ex vivo Asc'−
Mori et al. [83, 84] have observed Asc'− in the circulatory blood of living rats with EPR. In these experiments a 1 mm tube was used to
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 \([\text{Asc}^-]\)ss. This increase in \([\text{Asc}^-]\)ss 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 \(\text{Asc}^-\) in myocardial blood within \(\approx 4-5\) s from leaving the heart. By following the intensity of the \(\text{Asc}^-\) signal versus time we can determine...
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 ≈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 \( \text{Asc}^- \) signal intensity by over 400% upon reperfusion, Figure 10. A 5-min occlusion produced substantially less \( \text{Asc}^- \); 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 marker of oxidative stress.

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References


