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Original Contribution

ASCORBATE FREE RADICAL AS A MARKER OF OXIDATIVE STRESS: AN EPR STUDY

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Abstract—The ascorbate free radical ($A^{\bullet -}$) is a resonance-stabilized tricarbonyl species that is readily formed from the one-electron oxidation of ascorbate, AH^-. Because of the low reduction potential of the $A^{\bullet -}/AH^-$ couple, $E^{o\prime} = +282$ mV, nearly every oxidizing radical that can arise in a biological system will bring about the one-electron oxidation of AH^-, forming $A^{\bullet -}$. Thus, the steady-state concentration of $A^{\bullet -}$ can be used as a measure of oxidative stress in chemical, biochemical, and biological systems. In this article, we discuss the energetics of ascorbate free radical reactions and demonstrate that the $A^{\bullet -}$ electron paramagnetic resonance (EPR) signal intensity can serve as a marker for the degree of oxidative stress occurring in a system.

Keywords—Free radical, Antioxidant, Ascorbate, Electron paramagnetic resonance, Ascorbate radical

INTRODUCTION

Free radicals are in general short-lived paramagnetic species. It is now realized that free radicals play a role in the metabolic and chemical changes that occur in biological systems, which may result in, or be the result of, disease states. Because of their high reactivity, absolute bimolecular rate constants for free radical reactions are most often in 10^6–10^9 M^{-1} s^{-1} range. Thus, radicals usually have short lifetimes, $t_{1/2} \approx 10^{-3}$–10^{-9} s, making them difficult to detect. Because of the reactive nature of free radicals, a myriad of methods has been developed to monitor and identify these species.[1] The techniques range from various chemiluminescence and UV-spectrophotometry methods to EPR. EPR is the only direct method to detect free radicals; however, as a result of working with room-temperature aqueous solutions, EPR has limitations. EPR spin-trapping techniques that have been developed to overcome these problems do so by extending the lifetime of free radicals.[2] For example, the $HO^{\bullet}$ radical is not detectable by EPR in room-temperature aqueous solution because of its broad linewidth and short lifetime, $t_{1/2} \approx \mu$s–ns. However, the hydroxyl radical reacts with a commonly used spin trap 5,5′-dimethyl-1-pyrioline-*N*-oxide (DMPO) to produce the spin adduct DMPO/$^{\bullet}$OH, which has a relatively narrow linewidth, $\Delta H_{pp} \approx 1$ G, and long lifetime, $t_{1/2} \approx$ seconds to hours, depending on experimental conditions. These characteristics allow easy EPR detection and quantitation of DMPO/$^{\bullet}$OH. The integrative nature of spin trapping has provided a wealth of information on free radical processes in the chemical, biochemical, and biological sciences.[2,3]

The ascorbate anion, AH^-, is an endogenous water-soluble antioxidant that is present in biological systems. The one-electron oxidation of ascorbate produces the ascorbate free radical, $A^{\bullet -}$, which is easily detectable by EPR even in room-temperature aqueous solution. The ascorbate radical has a relatively long lifetime compared to other free radicals, such as hydroxyl, peroxyl, alkoxyl, and carbon-centered lipid free radicals.* The purpose of these experi-

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* Ascorbate free radical dismutes with a second-order rate constant of approximately 2.0×10^5 M^{-1} s^{-1} at pH 7.4. Thus, if $[A^{\bullet -}] = 10^{-7}$ M, then $t_{1/2}$ would be 50 s, assuming no other decay processes. In contrast, the very reactive $HO^{\bullet}$ has a rate constant of approximately 10^{+9} M^{-1} s^{-1} with nearly all organic substrates.[33] Thus, the half-life of $HO^{\bullet}$ generated in the presence of 1 M substrate will be approximately 10^{-9} s.

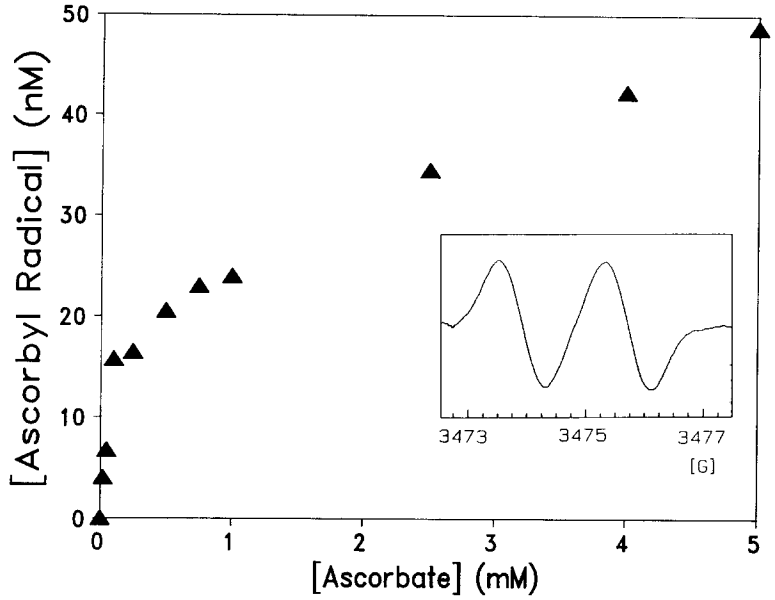

Fig. 1. Background [$A^{\cdot -}$] vs. [Ascorbate]: The solutions contained various concentrations of ascorbate in phosphate buffer pH 7.4. The points represent the $A^{\cdot -}$ concentration observed in the second of two scans, where the values had a standard deviation of less than 1 nanomolar. Inset: An example of the ascorbate radical doublet EPR signal observed in pH 7.4 buffer.

ments is to demonstrate that the ascorbate free radical EPR signal intensity can serve as a marker for the degree of ongoing free radical oxidative stress.

MATERIALS AND METHODS

Hypoxanthine, xanthine oxidase, ascorbic acid, DMPO, desferoxamine mesylate, chelating resin, and riboflavin were from Sigma Chemical Co., St. Louis, MO. The DMPO was purified with charcoal and stored as a 1.0 M aqueous solution.[4] 2,2'-Azobis(2-amidinopropane)dihydrochloride (AAPH) was obtained from PolySciences Company, Warrington, PA. All buffers were treated with chelating resin using the batch method;[5] absence of adventitious catalytic metals was verified with ascorbate.[5]

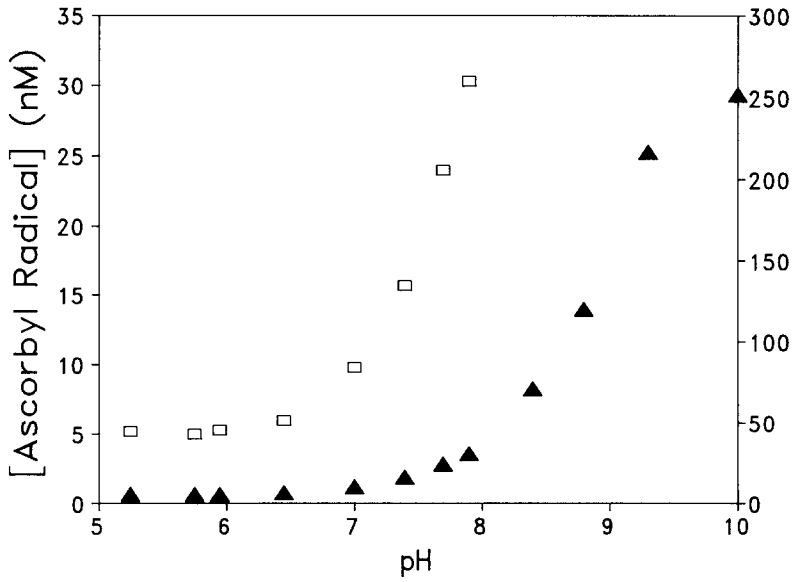

Fig. 2. Background [$A^{\cdot -}$] vs. pH: The solutions contained 500 μM ascorbate, 50 mM phosphate buffer, and 50 μM deferoxamine mesylate. The points represent the $A^{\cdot -}$ concentration observed in the second of three scans, where the values had a standard deviation of less than 1 nanomolar. □, left ordinate scale; ▲, right ordinate scale.

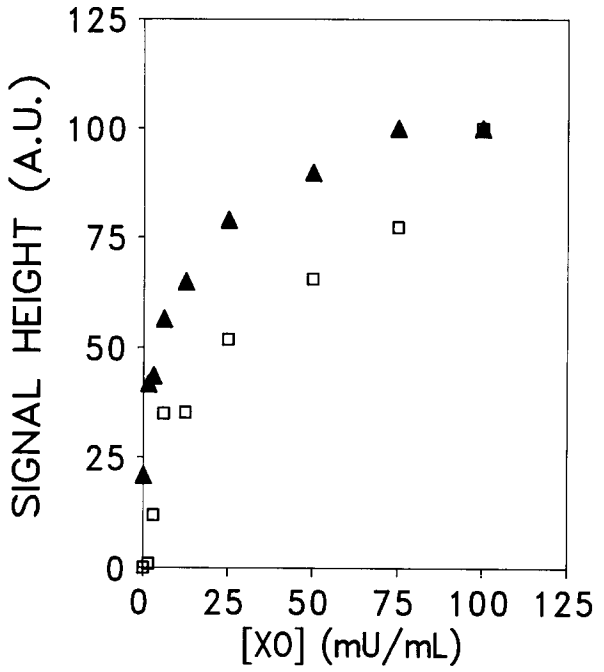

Fig. 3. Xanthine oxidase-produced radicals: $A^{\cdot -}$, ▲, and DMPO/$^{\cdot}$OOH, □, EPR signal height (arbitrary units) versus XO concentration. The solutions contained 500 μM ascorbate or 50 mM DMPO; 500 μM hypoxanthine; 500 μM or 250 μM DETAPAC; and various concentrations of XO in 50 mM phosphate buffer, pH 7.8 for DMPO or pH 7.4 for $A^{\cdot -}$. Each curve represents the median of three separate experiments. The results have been normalized to the highest value of each curve ([$A^{\cdot -}$] = 53 nM at 100; [DMPO/$^{\cdot}$OOH] = 2.0 μM at 100). At least three consecutive EPR spectra were collected for each solution. The plotted data represent signal height for the high field line of DMPO/$^{\cdot}$OOH or $A^{\cdot -}$ from the second scan of each sample.

The concentrations of $A^{\cdot -}$ and DMPO/$^{\cdot}$OOH were determined by double integration of the EPR spectra using 3-carboxy proxyl (Aldrich Chem. Co., Milwaukee, WI) as a standard. The spectra were obtained with the same instrument settings as used in collecting the experimental spectra, except for receiver gain. Saturation effects were accounted for as necessary.[6] EPR spectra were collected with a Bruker ESP 300 ESR spectrometer (Bruner Instruments; Karlsrube, Germany), using a TM_{110} cavity and aqueous flat cell.

Ascorbate radical background signals

In the experiments where the [$A^{\cdot -}$] was determined with varying amounts of ascorbate, the solutions contained 50 mM phosphate buffer, pH 7.4; 50 μM deferoxamine mesylate; and various concentrations of ascorbate. The deferoxamine mesylate was present in the buffer overnight to ensure complete chelation of adventitious iron.[7] In the experiments where the [$A^{\cdot -}$] was determined at various pH values, the solutions contained 50 mM phosphate buffer, 50 μM deferoxamine mesylate (overnight), and 500 μM ascorbate.

For the detection of the ascorbate free radical, EPR instrument settings were modulation amplitude, 0.71 G; scan rate, 6.0 G/84 s; time constant, 1.3 s; power, 40 mW; and receiver gain, 5×10^5.

Xanthine oxidase

In the experiments where xanthine oxidase (XO) was used as a source of oxyradicals, the solutions contained 500 μM ascorbate or 50 mM DMPO; 500 μM hypoxanthine; 50 μM or 250 μM diethylenetriamine pentaacetic acid (DETAPAC); and various concentrations of XO in 50 mM phosphate buffer, pH 7.8, for DMPO or pH 7.4 for $A^{\cdot -}$.

EPR spectral scans were initiated approximately 1 min after addition of XO. For the DMPO spin-trapping experiments, instrument settings were modulation amplitude, 0.96 G; scan rate, 80 G/168 s; time constant, 0.33 s; power, 40 mW; and receiver gain, 5×10^5. For the detection of the ascorbate free radical, instrument settings were modulation amplitude, 0.71 G; scan rate, 6.0 G/84 s; time constant, 1.3 s; power, 20 mW; and receiver gain, 5×10^5.

Riboflavin

In the experiments where riboflavin and light were used as a source of oxyradicals, the solution contained 500 μM ascorbate or 50 mM DMPO; 1 mM DETAPAC; various concentrations of riboflavin in 50 mM phosphate buffer, pH 7.8, for DMPO or pH 7.4 for $A^{\cdot -}$.

EPR spectra were collected at the same instrument settings as the XO experiment except for receiver gain. The light source was a 100-W tungsten microscope light focused on the TM_{110} cavity grid. The light fluence rate at the sample, as measured using a Yellow Springs Instrument (Yellow Springs, OH) model 65A radiometer with a 6551 probe, was $\approx$ 150 Wm^{-2}, assuming the cavity grid transmits 75% of the incident light. Infrared radiation from the light was removed by a 3-cm water filter.

Azo initiator, AAPH

In the chemical system where AAPH was used as a source of oxyradicals, the solution contained 500 μM ascorbate or 50 mM DMPO, 50 μM DETAPAC, and various concentrations of AAPH in 50 mM phos-

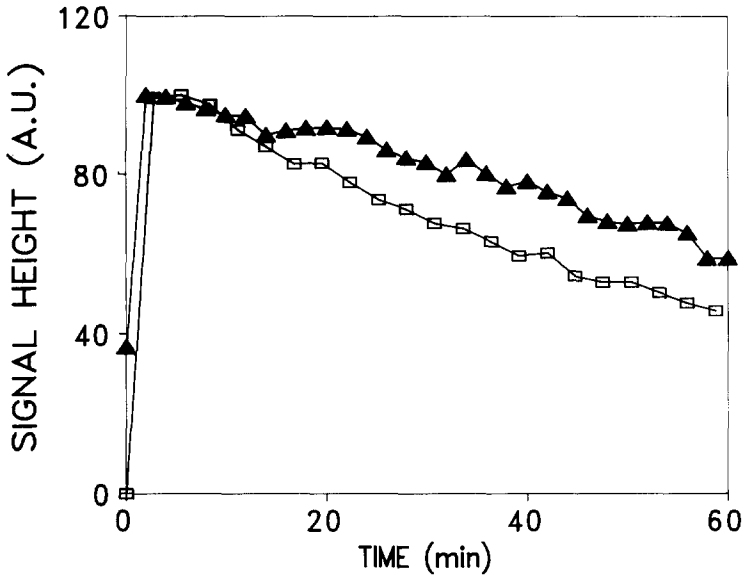

Fig. 4. Xanthine oxidase: $A^{\bullet -}$, ▲, and DMPO/$^{\bullet}$OOH, □, EPR signal height (arbitrary units) versus time in minutes. The solutions contained 500 μM ascorbate or 50 mM DMPO; 500 μM hypoxanthine; 50 μM or 250 μM DETAPAC; and 25 mU/ml of XO in 50 mM phosphate buffer, pH 7.8 for DMPO or pH 7.4 for $A^{\bullet -}$. Each curve shows the temporal steady-state EPR signal intensity of the radical signal after the introduction of XO. Spectra were acquired approximately every 2 min.

phate buffer, pH 7.4. In the experiment using human plasma, the concentration of ascorbate was 58 μM.

EPR spectra were collected at the same settings as the XO and riboflavin experiments except for receiver gain. EPR spectra were collected 10 min after the introduction of AAPH in the chemical system and 6 min after in the plasma system.

RESULTS

The DMPO nitrone spin-trapping system is EPR silent in the absence of impurities or a free-radical-generating system. However, background levels of the ascorbate radical are observable in an ascorbate solution even in metal-free pH 7.4 buffer.[7] This background $A^{\bullet -}$, produced by the one-electron oxidation of ascorbate, is thought to arise from the autooxidation of the ascorbate dianion;[7] thus, the $A^{\bullet -}$ concentration will vary as a function of ascorbate concentration and pH.

At pH 7.4, the concentration of $A^{\bullet -}$ increases as the concentration of ascorbate is increased (Fig. 1). At low concentrations of ascorbate (i.e., less than $\approx$ 100 μM) the $[A^{\bullet -}]_{ss}$ varies $\approx$ exponentially, whereas above 100 μM, $[A^{\bullet -}]_{ss}$ varies $\approx$ linearly, within the noise of the experiment.

Each line of the ascorbate radical doublet (Fig. 1, inset) is actually a triplet of doublets; in aqueous solution the EPR spectral parameters of $A^{\bullet -}$ are g value = 2.00518; $a^{H4} = 1.76$ G; $a^{H6}(2) = 0.19$ G; $a^{H5} = 0.07$ G.[8] However, our instrument settings were chosen for maximum sensitivity; thus, the hyperfine splittings for H5 and H6 are within the linewidth, and only a doublet with $a^{H4} \approx 1.8$ G is observed.[6]

Ascorbic acid is a diacid with $pK_1 = 4.2$ and $pK_2 = 11.6$.[9] As the pH rises, the concentration of the dianion will increase, resulting in an increase in the rate of ascorbate oxidation. This higher rate of ascorbate autooxidation will yield a higher steady state level of $A^{\bullet -}$ (Fig. 2). At pH values greater than $\approx$ 8, there is significant background ascorbate oxidation, which may limit the use of $[A^{\bullet -}]_{ss}$ as a measure of oxidation stress. At near neutral pH, the background $[A^{\bullet -}]_{ss}$ is minor; however, if the pH is held constant, then $[A^{\bullet -}]_{ss}$ can be used as a marker of oxidative stress.

Xanthine oxidase

Xanthine oxidase catalyzes the oxidation of hypoxanthine to uric acid producing hydrogen peroxide and superoxide, $O_2^{\bullet -}$. The flux of superoxide in this system can be varied by changing the concentration of xanthine oxidase while holding the other variables constant. As seen in Fig. 3, when the concentration of xanthine oxidase is increased, producing more superoxide, we see nearly parallel increases in both the ascorbate free radical and DMPO/$^{\bullet}$OOH EPR signals. Both the ascorbate free radical and DMPO/$^{\bullet}$OOH EPR signal intensities vary with time (Fig. 4). Naturally, the form of these curves will change as a function of the XO concentration (not shown).

Riboflavin

Riboflavin is a photosensitizer that produces a strongly oxidizing triplet. This triplet is reduced by electron donors; both ethylenediaminetetraacetic acid (EDTA) and DETAPAC can be oxidized by the triplet state of riboflavin. The reduced riboflavin can in turn convert dioxygen to superoxide. Thus, riboflavin can serve as a source of superoxide but will only generate superoxide in the presence of light. The flux of superoxide can be varied in a riboflavin system by changing the riboflavin concentration while holding the other variables and light fluence rate constant. Using riboflavin in the presence of light as a source of superoxide, we observed nearly parallel increases in both $A^{\bullet -}$ and DMPO/$^{\bullet}$OOH EPR signal intensity as the amount of riboflavin was increased (Fig. 5).

Azo initiator, AAPH

The free radical initiator AAPH undergoes thermal decomposition at a constant rate, producing carbon-centered sigma radicals that react with O_2 at nearly diffusion-controlled rates yielding peroxyl radicals.[10] Thus, AAPH, in an oxygen-containing system, produces a constant flux of oxidizing free radicals that can oxidize ascorbate or produce spin adducts with DMPO. When using AAPH as a source of oxidizing radicals in simple buffer solution, we observed nearly linear increases in both the DMPO/$^{\bullet}$OH adduct, $a^N = a^H = 14.8$ G, and ascorbate free radical EPR signal intensities as the concentration of AAPH was increased (Fig. 6). In the experiment using plasma containing 58 μM ascorbate, a value typical of physiological conditions, the $A^{\bullet -}$ EPR signal intensity was also found to increase as the concentration of AAPH increases (Fig. 7).

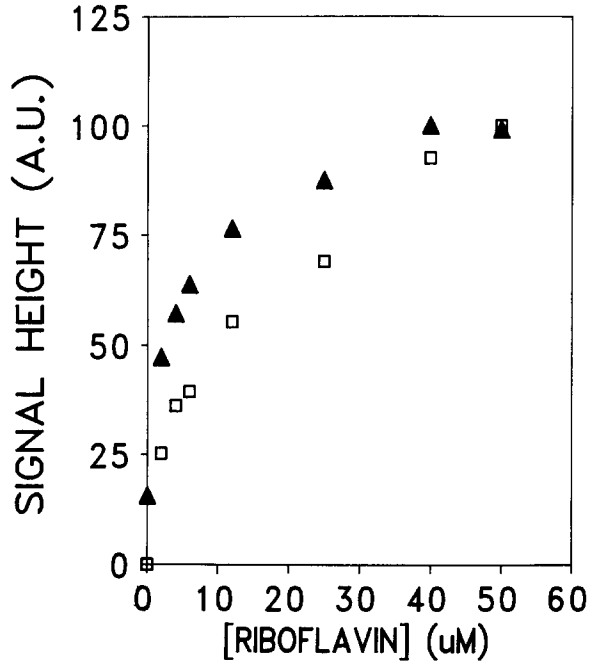

Fig. 5. Riboflavin-produced radicals: $A^{\bullet -}$, ▲, and DMPO/$^{\bullet}$OOH, □, EPR signal height (arbitrary units) versus riboflavin concentration. The solution contained 500 μM ascorbate or 50 mM DMPO; 1 mM DETAPAC; various concentrations of riboflavin in 50 mM phosphate buffer, pH 7.8 for DMPO or pH 7.4 for $A^{\bullet -}$. Each data point of each curve represents the median of three separate experiments. For each experiment, the EPR signal height was determined as the average of two consecutive scans, using the high field lines of DMPO/$^{\bullet}$OOH or $A^{\bullet -}$. Each curve represents the median of three experiments where the results were normalized to the highest value of each curve ([$A^{\bullet -}$] = 30 nM at 100; [DMPO/$^{\bullet}$OOH] = 1.8 μM at 100).

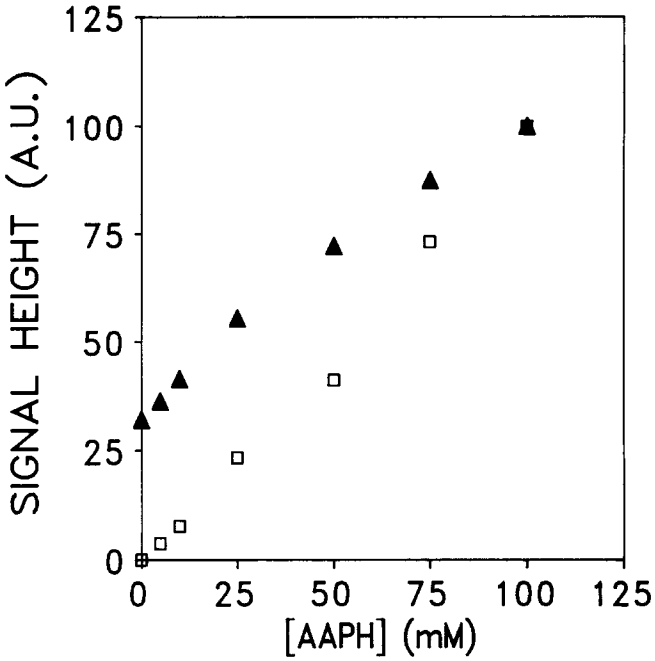

Fig. 6. Azo initiator-produced radicals in buffer: $A^{\bullet -}$, ▲, and DMPO/$^{\bullet}$OH, □, EPR signal height (arbitrary units) versus AAPH concentration. The solutions contained 500 μM ascorbate or 50 mM DMPO; 50 μM DETAPAC, and various concentrations of AAPH in 50 mM buffer, pH 7.4. Each curve represents the median of three separate experiments. The results have been normalized to the highest value of each curve ([$A^{\bullet -}$] = 36 nM at 100; [DMPO/$^{\bullet}$OH] = 2.3 μM at 100).

DISCUSSION

Ascorbate free radical has been observed by EPR in numerous chemical and biological studies. For example, in biological systems, $A^{\bullet -}$ has been observed in lyophilized tissue,[11] xenobiotic metabolism in the liver,[12] and myocardial ischemia/reperfusion stud-

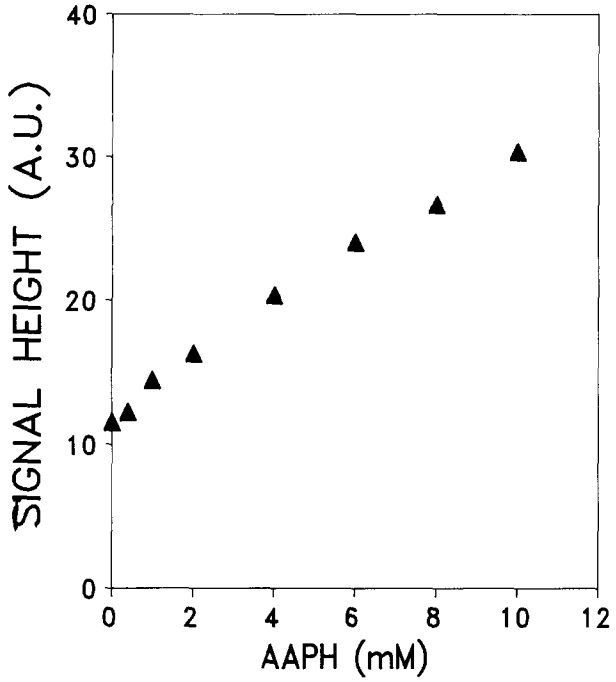

Fig. 7. Azo initiator-produced $A^{\bullet -}$ radicals in plasma: $A^{\bullet -}$, ▲, EPR signal height (arbitrary units) versus AAPH concentration. The solutions contained 58 μM ascorbate in plasma and various amounts of AAPH.

ies.[13-15] Since the first EPR observations of $A^{\bullet -}$ produced by oxidative enzymes,[16,17] the ascorbate radical has been used as an indicator of oxidative events. For example, increased $A^{\bullet -}$ concentrations have been observed in photochemical systems containing AH^-,[18] photosensitized reactions,[19,20] skin,[18] X-irradiated tissue,[21] and in organ endotoxic shock.[22]

In general, quantitative aspects of $A^{\bullet -}$ generation and decay have been limited principally to fast kinetic studies.[23,24] These studies have demonstrated that $A^{\bullet -}$ decays by a pH-dependent second-order dismutation process, k^{obs} (pH 7) = 2×10^5 M^{-1} s^{-1} (Ref. 23). A recent study has demonstrated that the steady-state level of $A^{\bullet -}$, as observed by EPR, can be used as a marker for the rate of the metal-catalyzed oxidation of ascorbate.[7]

Ascorbate has many functions in living systems, but a key feature is its ability to serve as a water-soluble antioxidant.[25] In fact, ascorbate is the most effective water-soluble antioxidant in human blood plasma.[26,27] These observations are consistent with the thermodynamic properties of the ascorbate radical/ascorbate couple (Table 1). Because of the low reduction potential of $A^{\bullet -}$, ascorbate will react as an antioxidant with nearly every oxidizing radical that could arise in a biological system. This one-electron oxidation of AH^- results in the production of $A^{\bullet -}$,

$$R^{\bullet} + AH^- \rightarrow A^{\bullet -} + R\text{-}H \quad \Delta E^{\circ\prime} > 0$$

a resonance stabilized tricarbonyl species that is easily observable by EPR.

In our experiments, using three different free-radical-generating systems, we have found that the EPR signal height of $A^{\bullet -}$ correlates with the flux of initiating free radicals. However, it must be kept in mind that there is always a background oxidation of ascorbate yielding a low level of $A^{\bullet -}$.[7] This background $A^{\bullet -}$ signal intensity is a function of pH, catalytic metal concentration, oxygen concentration, and ascorbate concentration. As demonstrated in our experiments, when these variables are controlled, the steady-state $A^{\bullet -}$ EPR signal intensity serves as a marker for the degree of free radical oxidative stress in the system.

In a biological system, it may be difficult to control all the variables that influence the $A^{\bullet -}$ EPR signal intensity. Thus, it is important that control experiments be carefully planned; a paired comparison design would be ideal.

EPR spin trapping is a widely used technique that has provided both structural and quantitative information about reactive free radical formation, whereas the ascorbate free radical does not provide structural information on the precursor radicals. However, spin traps must be added to the system being measured and may be toxic to living species when used at the concentrations of typical biochemical investigations. In addition, they are relatively expensive, whereas ascorbate is a natural, nontoxic endogenous biological compound. Because of the limitations of spin trapping, there is a need for complementary methods to detect oxidative radical production.

We propose 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. The ascorbate radical is a relatively stable, nontoxic radical species that is easily detectable. As oxidative stress increases in a system, the steady-state

Table 1. One-Electron Reduction Potentials

Couple	$E^{\circ\prime}$/mV	Ref.
$HO^{\bullet}$, H^+/H_2O	2310	28
$RO^{\bullet}$, H^+/ROH (aliphatic alkoxyl radical)	1600	29
$ROO^{\bullet}$, H^+/ROOH (alkyl peroxyl radical)	1000	29
$GS^{\bullet}/GS^-$ (glutathione)	920	30
$PUFA^{\bullet}$, H^+/PUFA-H (polyunsaturated fatty acid, *bis*-allylic-H)	600	29
α-chromanoxyl$^{\bullet}$, H^+/α-tocopherol ($TO^{\bullet}$, H^+/TOH)	480	31
$Ascorbate^{\bullet -}$, H^+/ascorbate monoanion	282	32

$A^{\bullet-}$ concentration increases. Thus, the EPR intensity of this radical signal can serve as an indicator of the degree of free radical oxidative processes taking place in chemical, biochemical, or biological systems.

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REFERENCES

1. Weber, G. F. The measurement of oxygen-derived free radicals and related substances in medicine. *J. Clin. Chem. Clin. Biochem.* **28**:569–603; 1990.
2. Janzen, E. G.; Haire, D. L. Two decades of spin trapping. In: Tanner, D., ed. *Advances in Free Radial Chemistry* Vol. 1. Greenwich, CT: JAI Press; 1990:253–295.
3. Buettner, G. R. Spin trapping: ESR parameters of spin adducts. *Free Radic. Biol. Med.* **3**:259–303; 1987.
4. Buettner, G. R.; Oberley, L. W. Considerations in the spin trapping of superoxide and hydroxyl radicals in aqueous solutions using 5,5-dimethyl-1-pyrroline-1-oxide. *Biochem. Biophys. Res. Commun.* **83**:69–74; 1978.
5. Buettner, G. R. 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; 1988.
6. Buettner, G. R.; Kiminyo, K. P. Optimal EPR detection of weak nitroxide spin adduct and ascorbyl free radical signals. *J. Biochem. Biophys. Meth.* **24**:147–151, 1992.
7. Buettner, G. R. Ascorbate oxidation: UV absorbance of ascorbate and ESR spectroscopy of the ascorbyl radical as assays for iron. *Free Rad. Res. Comm.* **10**:5–9; 1980.
8. Laroff, G. P.; Fessenden, R. W.; Schuler, R. H. The electron spin resonance spectra of radical intermediates in the oxidation of ascorbic acid and related substances. *J. Am. Chem. Soc.* **94**:26:9062–9073; 1972.
9. Lewin, S. *Vitamin C, its molecular biology and medical potential.* London: Academic Press; 1976.
10. Niki, E. Free radical initiators as source of water- or lipid-soluble peroxyl radicals. *Meth. Enzymol.* **186**:100–108; 1990.
11. Lohmann, W. Ascorbate acid and cancer. *Ann. N.Y. Acad. Sci.* **498**:402–417; 1987.
12. Thurman, R. G.; Connor, H. D.; Knecht, K. T.; Lacagnin, L. B.; O'Brien, P.; Mason, R. P. Spin trapping in the perfused liver: Carbon tetrachloride as a model system. In: Ballet, F.; Thurman, R. G., eds. *Perfused liver.* INSERM/John Libbey; 1991:69–82.
13. Arroyo, C. M.; Kramer, J. H.; Dickens, B. F.; Weglicki, W. B. Identification of free radicals in myocardial ischemia/reprefusion by spin trapping with nitrone DMPO. *FEB. Paris,* **221**:101–104; 1987.
14. Pietri, S.; Culcasi, M.; Stella, L.; Cozzone, P. J. Ascorbate free radical as a reliable indication of free-radical-mediated myocardial ischemia and post-aschemic injury. *Eur. J. Biochem.* **193**:845–854; 1990.
15. Nohl, H.; Stolze, K.; Napetschnig, S.; Ishikawa, T. Is oxidative stress primarily involved in reprefusion injury of the ischemic heart? *Free Radic. Biol. Med.* **11**:581–588; 1991.
16. Yamazaki, I.; Mason, H. S.; Piette, L. H. Identification, by electron paramagnetic resonance spectroscopy, of free radicals generated from substrates by peroxidase. *J. Biol. Chem.* **235**:2444–2449; 1959.
17. Yamazaki, I.; Piette, L. H. Mechanism of free radical formation and disappearance during the ascorbic acid oxidase and peroxidase reactions. *Biochim. Biophys. Acta* **50**:62–69; 1961.
18. Buettner, G. R.; Motten, A. G.; Hall, R. D.; Chignell, C. F. ESR detection of endogenous ascorbate free radical production during UV irradiation following topical application of chloropromazine. *Photochem. Photobiol.* **46**:161–164; 1987.
19. Felix, C. C.; Reszka, K.; Sealy, R. C. Free radicals from photoreduction of hematoporphyrin in aqueous solution. *Photochem. Photobiol.* **37**:141–147; 1983.
20. Buettner, G. R.; Doherty, T. P.; Bannister, T. D. Hydrogen peroxide and hydroxyl radical formation of methylene blue in the presence of ascorbic acid. *Rad. Environ. Biophys.* **23**:235–243; 1984.
21. Floyd, R. A.; Bransdon, A.; Commoner, B. ESR signals during x-irradiation of tissue: Their characteristics and relationship to the cancerous state. *Ann. N.Y. Acad. Sci.* **222**:1077–1086; 1973.
22. Stark, J. M.; Jackson, S. K.; Rowlands, C. C.; Evans, J. C. Increases in ascorbate free radical concentration after endotoxin in mice. In: Evans, C. R.; Halliwell, B. *Free Radicals, Methodology and Concepts.* Richelieu Press, London; 1988:201–209.
23. Bielski, B. H. Chemistry of ascorbic acid radicals. In: Seib, P. A.; Tolbert, B. M., eds. *Ascorbic acid: Chemistry, metabolism, and uses.* Washington, DC: American Chemical Society; 1982:81–100.
24. Burkitt, M. J.; Gilbert, B. C. Model studies of the iron-catalysed Haber-Weiss cycle and the ascorbate driven Fenton reaction. *Free Rad. Res. Comm.* **10**:265–280; 1990.
25. Bendich, A.; Machlin, L. J.; Scandurr, O. The antioxidant role of Vitamin C. *Adv. Free Rad. Biol. Med.* **2**:419–444; 1986.
26. Frei, B.; England, L.; Ames, B. N. Ascorbate is an outstanding antioxidant in human blood plasma. *Proc. Nat. Acad. Sci.* **86**:6377–6381; 1989.
27. Frei, B.; Stocker, R.; Ames, B. N. Antioxidant defenses and lipid peroxidation in human blood plasma. *Proc. Nat. Acad. Sci.* **85**:9748–9752; 1988.
28. Koppenol, W. H. Generation and thermodynamic properties of oxyradicals. *Membrane Lipid Oxidation* **1**:1–13; 1989.
29. Koppenol, W. H. Oxyradical reactions: From bond-dissociation energies to reduction potentials. *FEBS Lett.* **264**:165–167; 1990.
30. Surdhar, P. S.; Armstrong, D. A. Redox potentials of some sulfur-containing radicals. *J. Phys. Chem.* **90**:5915–5917; 1990.
31. Simic, M. G. Pulse radiolysis in study of oxygen radicals. *Meth. Enzymol.* **186**:89–100; 1990.
32. Williams, N. H.; Yandell, J. K. Outer-sphere electron-transfer reactions of ascorbate anions. *Aust. J. Chem.* **35**:1133–1144; 1982.
33. Buxton, G. V.; Greenstock, C. L.; Helman, W. P.; Ross, A. B. Critical review of rate constants for reactions of hydrated electrons, hydrogen atoms and hydroxyl radicals ($^{\bullet}OH/O^{\bullet-}$) in aqueous solutions. *J. Phy. Chem. Ref. Data* **17**:513–886; 1988.

ABBREVIATIONS

$A^{\bullet-}$—monodehydroascorbate radical
$[A^{\bullet-}]_{ss}$—steady-state $A^{\bullet-}$ concentration
AAPH—2,2′-azo*bis*(2-amidinopropane)dihydrochloride
AH^-—ascorbate monoanion
DETAPAC—diethylenetriaminepentaacetic acid
DMPO—5,5-dimethylpyrroline-1-oxide
$O_2^{\bullet-}$—the equilibrium mixture of $O_2^{\bullet-}$ and $HO_2^{\bullet}$
XO—xanthine oxidase