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The Vitamin C Radical and Its Reactions

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PROPERTIES OF THE ASCORBATE RADICAL

The ascorbate radical is an important intermediate in reactions involving the antioxidant function of ascorbate. However, its generation by and reaction with enzymes are also known. In this chapter, we discuss: (1) the physicochemical properties and reactions of the ascorbate radical, (2) its formation during antioxidant interactions, and (3) the in vivo detection of ascorbate radical by electron paramagnetic resonance (EPR) spectroscopy as a promising tool for noninvasive monitoring of oxidative stress. But first, we wish to clear up confusion and offer some suggestions for order to the multitude of descriptions and abbreviations used in the literature for the compounds involved.

Nomenclature

In the literature there exist many different names and abbreviations for the various species involved in the chemistry of vitamin C. For example, SDA, MDAA, AR, AFR, plus others, have been used for the ascorbate radical alone. In this review we use $AscH_2$, $AscH^-$, and Asc^{2-} to denote the undissociated form of ascorbic acid, the physiologically dominant ascorbate monoanion ($pK_1 = 4.1$) (1), and the ascorbate dianion ($pK_2 = 11.79$) (1), respectively; AscH$^{\cdot}$ and $Asc^{\cdot -}$ for the neutral ascorbyl and the anionic ascorbate radicals (with a pK value of -0.86 (2) only $Asc^{\cdot -}$ is relevant in biology); DHA for dehydroascorbic and DHAA for its hydrolyzed form, both products of the two-electron oxidation of vitamin C (3) (see Fig. 1 for the structures of each of these species.). Although there is no International Union of Pure and Applied Chemistry (IUPAC) convention for these abbreviations, we suggest that researchers use them because they are simple, yet they convey accurately the chemical aspects of the species being discussed.

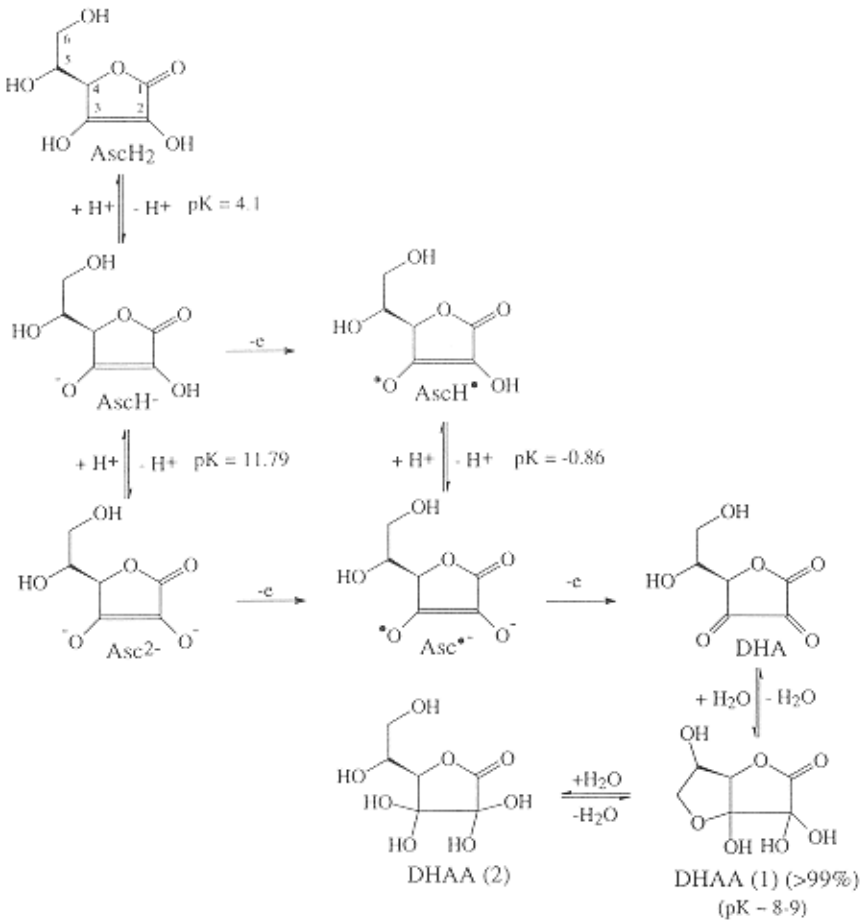

Figure 1 The equilibrium and redox species in the ascorbic acid–dehydroascorbic acid system.

Physicochemical Properties

The most detailed studies on the kinetics of ascorbate and ascorbate radical reactions have been carried out using pulse radiolysis (4–12). Photosensitized generation of $Asc^{\bullet-}$ is less suitable for these types of kinetics studies as it is prone to side reactions diminishing the yield of $Asc^{\bullet-}$ (13,14). The reaction of ascorbate with $HO^{\bullet}$ is quite complex as a result of formation of intermediates (4,7,12); thus generation of $Asc^{\bullet-}$ for kinetic studies is preferably done with other electrophilic species such as halogen radical (4,7) or especially azidyl radicals (15–17).

The National Institute of Standards and Technology (NIST) Solution Kinetics Database (18) contains quite a number of rate constants for the generation of $Asc^{\bullet-}$, almost two-thirds of them reactions of $AscH^-$ with peroxyl radicals (19–21), most others with inorganic radicals. However, the NIST Database has only a limited number of the reactions of $AscH^-$ with phenoxyl radicals (15,17,22–26); these are included in Table 1. Table 1 also contains four rate constants for reductive reactions of DHA with flavonoid aroxyl radicals, leading to

Table 1 Rate Constants for Ascorbate Radical ($Asc^{\cdot-}$) Formation

Radical	Substrate	pH	$k/M^{-1}s^{-1}$	Comment	Ref.
		Substrate: ascorbic acid ($AscH_2$)			
$HO^{\cdot}$	$AscH_2$	1.5	8.25×10^9	See text	4
$Br_2^{\cdot-}$	$AscH_2$	1.5	1.1×10^8		4
$I_2^{\cdot-}$	$AscH_2$	1.5	5.0×10^6		4
$(SCN)_2^{\cdot-}$	$AscH_2$	1.5	1.0×10^7		4
$HO_2^{\cdot}$	$AscH_2$	0.3–1	1.6×10^4		9
$SO_3^{\cdot-}$	$AscH_2$	3.6	2.3×10^6		27
$SO_5^{\cdot-}$	$AscH_2$	3.6	1.3×10^7		27
$CCl_3OO^{\cdot}$	$AscH_2$	1.0	1.4×10^7	H_2O/iPOH (8:1)	28
$CH_3OO^{\cdot}$	$AscH_2$	3.1	4.0×10^5	10% DMSO	21
		Substrate: ascorbate monoanion ($AscH^-$)			
$HO^{\cdot}$	$AscH^-$	7.0	1.28×10^{10}	See text	4
$Br_2^{\cdot-}$	$AscH^-$	7.0	1.1×10^9		4
$I_2^{\cdot-}$	$AscH^-$	7.0	1.4×10^8		4
$(SCN)_2^{\cdot-}$	$AscH^-$	7.0	6.0×10^8		4
$O_2^{\cdot-}$	$AscH^-$	≥8.0	5.0×10^4	pH-dep. 0.1–11	9
$SO_3^{\cdot-}$	$AscH^-$	6.8	9.2×10^6	pH 2–12	27
$SO_5^{\cdot-}$	$AscH^-$	6.7	1.4×10^8		27
$CCl_3OO^{\cdot}$	$AscH^-$	7.0	5.3×10^8	H_2O/iPOH (8:1)	28
$CH_3OO^{\cdot}$	$AscH^-$	7.0	1.8×10^6	40% DMSO	21
α-Tocopheroxyl	$AscH^-$	?	1.55×10^6	H_2O/iPOH/acetone	23
α-Tocopheroxyl	$AscH^-$	?	9.0×10^5	CTAB micelles-EPR	22
α-Tocopheroxyl	$AscH^-$	≥7.0	3.0×10^5[a]	DMPC bilayer; LFP	24
Trolox-$O^{\cdot}$	$AscH^-$	7.0	1.45×10^7	Also thermodynamic data	24
Trolox-$O^{\cdot}$	$AscH^-$	8.5	1.12×10^7	Kinetic modeling	17
Hydroxyaceto-phenone-$O^{\cdot}$	$AscH^-$	9.5	1.7×10^9	Also *iso*-$AscH^-$	25
Trp-$O^{\cdot}$	$AscH^-$	7.0	9.3×10^7	Phosphate buffer	15
VP-16-$O^{\cdot}$	$AscH^-$	8.5	3.5×10^7	Kinetic modeling	Unpublished
Fisetin-$O^{\cdot}$	$AscH^-$	8.5	8.65×10^4	Kinetic modeling	17
Dihydroquer-$O^{\cdot}$	$AscH^-$	8.5	1.6×10^5	Kinetic modeling	17
Rutin-$O^{\cdot}$	$AscH^-$	8.5	1.25×10^6		17
Quercetin-$O^{\cdot}$	$AscH^-$	8.5	4.75×10^6		17
Kaempferol-$O^{\cdot}$	$AscH^-$	8.5	5.2×10^6		17
Luteolin-$O^{\cdot}$	$AscH^-$	8.5	9.9×10^6		17
		Substrate: dehydroascorbic			
Fisetin-$O^{\cdot}$	DHA	8.5	6.9×10^4	Kinetic modeling	17
Rutin-$O^{\cdot}$	DHA	8.5	1.7×10^5	Kinetic modeling	17
Luteolin-$O^{\cdot}$	DHA	8.5	1.65×10^6	Kinetic modeling	17
Quercetin-$O^{\cdot}$	DHA	8.5	1.2×10^7	Kinetic modeling	17

[a]This is the best estimate for the rate constant of ascorbate with the tocopheroxyl radical in a biological membrane.

Table 2 Rate Constants for Formation of Derivatives of Ascorbate Radical from Derivatives of Ascorbate

Radical	Substrate	pH	$k/M^{-1}s^{-1}$	Comment	Ref.
		Substrates: O-methylated derivatives			
$HO^{\cdot}$	1-O-methyl	6.8	2.5×10^{10}		10
$HO^{\cdot}$	2-O-methyl	3.5–6.8	2.7×10^{9}		10
$HO^{\cdot}$	3-O-methyl	6.4–7.9	3.0×10^{10}		10
$HO^{\cdot}$	2,3-O-dimethyl	6.8	4.3×10^{9}		10
$Br_2^{\cdot -}$	1-O-methyl	6.8	3.7×10^{8}		10
$Br_2^{\cdot -}$	2-O-methyl	3.5–6.8	6.1×10^{8}		10
$Br_2^{\cdot -}$	3-O-methyl	6.4–7.9	7.5×10^{7}		10
		Substrates: 6-O-galactosyl derivatives			
$HO^{\cdot}$	6-O-α-gal.	6.5	6.1×10^{9}	Also decay rates	12
$HO^{\cdot}$	6-O-β-gal.	6.8	5.8×10^{9}	Also decay rates	12
$N_3^{\cdot}$	6-O-α-gal.	7.9	3.3×10^{9}	Also decay rates	12
$N_3^{\cdot}$	6-O-β-gal.	7.8	2.7×10^{9}	Also decay rates	12
		Substrates: 6-O-fatty acid derivatives			
α-Tocopheroxyl	6-Caprylate (8)	?	3.0×10^{5}	CTAB micelles; stop-flow EPR	22
α-Tocopheroxyl	6-Laurate (12)	?	7.0×10^{4}	CTAB micelles; stop-flow EPR	22
α-Tocopheroxyl	6-Palmitate (16)	?	3.0×10^{3}	CTAB micelles; stop-flow EPR	22

$Asc^{\cdot -}$. Listed in Table 2 are the rate constants for radical formation of various ascorbate derivatives (10,12,22). When comparing the reactivity of $AscH_2$ with that of $AscH^-$, we see that in nearly every case ascorbate monoanion demonstrates much greater reactivity for electron (hydrogen atom) transfer than the diacid (4,9,21,27,28).

Once formed, $Asc^{\cdot -}$ decays relatively slowly. In simple buffered solutions, the nearly exclusive mode of decay is disproportionation. This somewhat "slow" decay of $Asc^{\cdot -}$ may seem rather unusual, for example, slow compared to decay of $HO^{\cdot}$ or $RO^{\cdot}$. According to the most detailed study by Bielski et al. (7), the stability of $Asc^{\cdot -}$ is proposed to be due to obligatory dimer formation as an intermediate during the disproportionation reaction.

$$2\ Asc^{\cdot -} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (Asc)_2^{2-} \tag{1}$$

$$(Asc)_2^{2-} + H^+ \xrightarrow{k_2} AscH^- + DHA \tag{2}$$

$$(Asc)_2^{2-} + H_2O \xrightarrow{k_3} Asc^- + DHA + OH^- \tag{3}$$

$$\text{Net Reaction:}\quad 2\ Asc^{\cdot -} + H^+ \xrightarrow{k_{obs}} AscH^- + DHA \tag{4}$$

Thus, in the absence of phosphate (discussed later) we have

$$-d\frac{[\mathrm{Asc}^{\cdot-}]}{dt} = -2k_{\mathrm{obs}}[\mathrm{Asc}^{\cdot-}]^2$$

where

$$k_{\mathrm{obs}} = \frac{k_1}{[1 + (k_1/k_2\,[\mathrm{H}^+] + k_3)]}$$

An increase in ionic strength has been observed both to increase and to decrease the rate of dismutation of $Asc^{\cdot-}$ (7,29). However, a most important observation is that phosphate buffer accelerates the dismutation (7). This acceleration is attributed to the ability of various protonated forms of phosphate to donate a proton efficiently to the radical dimer, reaction 2. It is quite possible that other buffers may similarly act as proton donors, thereby accelerating $Asc^{\cdot-}$ dismutation. Figure 2 shows the pH dependence of the rate of $Asc^{\cdot-}$ dimutation. At neutral pH, i.e., pH approximately 7.4, changes in ionic strength have an insignificant effect on this rate. However, the presence of 45 m*M* phosphate buffer shifts this curve up; at pH 7.4 the rate constant increases by a factor of approximately 10.3, from 1.41×10^5 to 1.45×10^6 ($M^{-1}s^{-1}$). As anticipated, derivatization of ascorbate alters the rate of der-$Asc^{\cdot-}$ decay. Considerable prolongation of the der-$Asc^{\cdot-}$ lifetime was observed when the 6-position was elongated by saturated fatty acids (22)—with a concomitant decrease for the scavenging of the α-tocopheroxyl radical by the respective parent compounds. In contrast, 6-substitution with α- or β-galactosyl residues caused a doubling of the decay rate (12).

Pulse-radiolytic studies of ascorbate radical reactions have been mainly concerned with the reactivity toward redox-active substrates (6,9,11,17). As shown in Table 3, the reaction

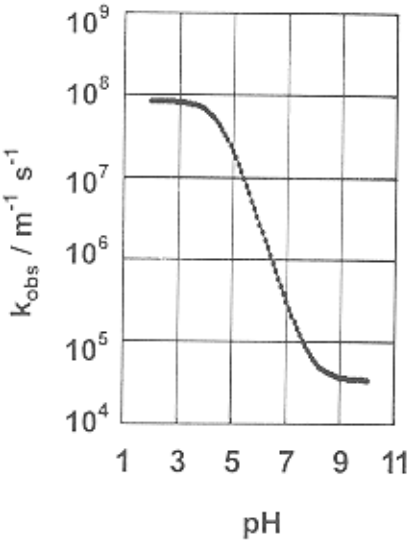

Figure 2 The calculated observed rate constants for the disproportionation of ascorbate radical as a function of pH. These observed rate constants were determined from data presented in Ref. 7.

Table 3 Rate Constants for Ascorbate Radical ($Asc^{\cdot-}$) Reactions

Reactant	pH	$k/M^{-1}s^{-1}$	$E^{\circ\prime}/mV^a$	Ref.
$HO_2^{\cdot}$	0.3–1	5×10^9	1060	9
$O_2^{\cdot-}$	≥8.0	2.6×10^8	950	9
O_2	8.6	$<5 \times 10^2$	−330	6
$Asc^{\cdot-}$	7.4	1.4×10^5	282	7[b,c]
$Asc^{\cdot-}$ in 45 m*M* phosphate	7.4	1.2×10^6	282	7
Ferric/ferrous iron complexes				
$[Fe(N)_6]^{3-}$	7.0	4.0×10^6	425	11
Fe(III)-EDTA	7.0	4.0×10^6	117	11
Cytochrome c (Fe^{3+})	7.4	6.6×10^3	262	6
Cytochrome b_5 (Fe^{2+})	7.0	$<10^4$	0	11
Phenolates				
Dopamine	8.4	3.6×10^2	?	6
Quercetin-O^-	8.5	1.55×10^3	398	17
Fisetin-O^-	8.5	3.85×10^4	214	17
Rutin-O^-	8.5	5.2×10^4	275	17
Luteolin-O^-	8.5	1.55×10^5	299	17
Kaempferol-O^-	8.5	2.8×10^6	209	17
Dihydroquer-O^-	8.5	1.2×10^7	83	17
Cytochrome b_5 reductase: E-$FADH^-$	7.0	4.3×10^6	−147	11
Aroxyl radicals/semiquinones				
Luteolin-$O^{\cdot}$	8.5	1.95×10^6	299	17
Quercetin-$O^{\cdot}$	8.5	1.45×10^7	398	17
Rutin-$O^{\cdot}$	8.5	3.5×10^7	275	17
Dihydroquercetin-$O^{\cdot}$	8.5	4.65×10^7	83	17
Fisetin-$O^{\cdot}$	8.5	7.1×10^7	214	17
Kaempferol-$O^{\cdot}$	8.5	3.4×10^8	209	17
Cytochrome b_5 reductase: E-$FAD^{\cdot-}$	7.0	3.7×10^5	−88	11
Quinoid structures				
Fisetin(=O)	8.5	3.75×10^3	−249	17
Rutin(=O)	8.5	4.1×10^4	−211	17
Quercetin(=O)	8.5	1.15×10^6	−233	17
Luteolin(=O)	8.5	1.65×10^7	−115	17

[a]$E^{\circ\prime}$ is the standard one-electron reduction potential of the reactant at neutral pH.
[b]This rate constant holds over a range of ionic strengths, e.g., 1–200 m*M*, but in the absence of proton donors such as phosphate.
[c]See Fig. 1.

rates are under strong control of the respective one-electron reduction potentials. The standard reduction potential of the ascorbate radical–ascorbate couple at pH 7 is + 282 mV and for the dehydroascorbic acid–ascorbate radical couple is −174 mV (pH-independent) (30–32). Most of the data in Table 3 were obtained by kinetic modeling of pulse-radiolytic data, i.e., calculations of the changes in the kinetics of the formation and decay of the flavonoid aroxyl radical (the only species that could be observed) by changing the concentrations of ascorbate. The iterative optimization program is based on a set of differential equations that is derived from all the pertinent reactions in the system (17). We have also investigated this system by observing either the flavonoid aroxyl or the ascorbate radical by EPR spectroscopy. Using these approaches we could verify that some of the fast radical–radical electron-exchange reactions are reversible on the slower time scale of EPR spectroscopy (Bors W, Michel C, and Stettmaier K, manuscript submitted).

Both $AscH^-$ and $Asc^{\cdot -}$ are considered to be strong reductants (vis-à-vis cytochrome b_5 and NADH-cytochrome b_5 reductase; $Asc^{\cdot -}$ can also be an oxidant) (31). However, ascorbate seems to be unable to reduce disulfides (33); that inability at first may seem inconsistent with its reducing ability. However, the first step of disulfide reduction (a one-electron transfer) would produce the disulfide radical anion. This is a very strong reducing species; the reduction potential ($E^{\circ\prime}$) for the $GSSG/GSSG^{\cdot -}$ couple is −1500 mV; Thus, its formation is thermodynamically difficult (32).

The weak (UV-visible) absorption spectrum of the ascorbate radical (at pH 6.4 λ_{max} = 360 nm, $\varepsilon_{360} = 3{,}300\ M^{-1}\,cm^{-1}$) (7,34) makes it impossible to observe it directly in steady-state experiments, especially in complex biological material. Consequently, EPR spectroscopy of $Asc^{\cdot -}$ is the preferred method for observing it (35). Detailed EPR studies of $Asc^{\cdot -}$ in 1972 (36) and 1973 (37) have accurately established the g-factor (2.00518) and coupling constants (1.76 G, 0.07 G, 0.19 G for a^{H4}, a^{H5}, and a^{H6}, respectively, in aqueous solution) (36) as well as the effect of different solvents (37). Although proton nuclear magnetic resonance (NMR) experiments have suggested a bicyclic structure for the ascorbate radical (38), analogous to that of DHAA (1) of Fig. 1 the EPR parameters are consistent with the structure of the ascorbate radical presented in Fig. 1. Corroborating the pulse-radiolytic and EPR data are quantum mechanical calculations on the optimized geometries of the various radical and ionic intermediates (39) and reaction pathways with hydroxyl radical (40). Both the quasi aromaticity of the radical and the lower pK values as compared to those of the model compound triose reductone are likely explanations of the stability of the ascorbate radical.

In most biological experiments the actual steady-state concentrations of $Asc^{\cdot -}$ observed are ~10^{-7} M, often 10^{-8}–10^{-10} M (41). These low concentrations dictate that for successful detection care must be taken to use the optimal EPR instrument settings, especially for the time resolution needed for kinetic for EPR/$Asc^{\cdot -}$ experiments. We have found that a modulation amplitude of 0.6–0.7 G produces the greatest EPR signal height for $Asc^{\cdot -}$ (42). When using a TM cavity we also have observed that a nominal microwave power of ≈40 mW also maximizes the signal height for $Asc^{\cdot -}$ in room temperature aqueous solutions (42). However, saturation effects begin at ≈16 mW nominal power. Thus, if absolute concentrations of $Asc^{\cdot -}$ are needed, then standardization and quantitation calculations must take into account these possible saturation effects.

The extreme sensitivity of EPR detection of $Asc^{\cdot -}$ coupled with the efficiency of metal catalysis of ascorbate oxidation have been used to estimate iron concentrations in reagents in the range of 1–1000 n*M* (41). Thus, the $Asc^{\cdot -}$ can provide investigators with information on several aspects of a system. But one must always keep in mind that what is being

monitored is $[Asc^{\cdot -}]_{ss}$. A kinetic argument has been made that the dominant route of $Asc^{\cdot -}$ decay will usually be via disproportionation (43). However, the relative stability of $Asc^{\cdot -}$, compared to that of radicals such as $HO^{\cdot}$ and $RO^{\cdot}$, gives a time resolution suitable for kinetic EPR spectroscopy (14,22,44,45,46).

Enzymology

The unusual stability of the ascorbate radical has apparently dictated that enzymatic systems are required to reduce the potential transient accumulation of $Asc^{\cdot -}$. An enzyme, NADH:monodehydroascorbate reductase (EC 1.6.5.4), has apparently evolved for that purpose. It is quite common in plants (47–51), where it plays a major role in stress-related responses. In animal organs it exists predominantly in the retina (52,53). Other sources indicate that the enzyme functions as a transmembrane electron-carrier system (54,55); e.g., in mitochondria and chromaffin granules, its activity depends on the thiol–disulfide redox balance (56). Recently, the presence of a soluble ascorbate radical reductase, which differs from the known membrane-bound enzyme, has been reported (57). In addition, a mitochondrial enzyme has been described, which has distinct properties such as molecular weight and catalytic parameters (58). In a timely report Kobayashi et al. (59) have determined the absolute rate constant of the cucumber ascorbate radical reductase at pH 7.4 to be $2.6 \times 10^8\ M^{-1}s^{-1}$. The fact that in animal tissues this enzyme is far less ubiquitous than in plant tissues has been explained by the predominant role of the glutathione-dependent dehydroascorbate reductase enzyme (EC 1.8.5.1) in controlling the ascorbate–dehydroascorbic redox balance (60). Furthermore, L-DHAA appears to be the major transport form of this vitamin across membranes (61). Thus, these enzymes play a major role in reducing DHAA to $AscH^-$, thereby keeping the antioxidant function of vitamin C operating at maximum efficiency.

The fact that several enzymes aside from ascorbate oxidase (47,62) also take advantage of the univalent redox cycling of ascorbate, i.e., generate ascorbate radicals during their turnover, for example, ascorbate peroxidase (EC 1.11.1.11) (48,63,64), dopamine-β-hydroxylase (EC 1.14.17.1) (62,65), and ascorbate-cytochrome b_5 reductase (EC 1.10.2.1) (66) indicates that "nature" considers this cycle as somewhat innocuous and not prone to severely toxic side reactions.

GENERATION AND REACTIVITY OF $Asc^{\cdot -}$ IN ANTIOXIDANT INTERACTIONS

Despite the fact that the original proposal of Szent-Györgyi and colleagues of certain flavonoids protecting the antiscorbutic effects of ascorbic acid (67–69) did not mention the involvement of ascorbate radicals, a recent study on flavonoid–ascorbate interactions by pulse radiolysis and kinetic modeling confirmed this intermediate as an essential link (17). This study, incidentally, was instigated by another observation of Szent-Györgyi and colleagues (70), where they showed a prooxidant and cytotoxic effect of ascorbate involving univalent reduction of quinones and a concomitant formation of $Asc^{\cdot -}$ by EPR spectroscopy. Because these reactions are evidently controlled by the respective redox potentials, the cytotoxicity of most biologically relevant quinones (e.g., antibiotics) may be partially explained by such reactions, in addition to potential futile redox cycling generating superoxide anion (71–73).

Quite recently, a report on the formation of $Asc^{\cdot -}$ by the reaction of ascorbate with

peroxynitrite, the presumed cytotoxic product of 'NO reacting with $O_2^{\cdot -}$, provided a potential link between these biologically relevant radicals and the vitamin C antioxidant systems (74–76).

$$AscH^- + O{=}NOOH \rightarrow Asc^{\cdot -} + {}^{\cdot}NO_2 + H_2O \quad (5)$$

$$AscH^- + {}^{\cdot}NO_2 \rightarrow Asc^{\cdot -} + NO_2^- \quad (6)$$

where

k_{obs} for reaction 5 at pH 7.4 is 47 $M^{-1}s^{-1}$ (7)

Phenols

One of the most important and intensely investigated reactions with ascorbate is the reduction of tocopheroxyl radicals forming $Asc^{\cdot -}$

$$AscH^- + \alpha\text{-}TO^{\cdot} \rightarrow Asc^{\cdot -} + \alpha\text{-}TOH \quad (7)$$

First observed in a pulse-radiolytic study (23) and since confirmed (22,24,77–80), rate constants for this reaction under various conditions (22–24) as well as EPR evidence of this redox cycling (22,77,79,80) have been reported; see Table 1. Even though superior analogues of α-tocopherol have been chemically synthesized (81–83) and lipophilic derivatives of ascorbate can be nearly as effective (with only a minor decrease in the rate constant with α-tocopheroxyl radical with lengthening of the C6-side chain) (22), the potential biological importance of reaction 7 must not be underestimated.

Because the tocopheroxyl radical is a good reactant for ascorbate, it is easily hypothesized that other phenoxyl radicals could also be reduced with the concomitant formation of $Asc^{\cdot -}$. This was first demonstrated by Schuler (34) and since corroborated for a number of pharmacologically relevant phenols (84–89), such as probucol (86,87) and etoposide (VP-16) (88,89). Yet the fact that certain *p*- (70) and *o*-semiquinones (25) are not reduced by ascorbate again points to the importance of the respective redox potentials of the radical species involved in controlling the reaction.

Thiols

A recurrent problem in the understanding of the interrelationships of biological antioxidative systems is the question of the extent to which the glutathione and the ascorbate redox cycling system may act synergistically in direct chemical reactions (90), in addition to the known interaction in their respective enzyme systems (56,63). Despite evidence to the contrary (89), it seems to be reasonable to expect such interaction, yet it may be difficult to prove as the primary link: the thiyl radical is hard to observe directly and has an altogether too short lifetime. Furthermore, with dihydrolipoic acid (DHLA) as a recently popular alternative thiol reductant (88,89), the thiyl radical, as a free radical, never actually occurs as cyclization to the radical disulfide anion is an extremely rapid intramolecular process ($\geq 1 \times 10^7$ s^{-1} (unpublished data from our laboratory). Whether this distinction between dihydrolipoic acid (DHLA) and reduced glutathione (GSH) is also the basis of the additive reducing effect of $AscH^-$ and GSH toward the etoposide phenoxyl radical (33)—whereas DHLA and $AscH^-$ do show a synergistic effect with both etoposide (88) and probucol phenoxyl radicals (86)—is the basis of ongoing kinetic studies in our laboratory. Since the thiols are capable of reducing the etoposide (88) but not the probucol phenoxyl radical (86), we are interested in whether this reactivity is kinetically or thermodynamically controlled.

ASCORBATE RADICALS IN PLANTS

The ascorbate radical was first observed by EPR spectroscopy over three decades ago. Since then interest in $Asc^{\cdot-}$ has grown. In plants and plant tissue the first reports of altered $Asc^{\cdot-}$ levels from environmental stress appeared in the early 1990s (91,92). Plants have an extensive system to handle ascorbate radicals (93,94) in addition to the redox cycling system mentioned (47–51). In addition to $Asc^{\cdot-}$ formation in response to oxidative stress (47,92,94), or during wounding (50), they may actually use $Asc^{\cdot-}$ formation as a signaling event in development (95). Because both the univalent and bivalent redox cycles of ascorbate are highly intertwined in plants, it augurs well to further explore the EPR technique for such studies.

ASCORBATE RADICAL EPR INTENSITY AS A MEASURE OF OXIDATIVE STRESS

The details of the EPR spectrum of $Asc^{\cdot-}$ in aqueous solutions were understood in 1972/73 (36,37) and soon thereafter studies of $Asc^{\cdot-}$ in cell cultures and animal organs were initiated (96,97). However, almost two decades passed before EPR spectroscopy of $Asc^{\cdot-}$ became an established method in cell and animal studies. It is now recognized that the monitoring of $Asc^{\cdot-}$ formation by EPR is an excellent marker of oxidative stress (98–102). Buettner and Jurkiewicz have provided thermodynamic and kinetic arguments for the use of $[Asc^{\cdot-}]_{ss}$ as a marker of oxidative stress (99). They have detailed some of the experimental considerations needed to allow a reasonable interpretation of the EPR data (41,99,102). In brief, an increase in $[Asc^{\cdot-}]_{ss}$ correlates with an increase in oxidative stress. However, to be able to make such an inference one must control pH and ascorbate concentration in the system. In addition, changes in oxygen levels as well as in catalytic metal activity must be considered. Examples of using the EPR $Asc^{\cdot-}$ signal to gain information on various biological systems follow.*

Cell Cultures

The study of $Asc^{\cdot-}$ in conjunction with cell cultures is proceeding on two fronts: (1) use of changes in $[Asc^{\cdot-}]_{ss}$ to reflect oxidative stress and (2) the possible involvement of $Asc^{\cdot-}$ in cell signaling and cell development.

Oxidative Stress

These investigations have used EPR to monitor $[Asc^{\cdot-}]_{ss}$ when the cell cultures are exposed to different stresses. The cells have ranged from isolated rat hepatocytes (98), to Ehrlich ascites tumor cells (103), L1210 murine leukemia cells (44), and even simple thyroid microsomes (104). Following the change in $[Asc^{\cdot-}]_{ss}$ with time via kinetic EPR spectroscopy can provide very useful information. Figure 3 provides an example of the change in $[Asc^{\cdot-}]_{ss}$ observed in a whole cell incubation of L1210 murine leukemia cells (44). Edelsfosine, an ether lipid anticancer drug that is membrane-active, is introduced at the arrow. Within a minute we observe a dramatic increase in oxidative flux as reflected by a rapid rise in $[Asc^{\cdot-}]_{ss}$. These results demonstrate the rapid response of the cells to this

*A recent study suggests that conjugated cytochrome c, which is not a substrate for either cytochrome c reductase or cytochrome c oxidase and also is not reduced by $O_2^{\cdot-}$, can be used indirectly to monitor $Asc^{\cdot-}$ levels in the circulation of rats (133).

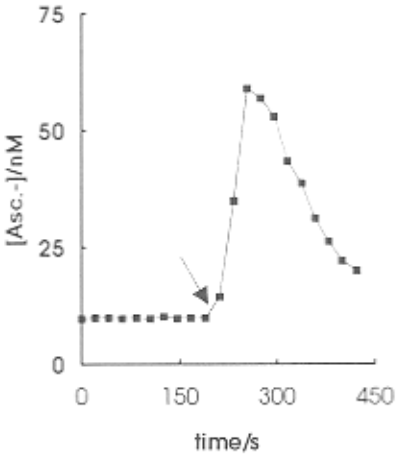

Figure 3 Edelfosine increases oxidative stress in L1210 murine leukemia cell suspensions as reflected by changes in $(Asc^{\cdot-})_{ss}$ (44). Docosahexaenonic acid enriched L1210 cells (5×10^6 cells/ml) were incubated in room temperature 0.9% NaCl solution that contained 20 μM Fe^{2+} and 100 μM ascorbate. Edelfosine 40 μM, a membrane-active ether lipid anticancer drug, was added at the arrow. Vehicle alone produced no significant change in $(Asc^{\cdot-})_{ss}$. EPR spectra were collected with a Bruker ESP 300 electron spin resonance spectrometer.

oxidative stress. The fall of $[Asc^{\cdot-}]_{ss}$ indicates a depletion of some necessary component: oxidizable substrate, ascorbate, or perhaps oxygen. Supporting experiments suggest oxidizable membrane lipids are being depleted (44).

$Asc^{\cdot-}$ and Cell Signaling

Ascorbate radical has been proposed to activate transplasma membrane electron transport systems (95,105–109). Cell-impermeable oxidants that can accept electrons from this electron transport system appear to stimulate cell growth (105). $Asc^{\cdot-}$ is poorly taken up by cells compared to $AscH^-$. Thus, $Asc^{\cdot-}$ appears to serve as an oxidant that can act as an external electron acceptor. $AscH^-$ is regenerated, but cell "signaling" can also occur, resulting in stimulated growth. Data that have been presented suggest this to be the case for HL-60 cells (105,109) and onion root meristems (107,108), two quite different eukaryotic cells.

Body Fluids

It would be ideal to learn something about free radical oxidations from body fluids. When freshly extracted fluids are examined by EPR, $Asc^{\cdot-}$ is quite likely observed. Fresh whole blood gives a very weak $Asc^{\cdot-}$ EPR signal (110). However, when plasma from this same blood is examined, a much stronger, easily detectable signal is observed (79,101,111–116). Fresh serum also yields detectable $Asc^{\cdot-}$ (111,117–119). The much lower $[Asc^{\cdot-}]_{ss}$ seen in whole blood compared to plasma or serum undoubtedly results from the ability of red cells to reduce $Asc^{\cdot-}$. There have been successes in gaining information on the "oxidative state" of plasma. However, careful attention to controls and most importantly the level of ascorbate present must be known (99,102). Other fluids also have $Asc^{\cdot-}$ present that may

provide information. $Asc^{\cdot-}$ in synovial fluid has been used to indicate the presence of catalytic metals (120). $Asc^{\cdot-}$ has been observed in semen and may provide an indication of sperm robustness (121). $Asc^{\cdot-}$ in cow's milk has potential to indicate the state of "freshness" (122,123). In a related application, $Asc^{\cdot-}$ has been used to study potential antioxidant components of infant formulas (123).

In all these examples, it is clear that to gain information from $Asc^{\cdot-}$, the mechanisms by which it may be formed must be understood.

Perfused Tissue/Organs

Changes in ascorbate radical EPR intensity have been observed when rabbit aorta or iliac artery has been subjected to changes in flow (115). These results implied that the endothelium was the principal source of flow-related free radical production. This radical production appears to be another example of the beneficial effects of physiological free radical production.

When isolated rat hearts were subjected to ischemic reperfusion episodes, an increased level of $Asc^{\cdot-}$ was observed in the perfusion buffer (112). The transient increase commenced on reperfusion and was dissipated usually within 30 min. These data were interpreted to indicate that at reperfusion ascorbate, probably mostly as DHAA, was released from the tissue, resulting in an increase in radical production. In similar experiments with isolated rat liver, an increase in $Asc^{\cdot-}$ has also been observed on reperfusion after ischemia (124). Thus, $Asc^{\cdot-}$ has potential to provide information about oxidative events during ischemia–reperfusion episodes.

Care must be exercised in designing and interpreting all experiments where perfusion buffers are used as it has been clearly shown that the heart can extract the adventitious metals present in the buffer. These metals, particularly copper, will alter the experimental results (125). It is best to remove these metals from all buffers used so that the best interpretation of the data is possible (126).

Whole Animals

Two approaches have been used to gain information from $Asc^{\cdot-}$ about oxidative events in whole animals. Both are ex vivo approaches in which fluid is routed from the body via tubing to the EPR for examination.

Mori et al. (127,128) have devised a method to route blood from a femoral artery of a rat through the EPR spectrometer and return it to the animal. This approach monitors $Asc^{\cdot-}$ in whole blood. The researchers observed that $[Asc^{\cdot-}]_{ss}$ increases, as predicted, when ascorbate or iron-citrate is infused into the animal.

At this same time, Sharma et al. (129) developed a similar system to circulate coronary venous blood from dogs through a flat cell in an EPR cavity and return it to the animal. An intravenous infusion pump is used to control the flow rate so that the transit time from heart to EPR is constant. These investigators have observed that $[Asc^{\cdot-}]_{ss}$ is increased on infusion of ascorbate or when metal-mediated oxidative stress is induced by infusion of iron or copper salts.

Of special interest is the observation that infusion of sodium nitroprusside, an antihypertensive drug, produced a dramatic rise in $[Asc^{\cdot-}]$ consistent with the initiation of oxidative stress by this compound. Myocardial ischemia reperfusion experiments demonstrated that on reperfusion after a 20 min ischemic episode the $[Asc^{\cdot-}]_{ss}$ in the blood from the coronary vein increased. This increase was blunted by superoxide dismutase–catalase and by metal

chelators, all consistent with radical formation on reoxygenation of the ischemic myocardium.

Still another approach uses microdialysis to produce an EPR sample for detection of $Asc^{\cdot-}$ (130–132). Microdialysis tubing is placed in the region of interest. The low-molecular-weight ascorbate-system species diffuse into the solution in the tubing and can then be examined by EPR. This approach has been used to monitor oxidative stress in the brain of rats. As predicted, the presence of iron salts or H_2O_2 increased the $Asc^{\cdot-}$ concentration in the dialysate. Other stresses such as cold injury also produced increases in $Asc^{\cdot-}$, indicating the potential of this system to prove oxidative events.

Humans

Investigations on the use of ascorbate radical to probe for human health status are exceedingly limited. Early work used lyphilized tissue samples for EPR examination (96,97). In these preparations, the EPR signals observed were those of the immobilized $Asc^{\cdot-}$. Most interesting was the observation of a very strong $Asc^{\cdot-}$ EPR signal from lyphilized erythrocytes of patients with acute lymphatic leukemia. A completely different EPR spectrum was obtained from erythrocytes of patients with acute myeloid leukemia. The unique spectrum obtained from acute lymphatic leukemia patients was never observed in erythrocytes from patients with other types of leukemia or other diseases of the hematopoietic or lymphatic system.

Recent work with human plasma ascorbate radical has as a goal probing for oxidative stress (112,116). In cardiac ischemia–reperfusion the depletion of plasma ascorbate was monitored via $Asc^{\cdot-}$ using EPR (112). This study suggests that transient changes in plasma ascorbate status induced during cardiac arrest and reperfusion may be a useful clinical marker for oxidative stress This change in ascorbate is reflected in the plasma $[Asc^{\cdot-}]_{ss}$.

Another study of $Asc^{\cdot-}$ in human plasma was directed toward monitoring intensive care patients with sepsis (116). The study found that sepsis patients have significantly more catalytic iron in serum than healthy control persons, while ascorbate in sepsis patients was less than in control patients. Yet $[Asc^{\cdot-}]_{ss}$ was nearly the same. However, a 1 g bolus injection of ascorbate to sepsis patients resulted in only a minor increase in $[Asc^{\cdot-}]_{ss}$ and plasma ascorbate, as compared to those of control patients. These results suggest that quite different ascorbate metabolism occurs in the sepsis patient. How to interpret this with respect to improvement of patient health is not yet known, but it suggests that quite a difference in ascorbate metabolism exists between healthy control and sepsis patients.

Skin

One of the most informative uses of $Asc^{\cdot-}$ has been in studies of skin. The first investigation of $Asc^{\cdot-}$ in this organ (SKH-1 hairless mouse) demonstrated that UV light increased endogenous $[Asc^{\cdot-}]_{ss}$ and that in skin treated with chlorpromazine, a photoactive drug, UV light produced an even greater increase in $[Asc^{\cdot-}]_{ss}$ (133). In complementary experiments using the EPR spin trapping agent α-(4-pyridyl 1-oxide)-*N*-*tert*-butylnitrone (POBN) applied to the skin, a carbon-centered radical presumably from lipid peroxidation products was detected. This radical signal was blunted when an iron chelating agent (Desferal) was applied prior to UV exposure (134). These results demonstrate that UV light does indeed produce free radicals in skin and that iron present in the skin may exacerbate the free radical oxidative stress.

If membrane-derived free radicals are involved in the deleterious effects of UV light on skin, then tocopherol-based antioxidants may reduce this damage. Indeed, Jurkiewicz et al. (135) in the SKH-1 mouse model have used EPR detection of $Asc^{\cdot-}$ as well as POBN spin trapping to demonstrate that topical application of tocopherol sorbate reduces UV light-mediated free radical formation. But most exciting is that they have also demonstrated that tocopherol sorbate protects against photoaging and UV light–induced skin tumor formation. This study is the first actually to correlate photoaging and UV light–induced tumor formation with free radical production. Thus, the development of effective antioxidants and the methods to deliver them to the skin for protection against UV light is well justified.

Timmons and Davies (136) have used these techniques to demonstrate that application of organic peroxides to murine skin results in an increase in the endogenous $Asc^{\cdot-}$ EPR signal. This radical formation requires that the peroxide penetrates the skin stratum corneum. These observations are the first steps needed to correlate peroxide-induced free radical formation with its deleterious effects in skin tissue.

SUMMARY

$Asc^{\cdot-}$ is both thermodynamically and kinetically a very domesticated free radical. It is a pi radical with low reactivity. Thus, it is an ideal radical to be formed as a product when ascorbate, a donor antioxidant, reacts to repair dangerously oxidizing radicals, such as peroxyl and alkoxyl radicals, and less dangerous, but potentially bothersome, phenolic radicals, such as those from flavonoids and tocopherol. Its low reactivity results in a "relatively" long lifetime, making it an ideal marker for ongoing free radical oxidations in solutions, cells, tissues, and even whole organisms. Perhaps one of the most powerful aspects of its use as a marker of oxidative stress is that it can provide information on the system in real time (99,102).

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