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Chemistry and Biochemistry of Ascorbic Acid

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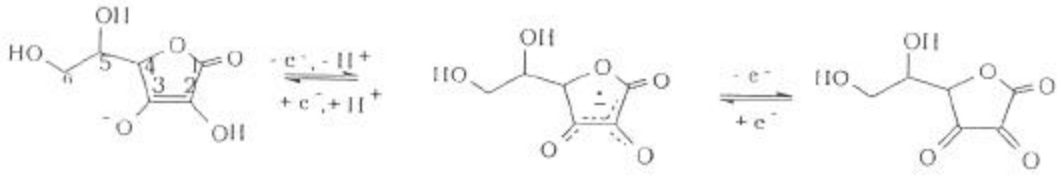
I. INTRODUCTION

Ascorbate is ubiquitous, yet there is still much to be learned about its chemistry, biochemistry, and biology. For the vast majority of mammals, ascorbic acid is not a vitamin because their liver enzymes have the ability to convert glucose to ascorbic acid. However, it is a vitamin for a fruit-eating bat from India, the guinea pig, and some primates, including humans. We are dependent on adequate intake of ascorbic acid for prevention of scurvy and for overall well-being. The lack of fresh foods in our diets may lead to scurvy. For example, many early sailors were lost at sea due to deficiency of vitamin C in their diet (1–3).

There were several early attempts to isolate this essential water-soluble antiscorbutic factor. It was not until 1922 that Albert Szent-Györgyi isolated a white crystalline substance from the adrenal cortex of cows, which he first believed to be a new adrenal hormone. He named this substance *hexuronic acid* and went on to demonstrate that this substance was vitamin C. He later discovered that Hungarian paprika was a much better source of this vitamin, and renamed the substance *ascorbic acid* because it prevented scurvy. In 1937 Szent-Györgyi was awarded the Nobel Prize for medicine "in recognition of his discoveries concerning the biological oxidation processes, with special reference to vitamin C" (1,2).



AscH⁻
I

Asc•⁻
II

DHA
III

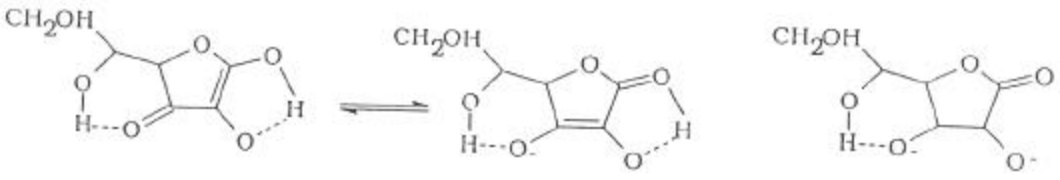
II. PHYSICAL–CHEMICAL PROPERTIES

Ascorbate (**I**) has a most unusual structure in that it has at its chemical core a five-membered lactone ring; on this ring is a bifunctional ene-diol group with an adjacent carbonyl group. This conjugated structure gives ascorbate a very rich chemistry. Ascorbate is an excellent reducing agent. The loss of one electron results in the semidehydroascorbate radical (**II**) (alias, monodehydroascorbate), a very stable π-radical with the unpaired electron delocalized over the highly conjugated tricarbonyl system. Loss of a second electron yields dehydroascorbic (**III**).

A. Structure and pKs

Ascorbic acid is a diacid. Its acidity ($pK_1 = 4.25$) exceeds that of weak carboxylic acids such as acetic acid ($pK_a = 4.75$) (4). This acidity is a result of the resonance stabilization of the monoanion form (**I**). Loss of a proton from the hydroxyl oxygen at the 3-position results in two possible resonance structures, **IV** and **V**, which distribute the negative charge between the oxygens at the 1- and 3-positions of the ring. Also, intramolecular hydrogen bonding results in additional stabilization of the monoanion (5).

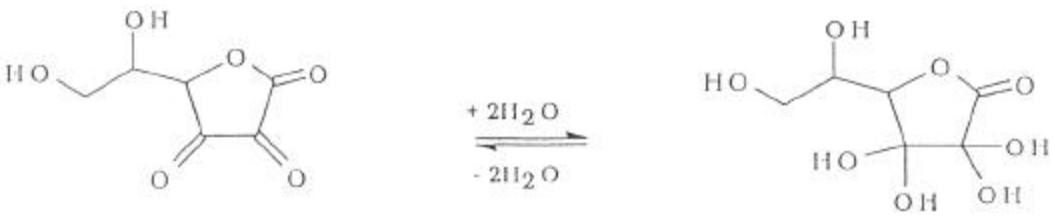
Ascorbic acid is to our knowledge unique in that the pK_2 (11.8) (4) of its diol is far removed from its pK_1. This is easily rationalized; the loss of the two stabilizing resonance structures, **IV** and **V**, to form **VI** results in a structure with additional negative charge to stabilize, yet there is a loss of intramolecular hydrogen bonding and thus less ability to delocalize the negative charge. Thus,



AscH⁻
IV

AscH⁻
V

Asc2-
VI



DHA
III

DHAA
VII

at pH 7.4, ≈99.9% of ascorbate will be present as the monoanion, with only ≈0.06% present as the diacid and ≈0.01% as the ascorbate dianion.

The ascorbate free radical is a strong acid, having a pK_a of −0.86 (6). Thus, it will exist as a monoanion over the entire biological pH range.*

DHA has a pK ≈ 9, however, structure **III** has no ionizable OH groups and therefore is really not an acid. Hydration of **III** at the C2 and C3 positions will result in the weakly acidic dehydroascorbic acid **VII**:

Thus **III** is usually referred to as dehydroascorbic, while **VII** is dehydroascorbic acid (DHAA) (3).

Б. Absorption Spectra

Pure ascorbic acid solutions are colorless as neither the diacid nor the monoanion has significant absorbance 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 $\epsilon_{244} = 10{,}800\ M^{-1} \cdot cm^{-1}$ in aqueous solution (3).
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 7,500 to 20,400 $M^{-1} \cdot cm^{-1}$ (3). We find that $\epsilon_{265} = 14{,}500\ M^{-1} \cdot cm^{-1}$ best reflects our experimental observations when doing experiments in near-neutral buffered aqueous solutions (7).
3. Ascorbate radical: The ascorbate free radical has an approximately sym-

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

metrical Gaussian-shaped absorption curve with $\epsilon_{360} = 3{,}300\ M^{-1} \cdot cm^{-1}$ and a half-width at half-maximum of about 50 nm (8).

4. Dehydroascorbic: Dehydroascorbic (acid) has a weak absorption at 300 nm, $\epsilon_{300} = 720\ M^{-1} \cdot cm^{-1}$ (3).

C. Solubility

Ascorbate is extremely soluble in water, ≈ 1 g dissolves in 3 ml water. It is insoluble in nonpolar organic solvents such as benzene, petroleum ether, fats and their solvents (9).

III. CHEMISTRY

A. Ascorbate: The Terminal Small-Molecule Antioxidant

Ascorbate is an excellent reducing agent. It readily undergoes two consecutive, yet reversible, one-electron oxidation processes to form the ascorbate radical ($Asc^{\bullet -}$) as an intermediate (see Sec. II). Because $Asc^{\bullet -}$ has its unpaired electron in a highly delocalized π-system, it is a relatively unreactive free radical. These properties make ascorbate a superior biological donor antioxidant (10–23).

Table 1 One-Electron Reduction Potentials at pH 7.0 for Selected Radical Couples

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

Source: Adapted from Refs. 14, 17, 25.

When biological fluids or tissues are examined by electron paramagnetic resonance spectroscopy (EPR), $Asc^{\bullet -}$ will most likely be observed (see Fig. 5). This is consistent with ascorbate's role as the terminal small-molecule antioxidant (17,24).

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 $HO^{\bullet}$, $RO^{\bullet}$, $LOO^{\bullet}$, $GS^{\bullet}$, urate, and even the tocopheroxyl radical ($TO^{\bullet}$), can be repaired by ascorbate. Therefore, we have:

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

where $X^{\bullet}$ is 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 damaging radical ($X^{\bullet}$) is replaced by the domesticated $Asc^{\bullet -}$. $Asc^{\bullet -}$ does not

Table 2 Rate Constants for Reaction of Equilibrium Mixture of $AscH_2/AscH^{-}/Asc^{2-}$ at pH 7.4 (unless otherwise noted)

Radical	k_{obs} ($M^{-1}s^{-1}$)	Ref.[a]
$HO^{\cdot}$	1.1×10^{10}	26
$RO^{\cdot}$(*tert*-butyl alkoxyl radical)	1.6×10^{9}	27
$ROO^{\cdot}$ (alkyl peroxyl radical, e.g., $CH_3OO^{\cdot}$)	$1–2 \times 10^{6}$	28
$Cl_3COO^{\cdot}$	1.8×10^{8}	29
$GS^{\cdot}$ (glutathiyl radical)	6×10^{8}(pH5.6)	30, 31
$PUFA^{\cdot}$	—[b]	
$UH^{\cdot -}$ (urate radical)	1×10^{6}	32
$TO^{\cdot}$ (tocopheroxyl radical)	2×10^{5}[c]	17
$Asc^{\cdot -}$ (dismutation)	2×10^{5}[d]	8
$CPZ^{\cdot +}$ (clorpromazine radical action)	1.4×10^{9}(pH5.9)	33
Fe(III)EDTA/ Fe(II)EDTA	10^{2}[e]	
$O_2^{\cdot -}/HO_2^{\cdot}$	1×10^{5}[e]	34, 35
	2.7×10^{5}	36
Fe(III)DFO/ Fe(II)DFO	Very slow	37, 38

[a] A complete summary of free radical solution kinetics can be found in Ref. 39.
[b] We were unable to find data that addresses this reaction directly.
[c] Estimated k_{obs} for $TO^{\cdot}$ when in a biological membrane.
[d] k is pH dependent, thus this is k_{obs} at pH 7.4.
[e] Estimated from data in Refs. 17, 40, 41.

react by an addition reaction with O_2 to form dangerous peroxyl radicals. Ascorbate (probably Asc^{2-}, vida infra) and/or $Asc^{\bullet -}$ appear to produce very low levels of superoxide (42,43). But, by removing $O_2^{\bullet -}$, superoxide dismutase provides protection from this possibility (44,45). Thus, the biological organism is protected from further free radical–mediated oxidations. In addition, $Asc^{\bullet -}$ as well as dehydroascorbic can be reduced back to ascorbate by enzyme systems, vida infra. 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.

B. Autoxidation

Before beginning this discussion it must be understood that we use the term *autoxidation* to mean oxidation in the absence of metal catalysts (46). 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 (7,37,38,40,41,47–50). The diacid is very slow to oxidize. Consequently, at low pH (<2 or 3), ascorbic acid solutions are quite stable, assuming catalytic transition metal ions are not introduced into the solutions. However, as the pH is raised above pK_1, $AscH^-$ becomes dominant and the stability of the ascorbate solution decreases. This loss of stability is usually the result of the presence of adventitious catalytic metals ($\sim$ 1 μM) in the buffers and salts that are typically employed in studies at near neutral pH (7). For example, we have found that in room temperature aerated, aqueous solutions at pH 7.0 (50 mM phosphate buffer), 10–30% of 125 μM ascorbate is lost in just 15 min. This large variation is the result of different sources and grades of phosphate used in the buffer preparation. However, if care is taken to remove these trace levels of transition metals, this rate of loss can be lowered to as little as 0.05% per 15 min (7), 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 6×10^{-7} s^{-1} under our experimental conditions (7). However, even in carefully demetaled solutions, as the pH is varied, the rate of oxidation increases (Fig. 1) (24).

We attribute this 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 (43):

$$Asc^{2-} + O_2 \xrightarrow{k \approx 10^2\ M^{-1} s^{-1}} Asc^{\bullet -} + O_2^{\bullet -}$$

Our experimental results are consistent with these estimates (7,24,38). Marcu theory would predict that the true autoxidation of $AscH^-$ would be muc

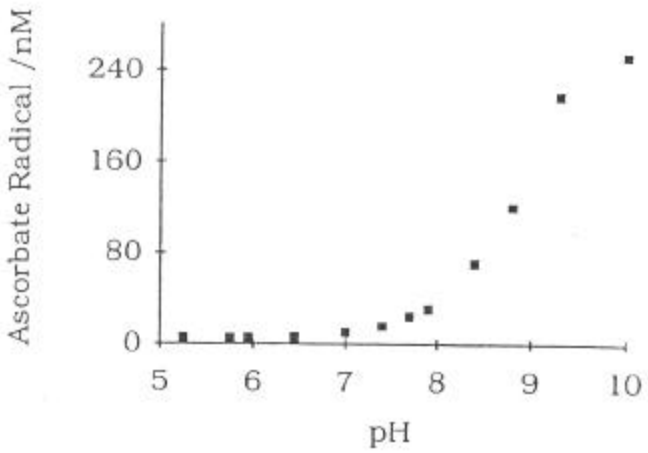


Figure 1 Background [$Asc^{\bullet -}$] *vs.* pH: Each solution was made with 50 mM demetalled phosphate buffer that contained 50 μM desferoxamine mesylate, for at least 12 h. To these solutions 500 μM ascorbate was added and the EPR spectra were collected. The points represent the $Asc^{\bullet -}$ concentration observed in the second of three EPR scans, where the values had a standard deviation of less than 1 nanomolar [adapted from (24)].

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

Typical buffers employed in biochemical and biological research have ~ 1 μM iron and <0.1 μM copper. 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 (7). We have developed two assays that take advantage of this chemistry.

1. 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 attention to detail to ensure that all glassware, pipets, and pipet tips are scrupulously clean, we can estimate iron levels in phosphate buffer to a lower limit of $\approx$100 nM using UV-Vis spectroscopy (7). However, using EPR spectroscopy this limit can be as low as $\approx$5 nM (38) (Figs. 2,3). 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 then introduce ascorbate and determine by EPR the steady-state concentration (i.e., signal height) of $Asc^{\bullet -}$. From a standard curve we can then estimate the iron concentration from $\approx$5 nM to $\approx$10 μM. To achieve estimates at the lowest end of this range, extreme care must be taken with each step and the EDTA must be pure—recrystalized 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 (7). This method is useful even if there is

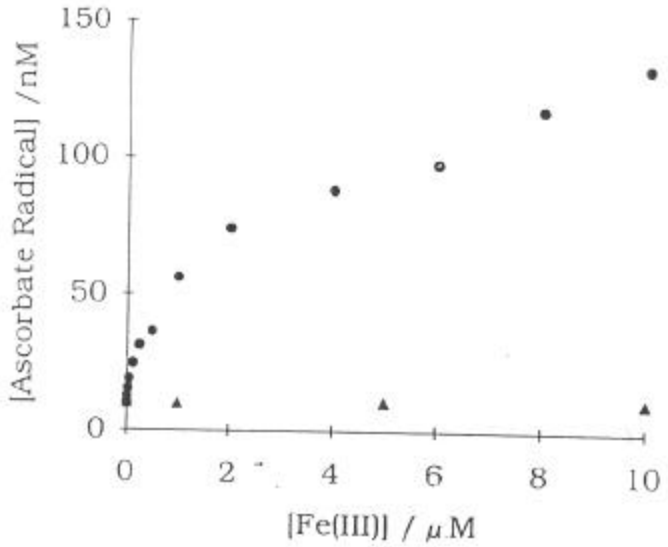


Figure 2 These data were gathered using EPR spectroscopy to quantitate the steady-state level of $Asc^{\cdot -}$. The curves were obtained in 50 mM demetalled phosphate buffer, pH 7.40 with 250 μM EDTA (•) or 50 μM Desferal (▲) with 125 μM ascorbate present [adapted from (38)].

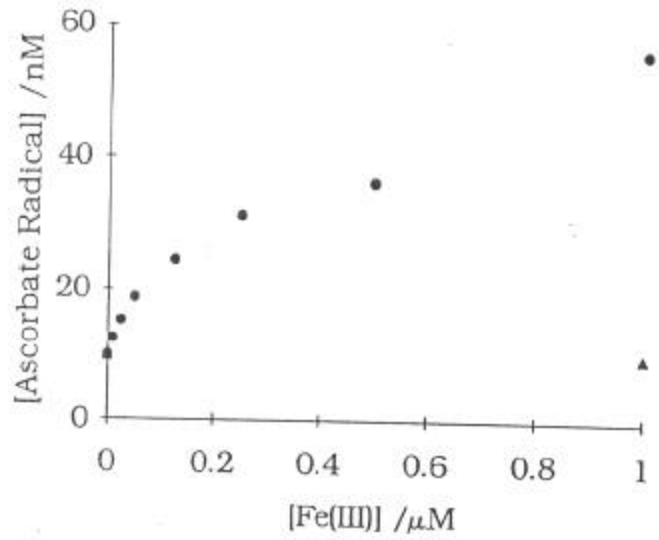


Figure 3 This figure is an expansion of the 0–1 μM [Fe(III)] region of Figure 2. The experimental conditions are the same as in Figure 2. The curves were obtained in 50 mM demetalled phosphate buffer, pH 7.40 with 250 μM EDTA (•) or 50 μM Desferal (▲) with 125 μM ascorbate present [adapted from (38)]

interference in standard colorimetric assays of iron or if only "loosely bound" iron is to be estimated (51).

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.

2. Removal of Trace Metals

We have also found (7,52) 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 ≈3.5 μl 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 ≈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.)

3. Stock Ascorbate Solutions

In our work with ascorbate we have found that the quality of the stock solution determines the quality and reproductivity of the results (7). We prepare ascorbate stock solutions using only the diacid. It is prepared as a 0.100 M stock solution (10 ml) using high-purity water. This solution is colorless, having a pH of ≈2. It is stored in a volumetric flask with a tight-fitting plastic stopper, thus oxygen is kept from the solution during long-term storage. As the solubility of oxygen in air-saturated water is ≈0.25 mM, the solution will become anaerobic with loss of <1% of the original ascorbate. If the flask is indeed clean, we have found that the solution can be kept for several weeks without significant loss of ascorbate due to the low pH and lack of oxygen. The appearance of a yellow color is an indication of ascorbate deterioration. We avoid the use of sodium ascorbate as it invariably contains substantial quantities of oxidation products as evidenced by the yellow color of the solution.

C. Equilibrium

The ascorbate free radical will be present in solutions due to both the autoxidation and the metal catalyzed oxidation of ascorbate. Foerster et al. observed that $Asc^{\bullet -}$ can also arise from comproportionation of $AscH^-$ and DHA (53):

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

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

Using EPR, they determined the equilibrium constant for this process and noted that it was pH dependent. The equilibrium constant K was found to vary from 5.6×10^{-12} at pH 4.0 to 5.1×10^{-9} at pH 6.4. Later, after the acid-base properties of ascorbic acid and ascorbate free radical were understood, it was then possible to develop an expression for K at any pH value (8):

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

where pK_1 is the first ionization constant of ascorbic acid and $[AscH_2]_{total} = [AscH_2] + [AscH^-]$.

D. Singlet Oxygen

Singlet oxygen undergoes an addition reaction with ascorbic acid (diacid) at C2 and C3 in cold methanol (−80°C) to form hydroperoxide products (54). However, in room temperature/neutral aqueous solutions, singlet oxygen oxidizes ascorbate to produce H_2O_2 (55,56). In fact, it has been found that one-half the oxygen consumed appears as H_2O_2. Thus, even though ascorbate efficiently quenches $^1O_2$ [$k = 8 \times 10^6\ M^{-1}s^{-1}$ (57–59)], this chemical quenching brings about the formation of another reactive oxygen species, H_2O_2. In the presence of iron, even at trace levels, this H_2O_2 will be reductively cleaved to form $HO^{\cdot}$ (56), which is perhaps the most oxidizing species that can arise in a biological setting. Consequently, quenching of singlet oxygen by ascorbate may not necessarily be of great benefit to an organism.

IV. BIOCHEMISTRY

A. Antioxidant and Prooxidant Properties

As discussed above, ascorbate is an excellent antioxidant. We consider it to be the terminal small-molecule antioxidant in biological systems. However, ascorbate is also widely used as a prooxidant (55,56,60–70). This paradoxical behavior results because it is an excellent reducing agent. As a reducing agent it is able to reduce catalytic metals such as Fe^{3+} and Cu^{2+} to Fe^{2+} and Cu^{+}. The redox cycling of these metals is essential to the oxidation of the vast majority of singlet state organic molecules (46). For the majority of organic molecules true autoxidation is negligible because of the minimum 23 kcal/mol activation energy required to overcome the spin restriction of dioxygen. However, redox active metals can overcome this spin restriction and thereby catalyze oxidations of otherwise stable organics. In the presence of oxygen the reduced metal can initiate these oxidation processes (46).

In general low concentrations of ascorbate are required for prooxidant conditions, while high concentrations are needed for antioxidant conditions. Thus,

there is a "crossover" effect (19); low and high are relative. In the literature there is a wide range of concentrations of ascorbate where this crossover from pro- to antioxidant action occurs (19,62,71–75). The crossover effect can be rationalized as follows: in the presence of ascorbate, catalytic metals will initiate radical chain oxidations. Because of the antioxidant chemistry of ascorbate, when the ascorbate concentration is high the chain length of these radical processes will be small. Thus, little damage is done. However, as the ascorbate concentration is lowered, the initiation processes are slowed somewhat, but more importantly the rate of the antioxidant reactions of ascorbate will be slowed, thus the radical reaction chain length will be longer and more oxidative damage will occur. We propose that the variability observed in the literature for the crossover effect is a result of the variability in the concentration and form of the catalytic metals present. Thus, at very low levels of catalytic metals, ascorbate will always serve as an antioxidant. However, if the levels of available metals should increase, then ascorbate could be dangerous. In fact, for patients suffering from iron overload, it would be expected that supplemental ascorbate would be detrimental.

B. $Asc^{\bullet -}$ as a Marker of Oxidative Stress

The ascorbate free radical is naturally detectable by EPR at low steady-state levels in biological samples, such as leaves from crops (76), plasma (22,67,75), synovial fluid (78), and skin (79,80). As oxidative stress increases in a system, the steady-state $Asc^{\bullet -}$ concentration increases. These findings are consistent with ascorbate's role as the terminal small-molecule antioxidant (see Table 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 (24,81). Based on our observations, we examined those parameters that would need to be considered to accurately use the ascorbate radical as a marker, such as pH and concentration (24).

The ascorbate radical as a marker of oxidative flux has been shown to be useful in the study of free radical reactions in many biological systems including mouse skin (79,80,82), hepatocytes (83), and ischemia reperfusion of hearts (84–86).* Human sera and rat plasma intoxicated with paraquat and diquat, known superoxide generators, have increased ascorbate radical levels (89). In animal experiments, sepsis has also been shown to increase $Asc^{\bullet -}$,

*In a quite different approach, Pietri et al. (87,88) have used $Asc^{\bullet -}$ as a probe for plasma ascorbate concentrations. In their approach, a 1:1 mixture of plasma and dimethylsulfoxide is examined for $Asc^{\bullet -}$ by EPR. They claim that the $Asc^{\bullet -}$ is an index of the transient changes in plasma ascorbate status during ischemia/reperfusion, whereas in our studies the $Asc^{\bullet -}$ levels reflect the ongoing free radical flux in the system being examined (24,80,86).

indicating the involvement of oxidative stress with this health problem (90). Sasaki et al. have investigated in human serum the use of $Asc^{\bullet -}$ 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 (91–95). Consequently, the ascorbate radical level in biological systems may be useful for monitoring free radical reactions in vivo, particularly when free radical production is low and other methods are insensitive.

C. Enzyme Recycling

There appear to be two ascorbate-regenerating enzymes: a monodehydroascorbate reductase (EC 1.6.5.4) that reduces $Asc^{\bullet -}$ back to $AscH^-$ and a dehydroascorbate reductase (EC 1.8.5.1) that reduces the two-electron deficient DHA back to $AscH^-$ (23,96–104). These enzymes appear to be quite labile as there has been only moderate success with their isolation and purification. The most progress in research on these enzymes has been made with plant systems (97).

Monodehydroascorbate reductase activity has been shown to be present in the outer membrane of mitochondria, microsomes, coated vesicles, and Golgi apparatus of mammalian cells. In the pioneering work on ascorbate radical chemistry from the University of Giessen, it has been reported that the apparent Michaelis constant for the monodehydroascorbate reductase from liver microsomes is approximately 4 μM (94,104). Monodehydroascorbate reductase from spinach is a flavoprotein, with one FAD/molecule. It is a monomer with $M_r = 47{,}000$ (97). It can use either NADH or NADPH, but NADH appears to be the better electron donor. The K_m values for NADH, NADPH, and the $Asc^{\bullet -}$ are 4.6, 23, and 1.4 μM, respectively.

$$2Asc^{\bullet -} + NADH + H^+ \xrightarrow{\text{Monodehydroascorbate Reductase}} NAD + 2AscH^-$$

Dehydroascorbate reductase has also been purified from spinach (97). The purified reductase in a single protein with no flavin or heme present. It is also a monomer with $M_r = 23{,}000$ and apparent K_m values of 2.5 and 0.07 mM for GSH and DHA, respectively (97). This work in plants suggests that the $Asc^{\bullet -}$ reductase may be the primary enzymatic means of $AscH^-$ regeneration (97). Work with human lens suggests a similar importance in mammals (99).

$$DHA + 2GSH \xrightarrow{\text{Dehydroascorbate Reductase}} NAD + 2AscH^-$$

D. Collagen Formation

Ascorbate is a reducing agent in hydroxylation reactions, as in the conversion of proline and lysine residues of collagen to hydroxyproline and hydroxylysine.

For proline, the enzyme prolyl hydroxylase is involved in its transformation, which has iron at its active site. By reducing the iron atom to its ferrous state, ascorbate maintains the enzyme in its active form. Collagen synthesized in the absence of ascorbate is insufficiently hydroxylated and hence cannot properly form fibers. This results in the skin and blood vessel fragility that is prevalent in scurvy (105). In addition to hydroxylating proline and lysine, ascorbate is involved in the formation of norepinephrine, serotonin, homogentisate, and carnitine.

E. Vitamin E Recycling

The first experimental evidence that ascorbate enhances the antioxidant action of vitamin E was gathered at The University of Iowa in the 1940s in a study of the antioxidant action of tocopherol on oils (106). Since this first observation, much work has been done to understand thermodynamically and kinetically the mechanism of this phenomenom (17,18,22,24,107). We now know that because ascorbate is at the bottom of the pecking order for oxidizing free radicals, it will serve as a donor antioxidant to repair each of the oxidizing radicals above it. The tocopheroxyl radical has a reduction potential of around +500 mV, thus it too will be repaired by ascorbate, $E^{\circ\prime} = +282$ mV (Table 1). However, ascorbate is water soluble, while tocopherol is lipid soluble. Thus, at first glance it would appear that in a biological setting there would be little or no interaction between these species. However, the phenol group of tocopherol, which is the basis of its antioxidant action, appears to be located at the water/membrane interface of a biological membrane (108–111) (Fig. 4).

This physical arrangement allows easy reaction between the tocopheroxyl free radical, $TO^{\bullet}$, and $AscH^{-}$:

$$TO^{\bullet} + AscH^{-} \xrightarrow{k = 2 \times 10^5\ M^{-1}s^{-1}} TOH + Asc^{\bullet -}$$

Although there are many measurements of the rate constant for this reaction in a variety of conditions and solvent systems, we believe that $k = 2 \times 10^5\ M^{-1}s^{-1}$ is the best estimate for this reaction when TOH is in a biological membrane (17).

Both the thermodynamics and kinetics of the $TO^{\bullet}$-ascorbate reaction are consistant with ascorbate being at the bottom of the pecking order for free radical reactions, not only in aqueous environments, but also in biological membranes. The reaction of $TO^{\bullet}$ with ascorbate is an avenue for the export of oxidative free radicals from the membrane. Thus, TOH protects the membrane by stopping the propagation reactions of lipid peroxyl radicals, while ascorbate protects the

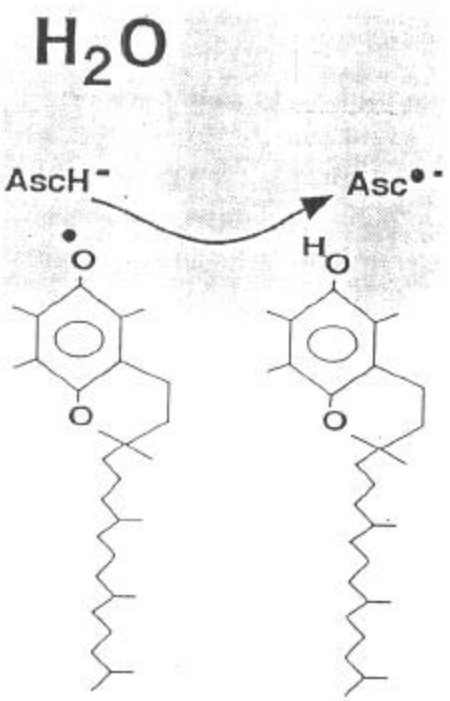


Figure 4 The phytyl tail of tocopherol lies parallel to the fatty acid acyl chains of the phospholipid components of biological membranes (110). The polar nature of the phenol group of TOH results in this part of the molecule being at or near the water-membrane interface. Thus, after the formation of $TO^{\bullet}$, ascorbate has easy access to repair, i.e., recycle, vitamin E.

membrane against the possible prooxidant reactions of $TO^{\bullet}$ (112,113), simultaneously recycling vitamin E and protecting the membrane from $TO^{\bullet}$. This process is well established in liposomes and experimental membrane preparations. However, the importance of this process in cells and tissues is not yet known.

V. DETECTION AND QUANTITATION METHODS

A. EPR Detection of the Ascorbate Free Radical

The ascorbate free radical is usually detected by EPR as a doublet signal with $a^{H} = 1.8$ G, $\Delta H_{pp} \approx 0.6$ G, and $g = 2.0052$ (Fig. 5). However, each line of the ascorbate doublet is actually a triplet of doublets: $a^{H4} = 1.76$ G, $a^{H6}(2) = 0.19$ G, and $a^{H5} = 0.07$ G (114).

In most biological experiments where the $Asc^{\bullet -}$ 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

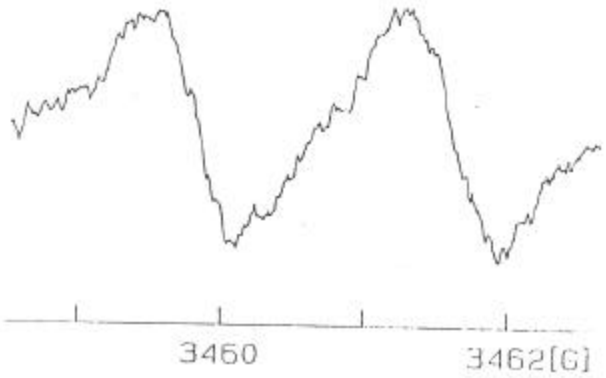


Figure 5 Ascorbate radical doublet EPR signal in mouse skin. A section of Skh-1 hairless mouse skin was placed into the well of an EPR tissue cell. The skin was exposed to UV light while in an EPR TM cavity. See (80) for details.

find that a modulation amplitude of ≈0.65 G maximizes the ascorbate free radical doublet peak-to-peak signal amplitude (115).

The EPR power saturation curve of $Asc^{\bullet -}$ in room-temperature aqueous solutions shows that saturation effects begin at ≈16 mW and maximum signal height is achieved at 40 mW nominal power when using an aqueous flat cell and a TM cavity (115). Thus, if quantitation of the $Asc^{\bullet -}$ levels is desired, appropriate corrections for saturation effects must be included in the calculations.

B. Quantitation of Ascorbate

1. Absorbance

Ascorbate has a relatively strong UV absorbance: $\epsilon_{265} = 14{,}500\ M^{-1} \cdot cm^{-1}$ (7). Thus, in principle it is possible to directly observe its presence over a range of ≈5 μM to ≈140 μM in near neutral aqueous environments. Unfortunately, in biochemical and biological settings, an immense number of substances absorb in this same wavelength region, making direct UV detection unfeasible. However, in simple chemical systems direct UV detection can be used to great advantage.

2. HPLC

Although many methods exist for monitoring ascorbic acid in biological fluids, high-performance liquid chromatography (HPLC) offers both simplicity and high sensitivity, making it a frequently used method for routine analyses. In HPLC the analytes are separated by column chromatography and quantitated using postcolumn detectors. The two most commonly applied detection techniques are ultraviolet and electrochemical detection. HPLC with UV detection is probably the easiest system for measuring ascorbic acid in biological systems

(116). UV detection is possible because of ascorbate's optical absorbance spectrum, which has peaks at 244 or 265 nm, depending on solvent and pH. Absorption at 254 nm is approximately 80% of the maximum (3). Usual limits of sensitivity for this approach are 50 pmol/injected sample for both standards and biological samples (116).

HPLC has been used to measure ascorbic acid in numerous biological samples, such as lymphocytes (117), urine (118), aqueous humor (119), and breast tissue (120). When extracting ascorbate from biological samples, antioxidants should be added to increase stability. However, antioxidants may not only stabilize ascorbate, but can also convert dehydroascorbic to ascorbic acid, resulting in the measurement of both reduced and oxidized forms. Thus, we recommend using addition methodology, where a known amount of ascorbate is added to serve as an index of depletion during sample processing. These techniques, as well as other assays for analysis of ascorbic acid and dehydroascorbic acid in biological samples, are reviewed extensively by Wasko et al. (116).

3. *Fluorescence*

The detailed method we present here is our adaptation and modification of that presented by Brubacher and Vuilleumier (121). This method of analysis for total ascorbate ($AscH^-$) is based on the condensation of dehydroascorbic (DHA) with *o*-phenylenediamine and the subsequent fluorescence detection of the product. Prior to introduction of *o*-phenylenediamine, reduced ascorbate is oxidized to DHA by ascorbate oxidase (Asc Ox). Thus, both ascorbate and DHA can be determined if ascorbate oxidase is omitted from one of the paired samples. The actual plasma concentration is determined by the method of standard additions—10 and 20 μM here.

Detailed Method

To assay for $AscH^-$ and DHA in fresh plasma:

1. To 2.00 ml of plasma, add 4.00 ml of a 5% meta-phosphoric acid solution (in 13 × 100 mm disposable glass culture tube)
2. Vortex and centrifuge at 3000 g for 5 min to yield a clear supernatant
3. Into each of 4 glass test tubes, pipette 1.00 ml of supernatant (12 × 75 mm disposable glass culture tubes, labeled A, B, C, and D)
4. To each tube, A–D, add reagent as follows:

   A. (DHA): +1.30 ml acetate buffer
   B. (Total ascorbate): +1.30 ml Asc Ox solution
   C. (Internal STD, 10 μM Asc): +5 μl 1.00 mM Asc
      +1.30 ml Asc Ox solution (this addition produces an increase in the ascorbate concentration of 10 μM as if in the original plasma, not the supernatant)

D. (Internal STD, 20 μM Asc): +10 μl 1.00 mM Asc
+1.30 ml Asc Ox solution (this addition produces an increase in the ascorbate concentration of 20 μM as if in the original plasma, not the supernatant)

5. Allow Asc Ox to work for 6 min
6. Transfer solutions to fluorescence cuvettes
7. Read background fluorescence in A–D:
Excitation 345 nm (10 nm slit width; this slit width may need to be varied). Emission 425 nm (10 nm slit width; this slit width may need to be varied)
8. Add 200 μl *o*-phenylenediamine stock to each, A–D.
9. Wait 10 min for the reaction to go to completion (very high ascorbate levels will require longer times and slight modification of this method, e.g., additional dilution)
10. Read fluorescence as in #7 above
11. The $AscH^-$ and DHA concentrations are determined from the fluorescence intensities of the samples with the standard additions after appropriate background corrections.

Reagents and Stock Solutions

5% Meta phosphoric acid (MPA): Wash solid MPA; prepare 5% (W/V) solution and store at 4°C. Prepare weekly.

Acetate buffer (2 M, pH 6.2): Dissolve 272 g/L of sodium acetate trihydrate (F.W. = 136) in high-grade H_2O. Adjust to pH 6.2 with acetic acid.

Ascorbate oxidase (Sigma # A-0157, 250 units): Prepare stock solution of 200 units/ml by adding 1.25 ml of acetate buffer to the Sigma vial. Store at 4°C until needed (keeps about 4 weeks). On the day of the experiment, add 14.4 μl of this 200 units/ml stock to 1 ml of the acetate buffer for each ml of ascorbate oxidase solution needed for the assay. (Note that 5.2 ml of ascorbate oxidase stock solution is required for each determination.) This produces a solution having 2.8 units/ml. Since 1.3 ml/2.5 ml is required, the final concentration will be ~1.5 units/ml.

o-Phenylenediamine (1,2-Phenylenediamine): Prepare a solution of 1 mg/ml on the day of use. Protect from light.

1.00 mM Ascorbate stock: Prepare 100 mM ascorbate stock by dissolving 176 mg of ascorbic acid in 10.00 ml of high-grade H_2O. Then dilute by 1/100. The exact concentration can be determined by a further 1/10 dilution in metal-free buffer (pH ~ 7) and determining its absorbance at 265 nm, $\epsilon_{265} = 14{,}500\ M^{-1}\ cm^{-1}$ (7).

VI. CONCLUDING REMARKS

Ascorbate is well known for its reducing properties. It is an excellent cosubstrate in monooxygenase reactions producing hydroxylated amino acids by keeping iron- and copper-containing enzymes in their reduced states. Ascorbate is also an excellent antioxidant; it is thermodynamically at the bottom of the pecking order, thus it is the terminal small-molecule antioxidant. Ascorbate protects cells from oxidative stress by scavenging free radicals and recycling other antioxidants, such as vitamin E. Using EPR, the ascorbate free radical can be used as a maker of oxidative stress.

Ascorbate is a simple molecule with numerous chemical and biochemical functions, many of which are probably unknown. It is essential for human life, yet the gene for its production was deleted from our ancestors millions of years ago. Thus, humans and other primates are forever (dietarily) at its mercy.

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