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Catalytic Metals, Ascorbate and Free Radicals: Combinations to Avoid[1]

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Trace levels of transition metals can participate in the metal-catalyzed Haber-Weiss reaction (superoxide-driven Fenton reac-tion), as well as catalyze the oxidization of ascorbate. Generally ascorbate is thought of as an excellent reducing agent; it is able to serve as a donor antioxidant in free radical-mediated oxidative processes. However, as a reducing agent it is also able to reduce redox-active metals such as copper and iron, thereby increasing the pro-oxidant chemistry of these metals. Thus ascorbate can serve both as a pro-oxidant and antioxidant. In general, at low ascorbate concentrations, ascorbate is prone to be a pro-oxidant, and at high concentrations, it will tend to be an antioxidant. Hence, there is a crossover effect. We propose that the "position" of this crossover effect is a function of the catalytic metal concentration. In this presentation, we discuss:
(1) the role of catalytic metals in free radical-mediated oxidations; (2) ascorbate as both a pro-oxidant and as an antioxidant; (3) catalytic metal catalysis of ascorbate oxidation;(4) Use of ascorbate to determine adventitious catalytic metal concentrations; (5) use of ascorbate radical as a marker of oxidative stress; and 6) use of ascorbate and iron as free radical pro-oxidants in photodynamic therapy of cancer.

INTRODUCTION

A major turning point in the research and understanding of free radicals in biology and medicine occurred in 1968-1969 with the discovery that erythrocuprein functions enzymatically as a superoxide dismutase *(1)*. However, perhaps just as important was the discovery that an enzyme, xanthine oxidase, produces superoxide *(2-4)*. Soon after these discoveries Beauchamp and Fridovich made the very important observation that hydroxyl radical appeared to be produced in the superoxide-generating system of xanthine-xanthine oxidase *(5)*. They proposed that the mechanism of hydroxyl radical production was that proffered by Haber, Weiss, and Willstätter *(6-8)*, i.e.

$$O_2^{\bullet-}/HO_2^{\bullet} + H_2O_2 \longrightarrow O_2 + H_2O + HO^{\bullet}.$$

However, studies of kinetics of this reaction found it to be very slow with measured rate constants of <0.0001 *(9)*, 0.13 *(10)*, and 2.3 M^{-1} s^{-1} at neutral pH values *(11)*. Thus, on a biological time scale, it is a very slow reaction at the cellular concentrations of these two reactants and thus can be considered to be a negligible process. So the question became—how can $HO^{\bullet}$ be formed in a superoxide-generating system?—Iron, or more generally catalytic transition metals!

It was soon realized that the addition of iron to a system generating superoxide enhances the peroxidation of membrane lipids *(12)*. Additional work demonstrated that superoxide could reduce ADP–Fe(III) to ADP–Fe(II) and that this iron facilitated the apparent production of $HO^{\bullet}$ *(13)*.

The role that iron plays in free radical oxidations was not appreciated until the Pinawa meeting[2] when it was presented that adventitious levels of iron in buffer solutions were on the order of 1 μ*M*, and that this level of iron would change the results observed in superoxide-generating systems *(14)*. However, just as important, it was demonstrated that chelating agents will alter the reactivity of iron in superoxide-generating systems. It was shown that EDTA[3] enhances the reactivity of iron toward $O_2^{\bullet-}$ while DETAPAC[4] drastically slows the $O_2^{\bullet-}$ reaction with iron *(14)*. Currently, chelating agents are being widely researched and used as tools to study free radical oxidations and as potential clinical agents to address human health problems.

[1]Presented at the Fenton Centennial Symposium at the 42nd Annual Meeting of the Radiation Research Society, San Jose, CA, April 1995

[2] International Conference on Singlet Oxygen and Related Species in Chemistry and Biology, 21-26 August 1977, Pinawa, Manitoba Canada. The symposium papers were published in *Photochem. Photobiol.* **28** (4, 5) (1978).

[3] Abbreviations: $Asc^{\bullet-}$, ascorbate free radical; Asc^{2-}, ascorbate dianion; $AscH^{-}$, ascorbate monanion; $AscH_2$, ascorbic acid; DETAPAC, diethylenetriaminepentaacetic acid; DTPA=DETAPAC; EDTA, ethylenediaminetetraacetic acid; POBN, α-[4-pyridyl 1-oxide]-N-*tert*-butyl nitrone; TBARS, thiobarbituric acid reactive substances; $TO^{\bullet}$, tocopheroxyl free radical; TOH, tocopherol.

[4] It was at the Pinawa meeting that the acronym DETAPAC was introduced. This was a simple name we used to label the container of our stock solution of this chelating agent. DTPA is the more accepted abbreviation, but nearly all researchers refer to it verbally as DETAPAC.

CAFTALYTIC METALS AND FREE RADICAL OXIDATIONS

Superoxide, H_2O_2, and $HO^\bullet$ produced from the autoxidation of biomolecules, such as ascorbate, catecholamines or thiols have been implicated in numerous human health problems. However, the direct reaction of dioxygen with the vast majority of biomolecules, including the above, is *spin-forbidden* because ground state dioxygen is a biradical (*15*). As a result, there is a severe limitation on the kinetics of the direct oxidation of these compounds by ground state dioxygen. Thus an alternate mechanism must be invoked to explain these "autoxidations".

Transition metals are efficient catalysts of redox reactions and their reactions with dioxygen are not spin restricted. Therefore, it is likely that the vast majority of so-called autoxidation reactions are, in fact, metal catalyzed (*15*).

Transition metals can exist in several spin-states; thus they are able to relieve the spin restriction of dioxygen, thereby enhancing the rate of biomolecule oxidation. This is especially important when it is realized that innumerable biomolecules bind transition metals, in particular protein moieties containing oxygen, nitrogen, or sulfur atoms. The coordination of biomolecules by transition metals almost always involves the d-orbitals of the metal (*16*). These orbitals may also allow the simultaneous binding of a biomolecule and dioxygen, thereby providing a bridge between O_2 and the biomolecule (*15,17*). Consequently, we have all the necessary ingredients to produce oxidative damage at a particular site.

It is important to remember that if transition metals are acting as true catalysts, then the thermodynamics of the oxidation reactions of the biomoleules is not changed. In essence, only the activation energy has been lowered, i.e. a reaction pathway is now available to overcome the spin restriction of O_2. Thus the rate by which equilibrium is achieved has been accelerated and as a result the rate of undesired biological oxidations could become quite significant.

Trace amounts of adventitious catalytic metals in buffer solutions can serve as catalysts for many oxidative processes. These adventitious metals have caused many problems for researchers as they explore the mechanistic aspects of free radical reactions. We now know that these trace levels of transition metals are significant players in ascorbate oxidation (*18-20*) as well as in the metal-catalyzed Haber-Weiss reaction, often referred to as the superoxide-driven Fenton reaction (*14*). Thus, for experiments in which low concentrations of catalytic metals are an important determinant of experimental results, these metals must be sequestered in an inactive form or removed from the reaction mixture.

Catalytic Metals—How Much Is There?

Trace catalytic metals are ubiquitous in salt and buffer solutions (Table I). Typical concentrations of adventitious iron are 1-10 μ*M* while that of copper is ~0.1 μ*M* (*18*). These levels of metals can make significant contributions to metal-mediated oxidative pathways. Thus, to appreciate the non-metal as well as the metal-mediated pathways, the activity of these metals need to be modulated.

TABLE I
Trace Iron and Copper Levels in Solutions[a]

Reagent	Treatment	[Fe](μM)	[Cu](μM)
50 mM phosphate, pH 7.0	none	0.3-0.7	≈ 0.13
50 mM phosphate, pH 7.0	Chelating resin	<0.1	≈ 0.001
100 mM KCl	none	2.5	
1 M NaCl	none		0.05
67.5 mM phosphate, pH 7.4/ 4.0 mM KCl	none	3 .5-8.9	
50 mM EDTA	none	9.7-19.4	
Xanthine Oxidase at 20 mU/mL	none	0.004-0.7 [b,c]	

[a] Adapted from ref. (18).
[b] This is redox active iron, not total iron.
[c] From ref. (21).

Chelating Agents

Chelating agents have been extensively used as tools to study the involvement of catalytic transition metals in free radical oxidative processes because they can modulate the catalytic activity of the metal (*14,22*). The prevailing hypothesis on how metals are involved in free radical oxidation suggests the oxidants, such as $HO^\bullet$, or perhaps perferryl ions (chelator-Fe-O_2 complexes), are involved in the observed oxidations. Typical scenarios would be:

$$Fe^{3+}\text{-chelate} + O_2^{\bullet-}/\text{AscH-} \rightarrow Fe^{2+}\text{-chelate} + O_2/\text{Asc}^{\bullet-} \quad (1)$$

$$Fe^{3+}\text{-chelate} + H_2O_2 \rightarrow Fe^{3+}\text{-chelate} + OH^- + HO^\bullet \quad \text{The Fenton Reaction} \quad (2)$$

Because of the extreme oxidizing nature of the hydroxyl radical it can initiate oxidations of biomolecules at will (*23*):

$$HO^\bullet + \text{Biomolecules} + O_2 \rightarrow \text{Oxidized Biomolecules} \quad (3)$$

Special attention has been given to the iron chelates DETAPAC and Desferal®; both appear to slow the Fe^{3+} to Fe^{2+} reduction reaction (*20, 24, 25*), reaction (1), significantly, rather than the Fenton Reaction, reaction (2) (*22,24*).

However, it must be recognized that metal chelates can protect biomolecules in two ways:

1. The formation of thermodynamically stable metal-chelate complexes can remove metals that are complexed to biomolecules, thereby protecting them from site-specific oxidation, *vide supra*.
2. Formation of complexes that hinder the redox cycling required for reactions (1) and (2) above.

In vivo, both protective mechanisms will be operable and the first may be very important in the pharmacologic use of chelates.

Both mechanisms are used naturally in biological systems. As an example, the iron proteins ferritin and transferrin

readily sequester loosely bound and therefore potentially damaging iron. Ferritin can hold up to ≈2500 irons/molecule, while transferrin can hold two ferric ions/molecule. On a per-iron basis each is very resistant to reaction with superoxide (*26,27*). The rate constant for the reaction of superoxide with ferritin[5] was found to be 2 x 106 M^{-1} s^{-1} or only ≈1 x 103 M^{-1} s^{-1} $(iron)^{-1}$, quite a slow process.

The reaction of superoxide with transferrin iron is also very slow, k < 105 M^{-1} s^{-1} (*27*). Even the ultimate of reducing free radicals, e_{aq}^-, is able to react with transferrin only by forming a protein radical with no significant reduction of iron (*27*). Thus, the iron of intact transferrin will not participate in the catalysis of free radical oxidation reactions.

The iron in both ferritin and transferrin can be prompted to participate in catalytic reactions. Reducing agents such as superoxide and ascorbate, or reducing radicals such as paraquat, adriamycin and alloxan radicals can reduce the Fe^{3+} in ferritin to Fe^{2+}. Fe^{2+} is much more soluble than Fe3+ and thus can be released from the core of the ferritin molecule. This iron can then participate in oxidation reactions (*28-30*).

Likewise, the iron in transferrin can become available for oxidation reactions, but it appears that this happens only when there is damage to the protein (*31*).

Removal of Metals

Although very useful, chelates cannot guarantee that metals are removed from the picture. For example, very reducing radicals, such as the paraquat free radical [E°‘ = –450 mV (*23*)], are able to reduce Fe(III)DETAPAC to the Fe2+ complex, thereby making it catalytically quite active (*32*). Thus it may be more prudent to remove these metals from the buffer solution being used. The most popular approach is the use of chelating resins such as Chelex 100®. When employing these approaches, there is always uncertainty in the success of producing a “metal free” buffer solution. We have found that the rate of oxidation of ascorbate can be used as a superb indicator of the catalytic metal content of near neutral buffer solutions (*18,19*).

ASCORBATE AS AN ANTIOXIDANT

Before discussing the use of ascorbate as a tool to measure very low levels of catalytic metals, we present a review of ascorbate antioxidant and pro-oxidant properties so that its use as a tool can be understood and put into perspective.

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. 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 (*23,33*).

TABLE II
One-Electron Reduction Potentials at pH 7.0 for Selected Radical Couples

Redox Couple	E°′ (mV)
$HO^{\bullet}$, H^+/H_2O	+2310
$RO^{\bullet}$, H^+/ROH (aliphatic alkoxyl radical)	+1600
$ROO^{\bullet}$, H_+/ROOH (alkyl peroxyl radical)	+1000
$GS^{\bullet}/GS^-$ (glutathione)	+920
$PUFA^{\bullet}$, H+/PUFA-H) (*bis*-allylic-H*)*	+600
$HU^{\bullet-}$, H+/UH^{2-} (urate)	+590
$TO^{\bullet}$, H^+/TOH (tocopherol)	+480
H_2O_2, H^+/H_2O, $HO^{\bullet}$	+320
$Ascorbate^{\bullet-}$, H^+/ascorbate monoanion	+282
Fe(III)EDTA/Fe(II)EDTA	+120
$O_2/O_2^{\bullet}$	–330
$Paraquat^{2+}$/$paraquat^{\bullet+}$	–448
Fe(III)Desferal®/Fe(II)Desferal®	–450
RSSR/$RSSR^{\bullet-}$ (GSH)	–1500
H_2O/e_{aq}^-	–2870

Note. This table is adapted from ref. (*23*).

When biological fluids or tissues are examined by electron paramagnetic resonance spectroscopy (EPR), $Asc^{\bullet-}$ will most likely be observed. This is consistent with ascorbate's role as the terminal small-molecule antioxidant (*23,34*).

As can be seen in Table II, 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:

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

where $X^{\bullet}$ is any of these oxidizing free radicals. From Table III we see that the kinetics of these electron (hydrogen atom) transfer reactions are rapid. Thus, both thermodynam-ically 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 react by an addition reaction with O_2 to form dangerous peroxyl radicals. Ascorbate (probably Asc^{2-}, and/or $Asc^{\bullet-}$) appears to produce very low levels of superoxide (*36, 37*). But by removing $O_2^{\bullet-}$ superoxide dismutase provides protection from this possibility (*38*). The biological organism is protected from further free radical-mediated oxidations. In addition, $Asc^{\bullet-}$ as well as the dehydroascorbic formed can be reduced back to ascorbate by enzyme systems. Thus, it is recycled.

ASCORBATE AS A PRO-OXIDANT

As discussed above, ascorbate is an excellent antioxidant. We consider it to be the terminal small-molecule

[5] These experiments were done with ferritin that contained ≈2500 irons/molecule. Thus, on a per iron basis this rate constant is ≈ 1 x 10^3 M^{-1} s^{-1} $(iron)^{-1}$.

TABLE III
Rate Constants for the Reaction of the Equilibrium Mixture of $AscH_2/AscH^-/Asc^{2-}$ at pH 7.4 Unless Noted Otherwise

Radical	$k_{obs}(M^{-1}\ s^{-1})$(pH 7.4)[a]
$HO^{\bullet}$	1.1×10^{10}
$RO^{\bullet}$ (*tert*-butyl alkoxyl radical)	1.6×10^{9}
$ROO^{\bullet}$ (alkyl peroxyl radical, e.g. $CH_3OO^{\bullet}$)	$1\text{-}2 \times 10^{6}$
$ClCOO^{\bullet}$	1.8×10^{8}
$GS^{\bullet}$(glutathiol radical)	6×10^{8}(5.6)
$UH^{\bullet-}$ (urate radical)	1×10^{6}
$TO^{\bullet}$ (tocopheroxyl radical)	2×10^{5}[b]
$Asc^{\bullet-}$ (dismutation)	2×10^{5}[c]
$CPZ^{\bullet+}$ (chlorpromazine radical action)	1.4×10^{9}(5.9)
Fe(III)EDTA/Fe(II)EDTA	$\approx 10^{2}$[d]
$O_2^{\bullet-}/HO^{\bullet}_2$	1×10^{5}[c]
	2.7×10^{5}
Fe(III)Desferal®/Fe(ii)Desferal®	Very slow

[a]A complete summary of free radical solution kinetics can be found in ref. (*35*).
[b]Estimated kobs for $TO^{\bullet}$ when in a biological membrane
[c]*k* is dependent on pH; thus this is k_{obs} at pH 7.4.
[d]Estimated from data in ref. (*18*)

antioxidant in biological systems. However, ascorbate is also widely used as a pro-oxidant (*36-48*). 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^{1+}. The redox cycling of these metals is essential to the oxidation of the vast majority of singlet state organic molecules (*15*). In nearly all the experimental systems where ascorbate has pro-oxidant properties, there is the simultaneous presence of redox active metals. Thus, catalytic metals and ascorbate are to be avoided.

THE CROSS-OVER EFFECT FOR ASCORBATE AS A PRO- OR ANTI-OXIDANT

In general, low concentrations of ascorbate are required for pro-oxidant conditions, while high concentrations are needed for antioxidant conditions. Thus there is a "crossover" effect (*49*); low and high are relative. In the literature there are a wide range of concentrations of ascorbate where this crossover from pro- to antioxidant action occurs (*39-49*). 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 nearly 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.

CATALYTIC METALS AND ASCORBATE OXIDATION

Autoxidation

Before beginning this discussion it must be understood that we use the term autoxidation to mean oxidation in the absence of metal catalysts (*15*). 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 (*18-20,50-52*). The diacid is very slow to oxidize. Consequently, at low pH, i.e. less than 2 or 3, ascorbic acid solutions are quite stable, assuming catalytic transition metal ions are not introduced into the solutions. However, as the pH is raised above pK_1 (4.2), AscH- becomes dominant and the stability of the ascorbate solution decreases. This loss of stability is usually the result of the presence of adventitious catalytic metals (on the order of 1 μ*M* iron) in the buffers and salts that are typically employed in studies at near-neutral pH (*18*). For example, we have found that in room-temperature, aerated, aqueous solutions at pH 7.0 (50 m*M* phosphate buffer) 10-30% of 125 μ*M* ascorbate is lost in just 15 min. The large variation in ascorbate loss is the result of different sources and grades of phosphate used in the buffer preparation. However, if care is taken to remove the trace levels of transition metals, this rate of loss can be lowered to as little as 0.05%/15 min (*18*), demonstrating the extreme importance of metals in controlling ascorbate stability. At pH 7.0 we have set an upper limit for the observed rate constant for the oxidation of ascorbate to be $6 \times 10^{-7}\ s^{-1}$ under our experimental conditions (*18*). However, even in solutions that have carefully been rendered metal-free, as the pH is increased the rate of oxidation increases, (Fig. 1; *34*). 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 is the only ascorbate species that would significantly undergo true autoxidation (*37*).

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

Our experimental results are consistent with these estimates (*18, 19, 34*). Marcus theory would predict that the true autoxidation of $AscH^-$ would be much slower, $\approx 10^6\ M^{-1}\ s^{-1}$ slower. Thus, at pH 7.4 the rate of autoxidation of an ascorbate solution is determined by the presence of Asc^{2-}.

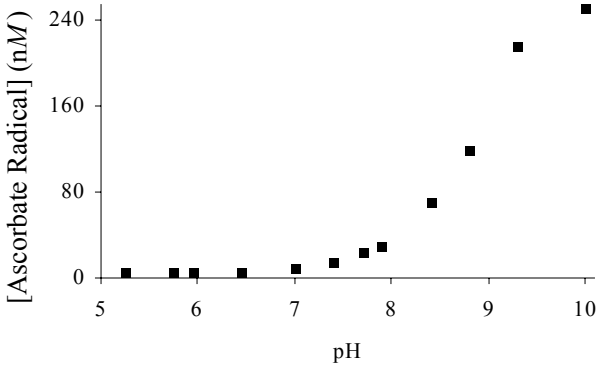

FIG. 1. Background [Asc$^{\bullet-}$] as a function of pH. Each solution was made with 50 m*M* phosphate buffer which had been rendered metal-free and which contained 50m*M* desferoxamine mesylate for at least 12 h. To these solutions 500 μ*M* ascorbate was added and the EPR spectra were collected. The 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 n*M* (adapted from ref. *34*).

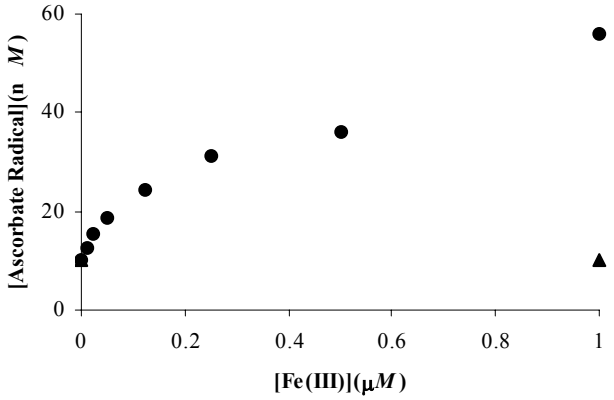

FIG.3. This figure is an expansion of the 0–1 mM [Fe(III)] region of Fig. 2. The experimental conditions are the same as in Fig. 2. The curves were obtained in 50 mM phosphate buffer, pH 7.4, which had been rendered metal free with 250 mM EDTA (•) or 50 μM Desferal (▲) with 125 μM ascorbate present (adapted from ref. 19).

Typical buffers employed in biochemical and biological research have on the order of 1 μ*M* iron and <0.1μ*M* copper. But because copper is ≈80 times more efficient as a catalyst for ascorbate oxidation than iron, in typical phosphate buffers it is the adventitious copper that is the biggest culprit in catalyzing ascorbate oxidation *(18)*!

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

Iron Analysis at the Nanomolar Level (18,19)

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, pipettes, and pipette tips are scrupulously clean we can estimate iron levels in phosphate buffer to a lower limit of ≈100 n*M* using UV-Vis spectroscopy *(18)*. However, using EPR spectroscopy this limit can be as low as ≈5 n*M* (ref.*38*; Figs. 2 and 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 ≈5 n*M* → ≈10 μ*M*. To achieve estimates at the lowest end of this range, extreme care must be taken with each step and the EDTA must be pure; 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 rendered metal-free using a chelating resin such as Chelex 100® *(18)*. This method is useful if there is interference for standard colorimetric assays of iron, or if only "loosely bound" iron is to be estimated *(21)*.

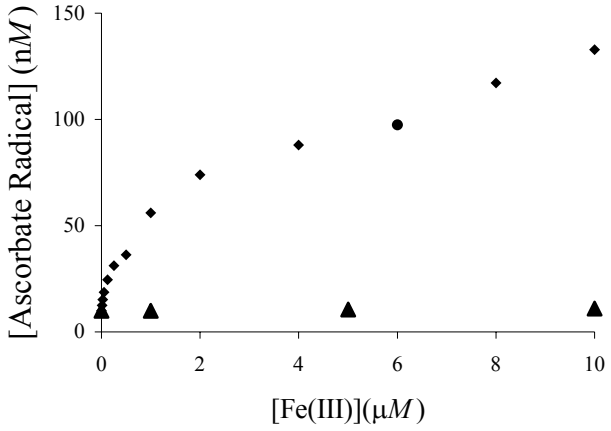

FIG. 2. These data were gathered using EPR spectroscopy to quantify the steady-state level of Asc$^{\bullet-}$. The curves were obtained in 50 m*M* phosphate buffer, pH 7.4, which had been rendered metal-free with 250 uM EDTA (•) or 50 mM Desferal (▲) with 125 mM ascorbate present (adapted from ref. *19*).

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

Removal of Trace Metals (18)

We have also found that ascorbate is an excellent tool to ascertain the effectiveness of adventitious catalytic metal removal from near-neutral buffer systems. In this method we follow the loss of ascorbate due to oxidation by monitoring its absorbance at 265 nm. In our standard test we add ≈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 photo-oxidation.)

Stock Ascorbate Solutions (18)

In our work with ascorbate we have found that the quality of the stock solution determines the quality and reproducibility of the results. We prepare ascorbate stock solutions using only the diacid. It is prepared as a 0.100 *M* stock solution (10 ml) using high purity water. This solution is colorless, having a pH of ≈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 m*M*, 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 shown by the yellow color of the solution.

ASCORBATE RADICAL AS A MARKER OF FREE RADICAL-MEDIATED OXIDATIVE STRESS

The ascorbate free radical is naturally detectable by EPR at low steady-state levels in biological samples, such as leaves from crops *(53),* plasma *(54-56)* synovial fluid *(57)* and skin *(58-60)*. 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 I). 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 *(34)*. 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 *(34)*.

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 the skin of the mouse *(58-61)*, hepatocytes in the rat *(62)* and ischemia reperfusion of hearts *(63-67)*[6]. Human sera and rat plasma intoxicated with paraquat and diquat, known superoxide generators, have increased ascorbate radical levels *(68)*. In animal experiments, sepsis has also been shown to increase $Asc^{\bullet-}$, indicating the involvement of oxidative stress with this health problem *(69)*. 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 *(70-74)*. 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.

TABLE IV

Metals from Syringes[a]

Treatment, pH 7.4 PO_4	[Fe](m*M*)
Chelex 100	≤0.01 (8)[b]
Hamilton, 705-N	5.0 ± 2.9 (5)
Gas-tight, Hamilton 1705-TEF (22S stainless steel needle)	0.18 ± 0.12 (5)
1705-TEF (Teflon needle)	0.14 ± 0.03 (5)
1725-TEF LL (stainless steel needle)	0.061 ± 0.008 (5)
1725-TEF LL (Teflon needle)	0.015 ± 0.007 (7)

[a] Adapted from ref. (*19*).

[b] Number of experiments

EXAMPLES

Syringes and Iron

Small glass syringes are used routinely in many laboratory settings as pipettes. However, these syringes can introduce substantial amounts of iron into the solutions they contain *(19)*. For example, the Hamilton 705-N syringe has a ground glass barrel and stainless steel plunger. Using the EPR/$Asc^{\bullet-}$ assay for iron, we found that when a 50 m*M* pH 7.4 phosphate buffer ([Fe] < 0.01 μ*M*) which has been rendered metal-free is introduced into this type of syringe the iron level can increase to 5 μ*M* or more (Table IV). If the buffer solution is allowed to reside in the syringe for a long time, >15 min, the iron concentration can easily be 10-50 μ*M*. This iron would be catastrophic to iron-sensitive agents that may be present in the buffer solution.

By comparing the iron levels of solutions that have passed through a 2 inch 22S stainless steel needle to the levels of solutions that have passed through a Teflon "needle", it can be deduced that a simple steel needle can introduce 40-50 n*M* iron into a solution.

The best glass syringes to use are the gas-tight syringes as they have a smooth barrel and the seal between plunger and barrel is provided by a Teflon plug. These materials provide little iron; we do not allow any solution to come into contact with ground glass. Ground glass will invariably be contaminated with metals and its large surface area ensures that our solutions that come into contact with it are contaminated as well.

This example demonstrates the need to consider carefully the nature of glassware and equipment used in free radical studies.

[6] In a quite different approach Pietri *et al.* (66,67) 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 temporal 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 (*34, 59, 65*).

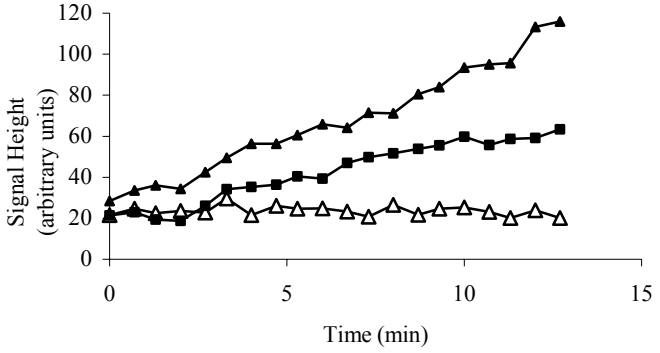

FIG.4. Skin treated with Desferal prior to UV-radiation exposure has decreased POBN adduct signal. (△) POBN spin adduct signal intensity from untreated skin that was exposed to room light only; (▲) POBN spin adduct signal intensity from untreated skin that was exposed to UV radiation; (■) POBN spin adduct signal intensity from skin treated with Desferal and exposed to UV radiation. The data represent the signal height in at least three separate experiments. After UV-radiation exposure, a paired comparison of each showed them to be statistically different, P<0.001 (adapted from ref. *59*).

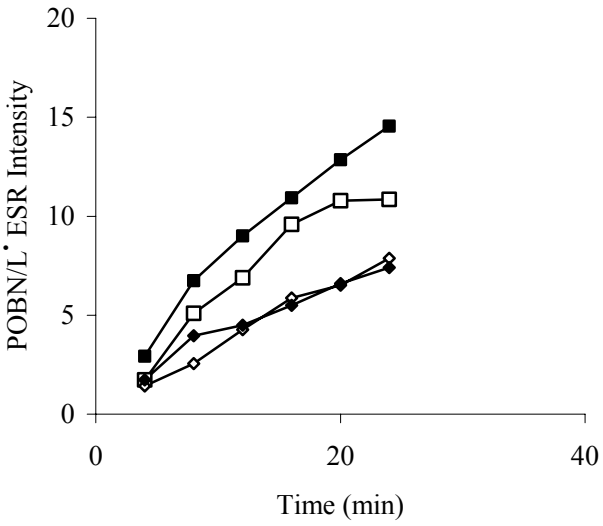

FIG. 5. Iron and ascorbate enhance lipid radical formation. These data show that the oxidative cofactors FE^{2+} (5 μ*M*) and ascorbate (100 μ*M*) synergistically enhance lipid radical formation from Photofrin-treated L1210 cells upon xposure to light. Because there were minor variations in the lipid radical EPR signal intensity prior to light exposure, the initial dark intensity of each sample was subtracted from the data points collected during exposure to light. Thus we have the changes in POBM/L$^{\bullet}$ as a funtion of light exposure. The values for no cofactors (◇) and ascorbate only (◆) are not statistically different at P = 0.05, whereas Fe^{2+} plus ascorbate (■) is different from Fe^{2+} only (□), P<0.05. Both curves are different from Photofrin only and ascorbate only, P< 0.01, using one-way analysis of covariance (adapted from ref. *39*).

Iron Produces Free Radicals in Skin

Skin is a significant site of iron excretion *(75)*. Chronic exposure of mouse skin to UV radiation increases basal amounts of non-hemoglobin iron. Topical application of iron chelators to skin is photoprotective with chronic exposure to UV radiation *(76)*. Thus, if iron is involved in UV-radiation-induced radical formation, then chelation of this iron will reduce this radical flux. Topical application of Desferal® to skin should chelate free iron in the skin and prevent the involvement of this iron in free radical reactions.

To test this possibility Desferal® (50 μl of a 10 m*M* stock) was topically applied to the dorsal skin of Skh-1 mice for approximately 10 min prior to treatment with the spin trap POBN (50 μl of a 250 m*M* stock). Skin samples were blotted and placed in the electron paramagnetic resonance spectrometer (EPR) cavity. The epidermal surface was exposed to UVA/B radiation while in the EPR cavity. The first doublet of the POBN radical adduct EPR spectrum was then measured.

Treatment with Desferal® resulted in a significant reduction of the POBN adduct signal increase (Fig. 4), suggesting a role for iron in UV-radiation-induced free radical formation. Desferal® has no significant UV absorption at wavelengths>280 nm. Therefore, its mechanism for protection cannot be simply due to blocking the UV radiation. These results indicate that iron is involved in UV-radiation-induced free radical formation in skin.

Iron in Photodynamic Therapy

The photodynamic action of the photosensitizers Photofrin and merocyanine 540 involves the generation of $^1O_2$ within cell membranes. This $^1O_2$ reacts with membrane lipids to form lipid hydroperoxides, which can be considered precursors to free radical chain reactions. The formation of oxidizing free radical intermediates can be signifcant if catalytic iron is present. Iron (II) will react with the lipid hydroperoxides in a reaction parallel to the Fenton reaction for highly oxidizing lipid alkoxyl radicals:

$$Fe^{2+} + LOOH \rightarrow Fe^{3+} + LO^{\bullet} + OH^{-}$$

We hypothesized that iron in the presence of ascorbate would serve as a pro-oxidant in a Photofrin photodynamic system. When Photofrin-treated L1210 cells were exposed to photosensitizing light, iron and ascorbate synergistically enhanced cellular lipid radical formation, (Fig. 5; ref. *39*). Bachowski *et. al. (77)*, using both TBARS and cholesterol oxidation products as markers, have also found that iron and ascorbate will significantly enhance radical-mediated oxidation in isolated plasma membranes subjected to merocyanine 540 photosensitization. Thus, iron could be used to advantage in pharmacological treatments that have oxidations involved in their mechanisms of action.

Metals in Reperfusion

Numerous studies have found that metal-chelating agents can ameliorate tissue damage resulting from ischemia/reperfusion episodes. Model systems such as the Langendorff system, in which isolated hearts are studied, have provided a great deal of information on myocardial events during ischemia and reperfusion.

However, these models use perfusion buffers such as the Krebs-Henseleit buffer, which contains metals, e.g. ≈0.3 μ*M* copper. Powell and Wapnir *(78)* have found that isolated hearts accumulate transition metals from the buffer, resulting in artificially high levels of redox iron and copper in the heart preparation. They found that the extracted copper was particularly detrimental; the copper laden hearts sustained moderate injury as determined by cystolic pressure, whereas when the perfusion buffer was rendered metal-free with chelating resin prior to use, no significant injury occurred. These results again demonstrate that adventitious redox active metals may be confounding factors in the interpretation of experimental results.

The study of the role of metals in biological free radical oxidations remains an important topic. We still need to improve our basic understanding of the reactions of metals in these processes, but more importantly, we need to investigate the importance of these metals in pathology. Metals are both too easily blamed and or often neglected in studies of free radical oxidations. Of particular importance will be to learn how to control metal catalysis in free radical-associated pathologies to ameliorate this potential damage. However, at the same time it may be possible to use metals to our advantage in treatment modalities. The example of Photofrin suggests that the effectiveness of a treatment scheme could be enhanced with the controlled use of metals.

One area that has only been examined briefly is the potential synergy of metals in oxidations. Our colleagues in environmental research have noted that iron and copper are co-conspirators in the oxidation of organics in atmospheric waters *(79)*. It was determined that reduced copper transferred an electron to iron, which in turn participates in the oxidation process.

$$k = 3 \times 10^{7}\ M^{-1} s^{-1} \quad (79)$$

$$Cu^{1+} + Fe^{3+} \rightarrow Cu^{2+} + Fe^{2+}$$

These same processes may be of significance in a biological setting.

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Note:

The below noted entry has been changed from the original publication to correct a typographical error:
[*excerpted from Table II page 534]*

RSSR/$RSSR^{\bullet-}$ (GSH) –1500