⊣ℝ· *Review Article*

TRANSITION METALS AS CATALYSTS OF "AUTOXIDATION" REACTIONS

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Abstract—Superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\cdot OH$) produced from the "autoxidation" of biomolecules, such as ascorbate, catecholamines, or thiols, have been implicated in numerous toxicities. However, the direct reaction of dioxygen with the vast majority of biomolecules, including those listed above, is spin forbidden, a condition which imposes a severe kinetic limitation on this reaction pathway. Therefore, an alternate mechanism must be invoked to explain the "autoxidations" reactions frequently reported.
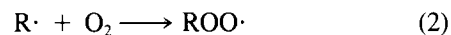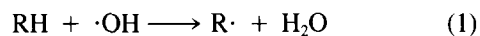
Transition metals are efficient catalysts of redox reactions and their reactions with dioxygen are not spin restricted. Therefore it is likely that the "autoxidation" observed for many biomolecules is, in fact, metal catalyzed. In this paper we discuss: 1) the quantum mechanic, thermodynamic, and kinetic aspects of the reactions of dioxygen with biomolecules; 2) the involvement of transition metals in biomolecule oxidation; and 3) the biological implications of metal catalyzed oxidations. We hypothesize that true autoxidation of biomolecules does not occur in biological systems, instead the "autoxidation" of biomolecules is the result of transition metals bound by the biomolecules.

Keywords—Free radicals, Iron, Superoxide, Hydroxyl radical, Peroxidation, Chelation, Autoxidation

I. INTRODUCTION

The metabolically uncoupled oxidation of polyunsaturated fatty acids, proteins, and DNA is fundamental to the development of numerous toxicities and pathologies.[1,2] Two general mechanisms of oxidation of these macromolecules have been postulated. The first, and probably most frequently invoked mechanism of oxidative damage, asserts that oxygen free radicals, in particular the hydroxyl radical ($\cdot OH$) initiate oxidation of lipids, protein, or DNA (reaction (1)). Subsequent propagation reactions (reactions (2,3)) further oxidize these molecules via numerous reaction pathways.[3]

$$RH + \cdot OH \longrightarrow R\cdot + H_2O \quad (1)$$

$$R\cdot + O_2 \longrightarrow ROO\cdot \quad (2)$$

$$ROO\cdot + RH \longrightarrow ROOH + R\cdot \quad (3)$$

The formation of $\cdot OH$ for reaction (1) requires the sequential one electron reduction of dioxygen to superoxide (O_2^-), hydrogen peroxide (H_2O_2), and finally to $\cdot OH$ before oxidation of the biomolecule occurs.

A second mechanism by which macromolecules may be oxidized involves the production and addition of singlet oxygen to these molecules generating endoperoxides (reaction (4)).


$$(4)$$
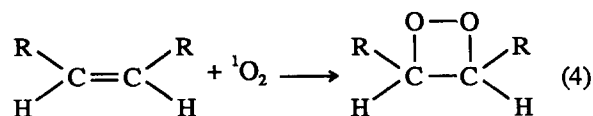
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This mechanism requires the input of energy, usually as light, to convert dioxygen to singlet oxygen.

Because ground state dioxygen is a triplet molecule, its reaction with most biomolecules is spin forbidden. Thus, the direct reaction of lipids, protein, DNA or the substances listed in Table 1 with dioxygen would be very slow (less than $10^{-5} M^{-1} s^{-1}$). Note that in both oxidation mechanisms above, activation of ground state dioxygen, either by reduction or by spin conversion is necessary before oxidation occurs. Nevertheless, several biomolecules (for examples, and references, see Table 1) have been reported to react directly with dioxygen, producing O_2^- or H_2O_2 or both.

$$RH_2 + O_2 \longrightarrow RH\cdot + O_2^- + H^+ \qquad (5)$$

$$RH_2 + O_2 \longrightarrow R + H_2O_2 \qquad (6)$$
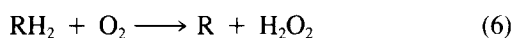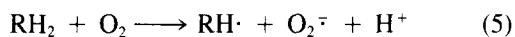
These types of reactions are termed "autoxidations," defined as the "...apparently uncatalyzed oxidation of a substance exposed to the oxygen of the air . . . ".[19] However, since it is unlikely that the direct reaction of dioxygen with these biomolecules occurs at significant rates, a catalyst, such as a transition metal, is needed.[20]

In this paper we detail the mechanisms of biomolecule oxidation and define the role of transition metals in these oxidations. Because iron and copper are the two most predominant transition metals in vivo, the chemistry and biochemistry of these metals is emphasized. Our hypothesis is that true autoxidation of biomolecules, such as those listed in Table 1, is negligible, and that these molecules will undergo significant oxidation in the presence of transition metals, such as copper or iron.
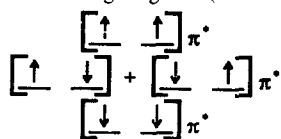
II. CHEMICAL CONSIDERATIONS

A. Chemistry of dioxygen

A simplified molecular orbital diagram for ground state dioxygen ($^3\Sigma g^-$) is shown below.[a]
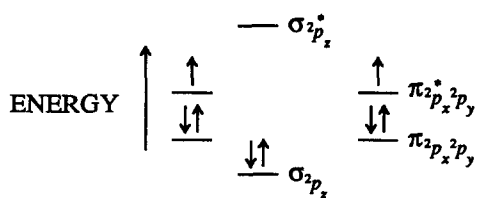
[a]A correct description of the 3-fold degenerate ground state of dioxygen is given in the following diagram. (See reference 96)

The two unpaired electrons give dioxygen a spin quantum number (S) of one (each unpaired electron contributing $1/2$) and a spin multiplicity of three ($2S + 1$), that is, dioxygen is a triplet molecule in the ground state. Valence bond theory depicts dioxygen as a double bonded species ($O = O$), but molecular orbital theory predicts that dioxygen is a diradical ($\cdot O \equiv O \cdot$), a structure which better accounts for the reactivity of dioxygen with radical molecules.

Dioxygen also exists in two singlet states, denoted as $^1\Delta_g$ and $^1\Sigma_g$, of higher energy than the ground state by 23.4 and 37.5 kcal per mole, respectively. Since $^1\Sigma_g$ rapidly interconverts to $^1\Delta_g$, it is probably the biologically relevant form of singlet oxygen.

Dioxygen also undergoes reduction reactions. The standard reduction potentials (at pH 7) for dioxygen and related species are listed in Table 2. The addition of one electron to dioxygen, forming the superoxide anion (O_2^-), occupies and fills one of the π^* orbitals, probably accounting for the unfavorable free energy change of this reaction. The addition of two electron to dioxygen, forming hydrogen peroxide (H_2O_2), results in significant electronic rearrangment yielding two sets of σ bonds for each of the oxygen atoms ($0 - 0$ and $O - H$); the remaining electrons occupy nonbonding orbitals on oxygen. Hydrogen peroxide has a spin multiplicity of zero, (i.e., a singlet molecule) and therefore is *not* an oxygen radical. The three electron reduction of dioxygen, or the one electron reduction of H_2O_2, results in scission of the oxygen — oxygen bond to give ^-OH and $\cdot OH$. The hydroxyl radical, like superoxide, is a doublet molecule in the ground state. Finally, the four electron reduction of dioxygen yields two moles of H_2O per mole of dioxygen.

B. Reactivity of dioxygen

In contrast to the triplet ground state of dioxygen, the vast majority of organic biomolecules are in a singlet ground state. The reaction of triplet molecules with singlet molecules is spin forbidden (see below), but these reactions can often be thermodynamically favorable. Many of the reactions of dioxygen with biomolecules, particularly those listed in Table 1, are thought to involve a direct one or two electron transfer to dioxygen, producing O_2^- or H_2O_2.[b]

[b]The free energy change and the equilibrium constant for these reactions are represented by:

$$\Delta G^{o\prime} = -nFE^{o\prime}$$

$$\Delta G^{o\prime} = -RT\ln K$$

$$\ln K = -\frac{nFE^{o\prime}}{RT}$$

where R is the gas constant (1.98 cal k^{-1} mole^{-1}) and F is Faradays constant (23061 cal V^{-1} mole^{-1}).

Table 1. Chemicals Reported to Autoxidize

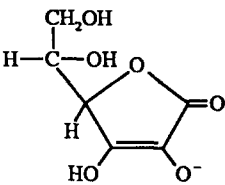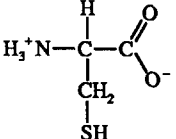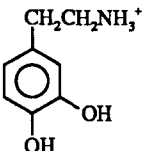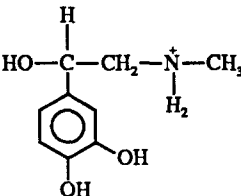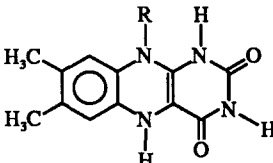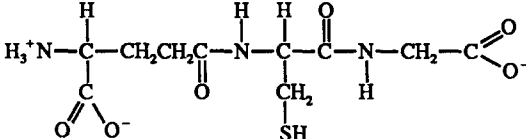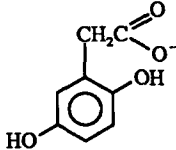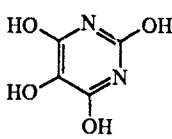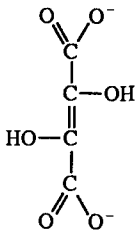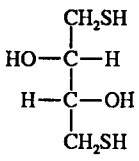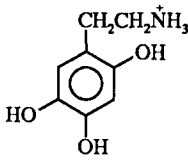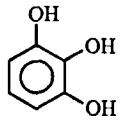| Name	Structure	Reference
Ascorbate		4,5
Cysteine		6
Dopamine		7,8
Epinephrine		9
Flavins		10
Glutathione		11
Homogenesic acid		12
Cysteamine*	$HSCH_2CH_2NH_2$	13

Table 1. (Continued)

Name	Structure	Reference
Dialuric Acid*	HO, N, OH / HO, N, OH (pyrimidine structure)	14
Dihydroxy fumarate*	structure	15,16
Dithiothreitol*	CH₂SH / HO—C—H / H—C—OH / CH₂SH	17
6-Hydroxydopamine*	CH₂CH₂N⁺H₃ with OH groups	14
Pyrogallol*	OH / OH / OH (trihydroxybenzene)	18

*These compounds, while not physiologic, are often used pharmacologically or for experimental biology.

$$RH_2 + O_2 \longrightarrow RH\cdot + O_2^{\cdot -} + H^+$$

$$\text{(1 electron transfer)} \quad (5)$$

$$RH_2 + O_2 \longrightarrow R + H_2O_2$$

$$\text{(2 electron transfer)} \quad (6)$$
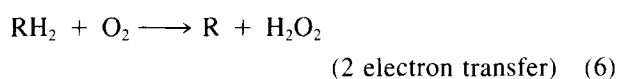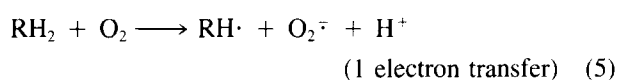
Tables 3 and 4 list the standard redox potentials for several of the compounds listed in Table 1 and other biomolecules pertinent to this discussion. By convention these redox potentials are shown as reduction potentials, and a negative $E^{o\prime}$ indicates that the *half* reaction is thermodynamically unfavored ($\Delta G^{o\prime} > 0$), although if coupled to a favored half reaction, the overall reaction may be thermodynamically favored. Thus, for reaction (5) to proceed (i.e., to have $O_2^{\cdot -}$ production favored), the reactant other than dioxygen (i.e., RH_2) must have an $E^{o\prime} < -160$ mV. Of the compounds listed in Table 3, the only molecules of physiological significance that satisfy this condition are the flavins.

In spite of the unfavorable free energy change for the one electron transfer of most biomolecules to dioxygen, these biomolecules are frequently said to "autoxidize." Consider the oxidation of ascorbate (AH^-) by dioxygen. It has been reported that $O_2^{\cdot -}$ is produced

Table 2. Standard Reduction Potentials for Dioxygen and Related Species*

Reaction	$E^{o\prime}$/Volts†	Reference
$O_2 + e^- \longrightarrow O_2^{\cdot}$	−0.33	21
$O_2(a) + e^- \longrightarrow O_2^{\cdot -}$	−0.16‡	22
$O_2 + 2 e^- + 2H^+ \longrightarrow H_2O_2$	0.33	23
$O_2 + 3 e^- + 3H^+ \longrightarrow H_2O + \cdot OH$	0.36	21
$O_2 + 4 e^- + 4H^+ \longrightarrow 2H_2O$	0.82	21
$O_2^{\cdot -} + e^- + 2H^+ \longrightarrow H_2O_2$	0.94	21
$H_2O_2 + e^- + H^+ \longrightarrow H_2O + \cdot OH$	0.33	21
$\cdot OH + e^- + H^+ \longrightarrow H_2O$	2.31	23

*These values of $E^{o\prime}$ are for aqueous solutions with O_2 at 1 atm, pH 7.
†Versus NHE.
‡The value of −0.16 volts results when unit concentration, that is, 1 molal, is used for the standard state of O_2 instead of 1 atm.
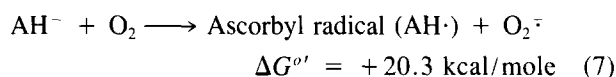
Table 3. Standard Reduction Potentials for Biomolecules and Other Selected Compounds*

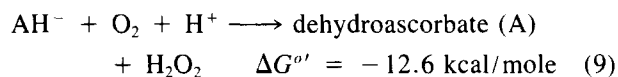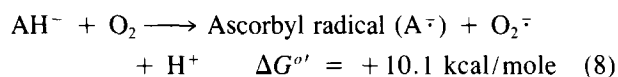| Reaction	$E^{o'}$/Volts†	Reference
Ascorbyl Radical (AH·) + e⁻ ⟶ Ascorbate (AH⁻)	+0.72	24
Ascorbyl Radical (A⁻) + e⁻ + H⁺ ⟶ Ascorbate (AH⁻)	+0.28	25
Cysteine − S· + e⁻ ⟶ Cysteine − S⁻	+0.84	26
FADH· + e⁻ + H⁺ ⟶ FADH₂	−0.24	27
NAD· + e⁻ + H⁺ ⟶ NADH	+0.30	28
Dehydroascorbate (A) + 2 e⁻ + H⁺ ⟶ Ascorbate (AH⁻)	+0.054	25
FAD⁺ + 2 e⁻ + 2H⁺ ⟶ FADH₂	−0.18	29
NAD⁺ + 2 e⁻ + H⁺ ⟶ NADH	−0.32	29
Adriamycin + e⁻ ⟶ Adriamycin Radical	−0.328	30
Alloxan + e⁻ + H⁺ ⟶ Alloxan H·	−0.44	31
Alloxan H· + e⁻ + H⁺ ⟶ Dialuric Acid	+0.64	31
Paraquat²⁺ + e⁻ ⟶ Paraquat⁺·	−0.45	32

*These values are for aqueous solutions, pH 7.
†Versus NHE.

from ascorbate autoxidation,[4,5] yet it has been shown that the aerobic oxidation of ascorbate strictly requires a metal catalyst.[41-43] Based on the redox potentials listed in Tables 2 and 3, O_2^- production from ascorbate is not thermodynamically favored,[41] whereas H_2O_2 formation is:

$$AH^- + O_2 \longrightarrow \text{Ascorbyl radical (AH·)} + O_2^-$$
$$\Delta G^{o'} = +20.3 \text{ kcal/mole} \quad (7)$$

or

$$AH^- + O_2 \longrightarrow \text{Ascorbyl radical (A⁻)} + O_2^-$$
$$+ H^+ \quad \Delta G^{o'} = +10.1 \text{ kcal/mole} \quad (8)$$

$$AH^- + O_2 + H^+ \longrightarrow \text{dehydroascorbate (A)}$$
$$+ H_2O_2 \quad \Delta G^{o'} = -12.6 \text{ kcal/mole} \quad (9)$$

Because a direct oxidation of ascorbate by dioxygen does not occur,[41-43] H_2O_2 production does not occur, even though H_2O_2 production from ascorbate and dioxygen is thermodynamically favored. Thus, the spin restriction of dioxygen is a kinetic barrier that prevents the oxidation of organic biomolecules regardless of thermodynamic considerations.

Because the spin restriction affects the kinetics of these reactions, it must manifest itself in the activation energy (E_a). The spin restriction may be conceptualized as follows: for a chemical reaction to occur, the molecular orbitals of the reactants must overlap to give a transition state species. Overlap of molecular orbitals requires that the orbitals have the appropriate symmetry and occupancy.[c] The molecular orbitals of dioxygen have the appropriate symmetry to overlap with most biomolecules. The occupancy of dioxygen's antibonding orbits (1 electron per σ* orbital), however,

is inappropriate for reactivity with singlet molecules (2 spin paired electrons in one orbital), but is appropriate for radical reactions (1 unpaired electron). Because spin angular momentum must be conserved, it is not possible to mix two unpaired electrons with the same spin (dioxygen) with two spin paired electrons and achieve two sets of spin paired orbitals. Thus, the activation energy barrier that prevents the thermodynamically favored production of H_2O_2 is largely due to the inability of spin angular momentums to appropriately couple. If the spin restriction largely accounts for the large activation energy of reaction of dioxygen with biomolecules, then by using the Arrhenius equation[d] and a value of 23.4 kcal/mole as an approximation of E_a (the energy needed to convert dioxygen to singlet oxygen, that is, relieve the spin restriction), one obtains an apparent rate constant of 10^{-5} $M^{-1}s^{-1}$ (if the frequency factor is assumed to 10^{13} $M^{-1}s^{-1}$, the largest possible value in aqueous solutions). Other factors may also contribute to the activation energy, and as such, the estimate of E_a of 23.4 kcal/mole may represent the minimum amount of energy needed (see, for example, ref. 44). Consistent with this, in the absence of transition metals, the rate constant for the reaction of dioxygen with ascorbate has been reported[41] to be 6 × 10^{-7} s^{-1}, which results in an observed second order rate constant[e] of approximately 2 × 10^{-3} $M^{-1}s^{-1}$.

It also could be envisioned that triplet state dioxygen could convert to a singlet state while bound to an organic biomolecule (i.e., the transition state). This is unlikely since the spontaneous conversion of triplet dioxygen to a singlet molecule can be estimated to

[d]The Arrhenius equation is represented by $k = A \exp(-E_a/RT)$ where A is the frequency factor, and E_a is the activation energy.
[e]This rate constant is for the reaction at pH 7 of:

$$AH^- + O_2 \longrightarrow O_2^- + A^- + H^+$$
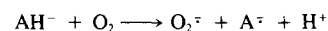
however, other species, such as the ascorbyl radical and the ascorbate dianion are present, yielding a complicated kinetic system.

[c]This approach has been successfully attempted to explain rates of reactions of oxygen and superoxide. (See Reference 97).

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Table 4. Standard Reduction Potentials of Metal Complexes at pH 7

Reactions	$E^{o\prime}$ / Volts*	Reference
$Fe^{3+} + e^- \longrightarrow Fe^{2+}$	0.11	33
EDTA—Fe(II) + $e^- \longrightarrow$ EDTA—Fe(II)	0.12	34
DETAPAC—Fe(III) + $e^- \longrightarrow$ DETAPAC—Fe(II)	0.03	21
Ferrioxamine—Fe(III) + $e^- \longrightarrow$ Ferrooxamine—Fe(II)†	−0.45	35
(1,10-phen)₃—Fe(III) + $e^- \longrightarrow$ (1,10 phen)₃—Fe(II)	+1.1	33
Cytochrome *c*—Fe(III) + $e^- \longrightarrow$ Cytochrome *c*—Fe(II)	0.27	36
Ferritin—Fe(III) + $e^- \longrightarrow$ Ferritin—Fe(II)	−0.19	37
$Cu^{2+} + e^- \longrightarrow Cu^+$	0.15	38
(1,10-phen)₃—Cu(II) + $e^- \longrightarrow$ (1,10 phen)₃—Cu(I)	0.17	38
SOD—Cu(II) + $e^- + H^+ \longrightarrow$ SOD—Cu(I) H	0.42	39
Transferrin—Fe(III) + $e^- \longrightarrow$ Transferrin—Fe(II)	−0.40‡	40

*Versus NHE.
†Ferrioxamine is desferrioxamine B chelated Fe(III), ferrooxamine is desferrioxamine B chelated Fe(II).
‡Value measured at pH 7.3.

occur at 10^{-7} s or longer that is, much longer than the lifetime of an excited triplet state). Because the lifetime of a typical transition state due to the collision of molecules is of the order of 10^{-12} s, the conversion of ground state triplet dioxygen to singlet state dioxygen does not occur within the time frame of the existence of a biomolecule-dioxygen transition state.[45]

It should be noted that in addition to the spin restriction, other barriers exist that prevent the reaction of dioxygen with biomolecules. This is exemplified by the reactivity of dioxygen with excited triplet state biomolecules; dioxygen does not always oxidize triplet molecules, but often physically quenches the excited state, producing the singlet ground state of the biomolecule and singlet oxygen. This is the well known Type II reaction of photochemistry which, through the use of photosensitizers, is used to generate singlet oxygen in vitro and in vivo.[46]

The reactions of biomolecules with singlet oxygen are not spin restricted and, as might be expected, singlet oxygen reacts as an electrophile with many biomolecules. For example, the biomolecular rate constants for the reactions of singlet oxygen with cysteine and histidine are $8.9 \times 10^6 M^{-1}s^{-1}$ and $5 \times 10^7 M^{-1}s^{-1}$, respectively.[46,47] As shown in Section I, endoperoxides are formed upon the addition of singlet oxygen to many biomolecules, or as in the case of cholesterol, the 5α-hydroperoxide is formed.[48]

In contrast to singlet ground state biomolecules, the radical forms of many biomolecules will react with dioxygen at near diffusion limited rates. For examples, the glutathionyl radical or polyunsaturated conjugated dienyl radicals react with dioxygen with rate constants of the order of $10^9 M^{-1}s^{-1}$.[49,50] Reactions of this type do not produce O_2^{-} or H_2O_2, instead the corresponding peroxyl radicals are formed. Conversely many of the radical forms of compounds of toxicological or pharmacological interest (see Table 3 for examples) react with dioxygen to produce superoxide. The paraquat radical $(PQ^+\cdot)$ will react with dioxygen to produce

O_2^{-} at near diffusion limited rates ($k = 8 \times 10^8$ $M^{-1}s^{-1}$).[51] On the other hand, the radical forms of some biomolecules, such as the ascorbyl radical,[23] react with dioxygen to produce O_2^{-} with a rate constant on the order of $10^2 M^{-1}s^{-1}$. This is approximately three orders of magnitude less than the rate of ascorbyl radical dismutation ($10^5 M^{-1}s^{-1}$, pH 7).[23] Thus, it should be apparent that the only significant *uncatalyzed* reactions of dioxygen are with radicals, but these radical reactions do not always result in O_2^{-} or H_2O_2 production.

C. Chemistry of transition metals

Although dioxygen cannot directly react with biomolecules at significant rates, many transition metals, which exist in several spin states, can relieve the spin restriction of dioxygen and enhance the rates of biomolecule oxidation. This becomes important since innumerable biomolecules bind transition metals. In particular, the oxygen, nitrogen, and sulphur atoms of biomolecules bind or ligate to transition metals. For an extensive review of metal chelation, consult the monograph by Bell.[52]

By definition, chelation requires the presence of two or more atoms on the same molecule capable of metal binding. For aliphatic compounds, a five membered ring involving the metal and the two chelating ligands is optimal for metal chelation. For example, glycine chelated Cu(II):
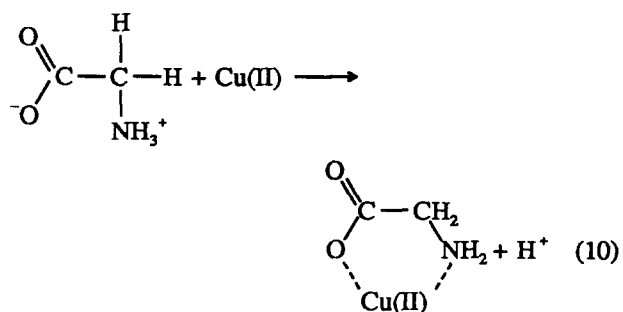
For aromatic compounds, or chelators containing double bonds, six membered rings tend to be the most stable. For example, salycilate chelated Fe(III):
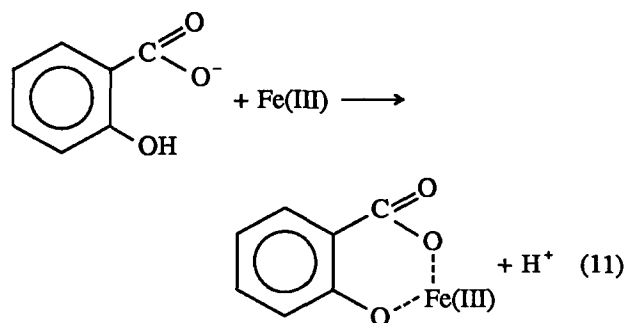
The chemicals listed in Table 1 all contain chelating functionalities. For example, the ortho hydroquinone moieties of dopamine or epinephrine are well suited for metal chelation:
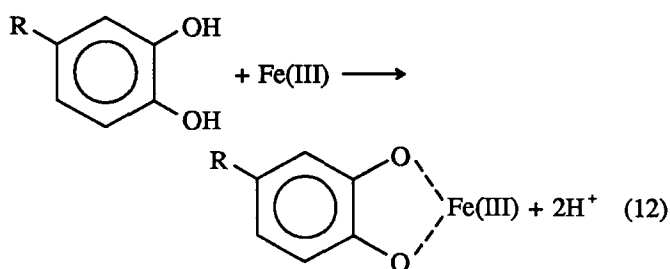
Coordination of transition metals to biomolecules almost always involves the d orbitals of the metal. In addition, dioxygen can also ligate to transition metals primarily through the d orbitals of the metal.[53] Therefore, transition metals may simultaneously bind to biomolecules and dioxygen, effectively serving as a bridge between the biomolecule and dioxygen. It has been postulated that ascorbate, iron, and dioxygen form such a complex.[54]

Although reduced flavins possess sufficiently negative redox potentials to reduce dioxygen, the reaction is still spin forbidden. However, flavins also possess a chelating functionality, and as such the "autoxidation" of many flavins may be due to metal-flavin-dioxygen interactions.

Reduced flavin

Oxidized flavin

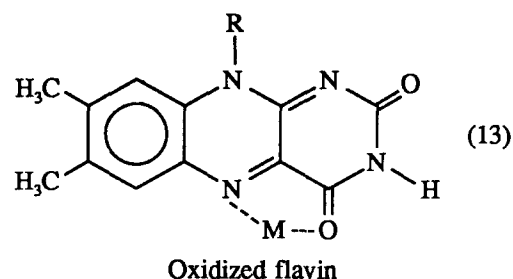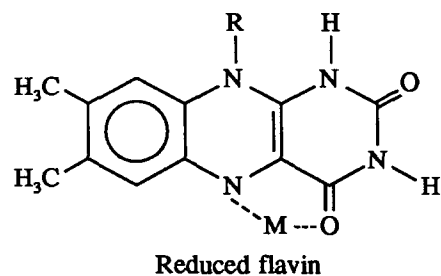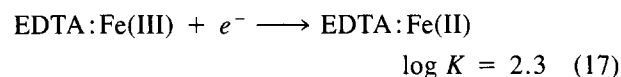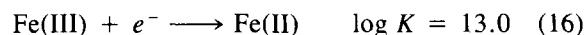Chelators in which oxygen atoms ligate the metal tend to prefer the oxidized forms of iron or copper, thus they decrease the redox potential of these metals. On the other hand, chelators in which nitrogen atoms primarily bind the metal prefer the reduced forms of iron or copper and tend to increase the redox potential of the metal. Inspection of the equilibrium constants for metal–ligand interactions, often called stability constants for the metal–ligand complex, gives an indication of the preference of a chelator for the various oxidation states of the metal. Stability constants for many biomolecules can be found in Smith and Martell.[55]

The stability constants of metal ligand interactions can be used to estimate the redox potential of the metal provided the same complexes are formed by both the oxidized and reduced form of the metal. For example, the log of the equilibrium constant for the formation of EDTA:Fe(III) is 25.0, and for EDTA:Fe(II) is 14.3.[55] From the standard reduction potential for the Fe(III)/Fe(II) couple ($E^o = 0.77$ volts) , a log K of 13.0 for reaction (16) is obtained:

$$\text{EDTA:Fe(III)} \longrightarrow \text{EDTA} + \text{Fe (III)}$$
$$\log K = -25.0 \quad (14)$$

$$\text{EDTA} + \text{Fe(II)} \longrightarrow \text{EDTA: Fe(II)}$$
$$\log K = 14.3 \quad (15)$$

$$\text{Fe(III)} + e^- \longrightarrow \text{Fe(II)} \quad \log K = 13.0 \quad (16)$$

$$\text{EDTA:Fe(III)} + e^- \longrightarrow \text{EDTA:Fe(II)}$$
$$\log K = 2.3 \quad (17)$$
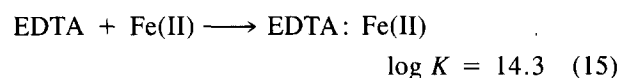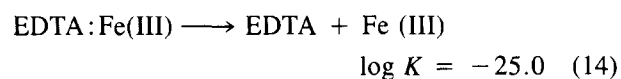
An $E^{o\prime}$ value of 0.14 volts is calculated for the EDTA:Fe(III)/EDTA:Fe(II) redox couple, in close agreement with the measured value of 0.12 volts.[33] Thus, the reduction potential of the Fe(III)/Fe(II) redox couple has been decreased by approximately 0.6 volts by chelation to EDTA, a chelator in which four oxygen and two nitrogen atoms bind the iron. One note of caution for this type of approximation is that hydrolysis of the metal ligand complex can greatly affect the stability of the complex, often making the effect of chelators on the redox chemistry of the metal difficult to predict.

Considerable information as to the products formed from the reactions of dioxygen with metal complexes can be obtained by examining the equilibria and the rate constants for the reactions. Again using EDTA chelated iron as an example, the following free energy charges can be calculated.

$$EDTA:Fe(II) + O_2 \longrightarrow EDTA:Fe(III) + O_2^{\bar{}}$$
$$\Delta G^{o\prime} = +6.2 \text{ kcal/mole} \quad (18)$$

$$2EDTA:Fe(II) + O_2 + 2H^+ \longrightarrow 2EDTA:Fe(III)$$
$$+ H_2O_2 \quad \Delta G^{o\prime} = -8.8 \text{ kcal/mole} \quad (19)$$

$$EDTA:Fe(II) + O_2^{\bar{}} + 2H^+ \longrightarrow EDTA:Fe(III)$$
$$+ H_2O_2 \quad \Delta G^{o\prime} = -19 \text{ kcal/mole} \quad (20)$$
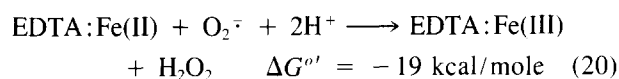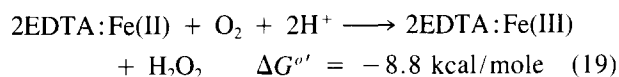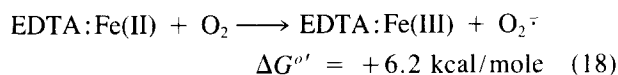
Although it is well known that EDTA:Fe(II) rapidly autoxidizes, it is unlikely that significant $O_2^{\bar{}}$ is produced, because $O_2^{\bar{}}$ production is thermodynamically unfavored. Any $O_2^{\bar{}}$ that may be produced would be rapidly converted to H_2O_2, either by $O_2^{\bar{}}$ dismutation or by reaction with EDTA:Fe(II) or EDTA:Fe(III) ($k \sim 10^6$ M^{-1}s^{-1} for either complex).[56-58]

Similarly, it is commonly believed that unchelated Fe(II) (i.e., Fe^{2+}) readily autoxidizes. However it has been observed that the rate of Fe^{2+} (e.g., FeCl$_2$) autoxidation is slow.[59] A common misconception is that the redox potential for Fe^{2+} at pH 7 is 0.77 volts, but significant $^{-}$OH ligation of the iron occurs, which decreases the redox potential by 0.66 volts.[32] Although the redox potential of Fe^{2+} at pH 7 is 0.11 volts, equivalent to that for EDTA chelated iron, the rates of the two autoxidations are markedly different. However, the rate expression for Fe^{2+} autoxidation is dependent on the square of the Fe^{2+} concentration,[45] similar to the EDTA:Fe(II) autoxidation rate expression.[60] Therefore autoxidation of unchelated Fe^{2+} by dioxygen will likely produce H_2O_2 rather than $O_2^{\bar{}}$ analogous to EDTA:Fe(II).

Based on reported stability constants for chemicals with chelating functionalities similar to those of the biomolecules listed in Table 1, it can be inferred that for the hydroquinones, chelation of the oxidized form of transition metals will be favored. However, these biomolecules also possess the appropriate redox potential to reduce Fe(III) or Cu(II).[61] Thus, molecules like 6-hydroxydopamine or epinephrine are capable of reducing Fe(III) to Fe(II), but because the ligand preferentially binds Fe(III) over Fe(II), the ligand also catalyzes the autoxidation of Fe(II), with concomitant oxidation of the ligand. A similar situation exists for thiol compounds, such as cysteine or glutathione, which are well suited for metal chelation, and have sufficient redox potential to reduce Fe(III) and Cu(II). While many of the thiol compounds do not strongly favor the

oxidized form of the metal, other factors, such as the reaction buffer (see Section IV) may contribute to oxidation of the reduced metal, establishing a redox cycle between the thiol and the metal. Indeed several recent reports have shown that a metal catalyst is required for oxidation of the ligand, and these reactions are greatly affected by factors such as buffers.[62-64]

It is important to remember, however, that if these metals are acting as true catalysts, then the thermodynamics of the reaction of dioxygen with catechols, thiols, etc. have not changed. That is, if the reaction of dioxygen with a thiol, for example, is thermodynamically unfavorable, then even in the presence of a metal ion, the reaction is still unfavored. It is often stated that thermodynamically unfavored reactions can be "pulled" to the right (i.e., towards product formation) if the products formed are unstable and hence the system constantly tries to achieve equilibrium. However, as was stated above, due to the large activation energy associated with a direct reaction of dioxygen with biomolecules, the rate at which equilibrium is established is so slow that these reactions can be considered insignificant. However, if a metal ion catalyst is present, the activation energy of the reaction has been lowered such that the rate at which equilibrium is established has been enhanced. Alternatively, the binding of metal ions to biomolecules can result in mixing of the ligand orbitals with the metal orbitals resulting in shifts in the redox potential of the ligand or metal or both, in effect a chemically distinct species different from the metal ion or ligand alone results.

Finally, certain chemical properties of copper must be remembered when considering redox reactions involving biomolecules. For example, the coordination geometry of Cu(I) and Cu(II) are frequently different. Copper (I) complexes are often tetrahedral, whereas Cu(II) complexes have square planar geometry. In contrast, both Fe(II) or Fe(III) complexes are almost always octahedral or distorted octahedral. The fact that the two valence states of copper usually have different coordination environments may impose a kinetic hinderance on redox reactions involving copper. Also, some Cu(I) complexes disproportionate to yield Cu(0) and Cu(II) in addition to Cu(I) oxidation by dioxygen.

In summary, the electronic structure of dioxygen imposes severe kinetic limitations on the direct reaction of dioxygen with biomolecules, and thus autoxidation of singlet biomolecules is, in general, not a significant process (rate constants $\sim 10^{-5}$ M^{-1}s^{-1}). Transition metals greatly influence the electronic structure of dioxygen, and thereby often facilitate the oxidation of biomolecules by dioxygen, but because these oxidations are metal catalyzed, they cannot be considered autoxidations. Finally, the chemical details of these reactions (e.g., rate, products formed, etc.) will vary

greatly, depending on the metal, the biomolecule ligand and other factors including pH and the type of buffer used.

III. BIOLOGICAL CONSIDERATIONS

Based on the low solubilities of copper or iron at physiological pH [10^{-9} M for $Cu(II)_{aq}$; 10^{-18} M for $Fe(III)_{aq}$], biological systems must use chelation to solubilize these metals so that they can be transported to and from storage proteins and incorporated into enzymes. Thus, a question of paramount importance to the biochemistry of these metals is, to what are these metals chelated? Unfortunately, the chemical identity of these chelators are unknown, although good evidence suggests that glutathione is the intracellular chelator of copper.[65] Because iron or copper are efficient catalysts of oxidation reactions, many of which may be irreversible and toxic, the concentrations of catalytic iron or copper chelates in biology is low, and the metabolism of these metals must be stringently controlled to prevent these oxidations.

A. Transition metal metabolism

The majority of intracellular biomolecules that participate in redox reactions in vivo exist in the reduced form. It is likely then that intracellular iron or copper complexes exist primarily as Fe(II) and Cu(I) chelates. Consistent with this, most enzymes that require iron as a cofactor or use iron as a substrate require the iron to be Fe(II). Therefore, it is unlikely that significant chelation of iron will occur in vivo by biomolecules such as the catecholamines, because they preferentially bind Fe(III). Chelation of iron in a reducing environment by chelates that prefer Fe(III) would result in a cycle of iron oxidation and reduction, and consumption of dioxygen, producing O_2^-, H_2O_2 or both. The same would be true for an Fe(III) chelator that was also a reductant, except that the chelator would also be oxidized. From an evolutionary standpoint, the incessant redox cycling of a biomolecule required for other biological functions would not be biologically advantageous.

Based on this, several criteria can be asserted for the chemical properties of the physiological chelator(s) of iron and copper:

1. the chelators must bind these metals such that autoxidation of the reduced metal occurs slowly,[66,67] if at all, thereby preventing O_2^- or H_2O_2 production as well as possible oxidation of the chelator;

2. the chelate must bind the metal with an affinity at least comparable to that of other biomolecules that are strong chelators, but based on criteria 1 are unlikely to bind metals physiologically, (e.g., epinephrine);

3. the in vivo chelator(s) must be kinetically inert to substitution reactions of the metal, that is, the metal must remain associated with the chelator until properly metabolized.

Taken together these criteria insure that the number of metal chelates formed are few, making metabolism of the metal easier to control. It is likely, perhaps even necessary, that the physiological chelators of iron and copper are different. Again this would make metabolic regulation of the metals easier, because saturation of the chelator for iron would not saturate the chelator for copper.
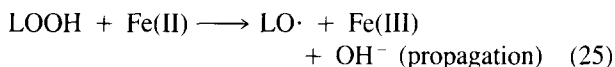
Additional regulation of iron and copper metabolism is achieved by storing these metals in the oxidized state, in ferritin and metallothionein, respectively. Once iron or copper are bound by ferritin or metallothionein, reactions of these metals with most biomolecules is prevented. In effect these metals have been made redox inert towards biological reductants. For example, in order to remove iron from ferritin, the iron must be reduced in the presence of a chelator, yet biologically relevant reductants such as ascorbate or glutathione do not reduce ferritin iron at significant rates.[68] Thus storage of iron by ferritin probably helps prevent useless oxidation of reductants such as ascorbate or glutathione. While the physiological reductants of ferritin iron are unknown, they are likely to be few. In addition, since ferritin is the major storage form of iron and regulates the concentration of low molecular weight chelatable iron, the deposition and release of ferritin iron must be tightly regulated and therefore probably controlled by enzymes.

B. Transition metal toxicities

Under physiological conditions, it is unlikely that the biomolecules in Table I chelate iron or copper. It is plausible, however, that chelation of iron or copper by these biomolecules may occur if the physiological chelate becomes saturated, as might happen under toxicological, pathological, or pharmacological conditions involved in "oxidative stress." For example as stated above ferritin iron is released upon reduction in the presence of a chelator. Experimentally it has been shown that redox cycling compounds, such as diquat catalyze the release of iron from ferritin in vitro,[69] and in vivo.[70] Thus, the toxicity of diquat may be related to a rapid rise in chelatable iron leading to saturation of the normal physiological chelator of iron, the establishment of other iron chelates, or both, caused by enhanced ferritin iron release. These other chelators of iron may catalyze free radical reactions such as lipid peroxidation, reactions probably not normally catalyzed by the physiological chelates of iron. If in fact

the physiologic chelators of iron or copper are satur-
able, then the likelihood that these chelators are present
at high concentration is slight. As such, it is unlikely
that chemicals that exist at relatively high concentra-
tions such as ADP, glutathione, citrate, etc. are the
physiological chelators of iron or copper. However,
significant metal chelation by these compounds may
occur under nonphysiological conditions.

The toxicities of chemicals such as diquat, paraquat,
adriamycin, alloxan, etc. have been attributed to "ox-
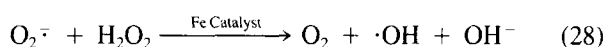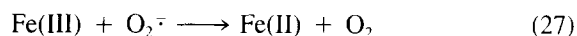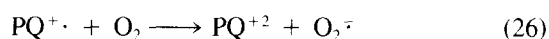idative stress." As stated above, the toxicities of these
compounds may arise from altered iron or copper me-
tabolism. A likely question then is, what is the role of
iron or copper in these toxicities? To date, the most
widely studied biomolecule oxidation reaction result-
ing from "oxidative stress" is lipid peroxidation. Al-
though the role of copper in the catalysis of lipid per-
oxidation is not clear,[71] iron is a known catalyst of
lipid peroxidation, as well as a catalyst for DNA and
protein oxidation.[41,72,73] The predominant theory of iron
catalyzed lipid peroxidation is that iron catalyzes $\cdot$OH
formation from H_2O_2, which reacts with polyunsatu-
rated fatty acids, initiating a chain reaction.

$$Fe(II) + H_2O_2 \longrightarrow Fe(III) + \cdot OH + OH^- \quad (21)$$

$$LH + \cdot OH \longrightarrow L\cdot + H_2O \text{ (initiation)} \quad (22)$$

$$L\cdot + O_2 \longrightarrow LOO\cdot \text{ (propagation)} \quad (23)$$

$$LOO\cdot + LH \longrightarrow LOOH + L\cdot \text{ (propagation)} \quad (24)$$

$$LOOH + Fe(II) \longrightarrow LO\cdot + Fe(III) \\ + OH^- \text{ (propagation)} \quad (25)$$
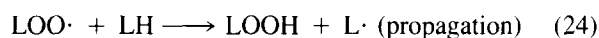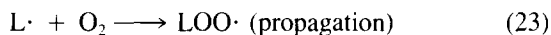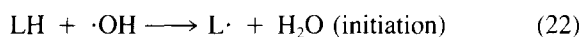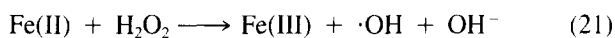
According to this theory, the role of iron in lipid
peroxidation is to catalyze either the formation of in-
itiators or catalyze hydroperoxide decomposition and
hence the propagation of lipid peroxidation. However,
based on several studies of model lipid peroxidation
systems,[59,74,75] an alternate proposal for the role of iron
in lipid peroxidation has been put forth. It was observed
that lipid peroxidation catalyzed by ADP chelated Fe(II)
occurred only after a significant lag phase during which
no lipid peroxidation was observed, however, this lag
phase could be eliminated by the addition of ADP che-
lated Fe(III).[74] The requirement for both Fe(II) and
Fe(III) for lipid peroxidation has subsequently been
observed several times either by directly adding Fe(II)
and Fe(III)[75] or by oxidation of added Fe(II)[59] or re-
duction of added Fe(III).[43] The predominance of iron
in either the Fe(II) or Fe(III) state results in no lipid
peroxidation. Thus, an Fe(II):Fe(III) complex, which
probably requires dioxygen, has been proposed as the
initiator of lipid peroxidation.[64] If this Fe(II):Fe(III)
complex is in fact the catalyst of lipid peroxidation,

then iron is directly involved in *both* the initiation and
propagation of lipid peroxidation.

As discussed in Section II, O_2^- or H_2O_2 production
from the radical forms of paraquat, adriamycin, etc.
is thermodynamically favored, occurs rapidly, and has
been attributed to the toxicity of these chemicals. The
toxicity of O_2^- has been related to the iron catalyzed
Haber-Weiss reaction (reaction (28)).

$$PQ^+\cdot + O_2 \longrightarrow PQ^{+2} + O_2^- \quad (26)$$

$$Fe(III) + O_2^- \longrightarrow Fe(II) + O_2 \quad (27)$$

$$Fe(II) + H_2O_2 \longrightarrow Fe(III) + \cdot OH + OH^- \quad (21)$$

$$O_2^- + H_2O_2 \xrightarrow{\text{Fe Catalyst}} O_2 + \cdot OH + OH^- \quad (28)$$

Since the concentration of Fe(III) in biology is low,
except in the iron storage protein ferritin, it can be
presumed then, that one major effect of O_2^- or other
radical species is to reduce ferritin iron, such that it
can be released as Fe(II). Once the iron has been re-
leased from ferritin it may be kept in the reduced state
by a number of cellular reductants, such as ascorbate,
or even by O_2^-. Alternatively, the iron may autoxidize
or be oxidized by O_2^-, or H_2O_2 produced by the dis-
mutation of O_2^-.

The rapid and indiscriminate manner with which
$\cdot$OH reacts has led several investigators to question the
role $\cdot$OH in the development of "oxidative stress."
Specifically, it has been argued that the lifetime of
$\cdot$OH is too short to cause significant damage to lipids,
unless $\cdot$OH is generated in very close proximity to the
lipid molecules. It is possible that $\cdot$OH could oxidize
DNA or proteins if iron is bound to these molecules.
However, it is important to note that oxidants weaker
than $\cdot$OH can damage lipids, DNA, and proteins. As
stated above, an Fe(II):Fe(III) complex has been pos-
tulated as the initiator of lipid peroxidation. In this
proposed model, the role of O_2^- as a ferritin iron re-
ductant is still valid. Once the iron is released from
ferritin, however, the importance of O_2^- and H_2O_2 no
longer reside with $\cdot$OH production, but with Fe(II)
oxidation. In other words, in reducing systems, such
as within the cell, oxidants of iron are required to form
an Fe(II):Fe(III) complex and hence for lipid peroxi-
dation to occur. Experimentally, if the rate of oxidation
of iron is marginal in comparison to its rate of reduction
(i.e., if Fe(II) predominates), then the formation of an
Fe(II):Fe(III) complex cannot occur, and lipid per-
oxidation does not occur. Similarly, if the rates of
Fe(II) oxidation by O_2, O_2^-, H_2O_2, or other oxidants
far exceed the cellular capacity to reduce Fe(III), again
an Fe(II):Fe(III) complex will not form. Partial oxi-
dation of the Fe(II) pool may produce the Fe(II):Fe(III)

complex and result in the initiation of lipid peroxidation. Oxidation of Fe(II) by H_2O_2 would also produce ·OH. A comparison of the rates of Fe(II) oxidation by O_2, O_2^-, or H_2O_2 shows that the chelation of iron greatly affects the rate at which Fe(II) is oxidized by these oxidants, however, in general O_2^- oxidizes Fe(II) much faster than O_2 or H_2O_2.** Thus, the relative importance of iron reduction versus oxidation will only be known when the physiological iron chelator(s) have been identified.

Many of the compounds in Table 1 are considered prooxidant or antioxidants or both (e.g., ascorbate). Often the prooxidant activity of these compounds is related to O_2^- or H_2O_2 production from these molecules. However, as discussed earlier, production of O_2^- and H_2O_2 cannot occur in the absence of transition metals. Because these molecules can be effective iron chelators, their prooxidant effect may be due to altered reactivity of iron towards O_2, O_2^-, H_2O_2. In addition, these compounds are iron reductants, and could promote ·OH production by providing a source of Fe(II), or promote the formation of an Fe(II):Fe(III) complex. Regardless, the prooxidant activity of these compounds must in some way be related to metals. It has also been shown that the antioxidant activity of many of these compounds is also metal dependent. Specifically, the antioxidant activity of ascorbate[43] and cysteine[82] has been related to complete reduction of iron. In this regard, the pro- and antioxidant activity of many of the compounds in Table 1 is more easily reconciled with the proposed Fe(II):Fe(III) model, than with the traditional Fenton reaction type model.

IV. EXPERIMENTAL CONSIDERATION

The undertaking of a study of metal catalyzed oxidation of biomolecules, while at first may seem quite straightforward, is frequently fraught with numerous complications. The two most commonly encountered and unrecognized problems associated with studying metal catalyzed oxidations are first, contamination of reagents with trace amounts of metals, and second, undesirable metal ligation by components of the reaction mixture.

The presence of contaminating metals in reagents used for experimental biology can lead to unpredictable changes in the redox chemistry of the metal under study, due to 1) exchange of contaminating metals with the metal of interest, 2) affects on the rates of metal oxidation or reduction. Additionally, the contaminating metals may serve as catalysts, and as such the observed

results might be incorrectly assigned to the metal of interest. The contaminating metals may also cause the disappearance or formation of chemical species (e.g., O_2^- scavenging or production), that might not otherwise be present.

Several strategies are available to remove metal contaminants from reagents. However, many of these techniques rely on the establishment of a competitive equilibrium between the reagent and the treatment process. For example, chelating resins, such as Chelex-100®, are frequently used to remove metals from reagents.[42,83] However, if the reagent possesses significant chelating capacity, or contains less metal than the resin, it is likely that treatment of the reagents with chelating resin will not remove contaminating metals, and may in fact increase the level of metal contamination in the reagent.[83] This is frequently the case for buffers such as phosphate or Tris, which either retain significant metal contamination after Chelex-100® treatment, or they may remove metals from the resin. Thus, it is necessary to determine the efficacy of the technique used to remove metals from the reagents. A simple test, which depends on the metal catalyzed oxidation of ascorbate, should be used to evaluate the level of iron or copper contamination both before and after any treatment procedure.[42] An alternative method to remove metals from reagents is to extract an aqueous solution of the metal with a nonpolar chelator in an organic solvent. For example, 4,7-diphenyl-1,10-phenanthroline can be used to extract metals from aqueous solutions because this chelator is only soluble in organic solvents, such as 1-pentanol. It should be noted that this method may leave residual organic solvent in the aqueous phase.

Alternatively, DETAPAC is frequently used to chelate contaminating metals from reagents in a form presumed to be redox inert.[84] Unfortunately, chelation of iron by DETAPAC does not render the iron redox inert, but alters the rate at which DETAPAC chelated iron is oxidized or reduced, in comparison to other metal chelates, such as EDTA chelated iron.[42,56,58,85,86] As such, it is not valid to assume that the addition of DETAPAC to reagents has converted contaminating metals, in particular iron, into a redox inert form and thereby eliminating any metal dependent effects.[86] In a similar vein, under appropriate conditions, it is possible that even the addition of deferrioxamine to chelate iron has not rendered the iron redox inert.[35,86,87] Thus, it appears that a "catch-all" chelator does not exist that will bind transition metals in such a way as to render the metals redox inert.

Many of the buffers used in experimental biology, such as phosphate or Tris, in addition to presenting a problem with metal contamination, also ligate metals, properties that are obviously related. Any chemical that strongly binds metals, so as to make removal from

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**For oxidation of Fe(II) by O_2^-, $k \sim 10^7$ $M^{-1}s^{-1}$,[57,58,76,77] while for the oxidation of Fe(II) by H_2O_2, $k \sim 10^2-10^4$ $M^{-1}s^{-1}$.[78-81]

reagents difficult, will also bind metals that have been added to a reaction mixture. Perhaps the most nefarious buffer used in studying metal catalyzed biomolecule oxidations is phosphate. Phosphate catalyzes rapid Fe(II) oxidation[88] (Table 5) because of its preference for Fe(III). Based on the rate expression for Fe(II) autoxidation in phosphate buffer, it is likely that significant O_2^- is produced (the rate expression depends on the concentration of Fe(II) to the first order),[45,88] unlike many chelator catalyzed Fe(II) autoxidation reactions. The Fe(III) formed by phosphate catalyzed Fe(II) autoxidation is insoluble ([Fe(III)aq] = 10^{-18} M), and in the presence of a weak iron chelator (i.e., log $K < 10$) phosphate will effectively compete with the chelator for iron. Although it has been argued that phosphate serves as part of the buffering capacity of the cell, its use experimentally for metal catalyzed oxidation reactions may be imprudent due to the wide array of undesirable or unpredictable effects.

Tris buffers also influence metal catalyzed reactions. Tris is an iron and copper chelator and seems to have its most pronounced effects when used in conjunction with weak metal chelators. For example, in a lipid peroxidation system relying on H_2O_2 dependent oxidation of Fe(II), Tris inhibited lipid peroxidation when no Fe(II) chelator was added except phospholipid.[64,89] However when the Fe(II) was chelated to citrate, Tris had no effect on the rate of lipid peroxidation.[64] In addition Tris is also an effective ·OH scavenger.[90] That is, it may possess metal independent effects on biomolecule oxidation studies.

The "Good" buffers also influence metal redox chemistry.[63] Although these buffers are not generally

Table 5. Effects of Some Buffers on Fe(II) Autoxidation

Buffer	30 Sec	5 Min
% Fe(II) oxidized		
Phosphate	87	98
HEPES	6	99
Tris	6	28
NaCl	0	5
% ADP chelated Fe(II) oxidized		
Phosphate	96	100
HEPES	11	63
Tris	17	70
NaCl	11	58

Note. Reaction mixtures (3 ml) contained the indicated buffer or salt solution at concentrations of 50 mM, pH 7.2. 200 μM ferrous chloride or 1 mM ADP and 200 μM ferrous chloride were added to initiate the reaction. Aliquots (0.35 ml) were removed at the time of Fe(II) addition, and 30 sec and five minutes after, mixed with 1 mM ferrozine (2 ml total volume) and the absorbance at 564 nm due to the tris ferrozine:Fe(II) complex was measured.

considered to be metal chelators, it is possible that these compounds ligate to chelator-metal complexes, thereby altering the redox chemistry of the metal chelate (see Table 5). In this regard, HEPES buffer is frequently used in studies involving ferritin. In particular, several investigators have examined the incorporation of iron into ferritin using a system comprised of Fe(II) as $Fe(NH_4)_2(SO_4)_2$, HEPES buffer, and apoferritin.[91,92] From these studies, it has been asserted that apoferritin possesses a ferroxidase activity. Other studies have shown that apoferritin or ferritin, however, does not appear to oxidize Fe(II).[93,94] Since HEPES buffer can affect iron redox chemistry,[63] it is possible that the observed Fe(II) oxidation in the presence of apoferritin was due to a HEPES—Fe(II) or HEPES—Fe(II)—apoferritin interaction, and thus may not reflect a true function of ferritin. Unfortunately, there does not seem to be a buffer suitable for studies of metal catalyzed or metal dependent processes.

Finally, in certain experimental systems, such as tissue culture studies, it may be impossible to remove metals or to avoid buffer problems, because both trace metals and buffers may be required. However, before definitive conclusions are drawn about observed effects, a series of preliminary studies on the effects of added reagents may be warranted. Measurement of a few key parameters such as oxygen uptake, H_2O_2 formation, etc., such as the studies of Biaglow et al.,[95] may assist in the interpretation of observed results.

V. CONCLUSIONS

True autoxidation is defined as the spontaneous oxidation in air of a substance not requiring catalysts. In this paper we have summarized experimental and theoretical evidence that true autoxidation is a kinetically limited process for singlet ground state molecules. Thus, we hypothesize that autoxidation of biomolecules is, in general, a negligible process (occurring in the time frame of days) and further we hypothesize that these molecules will undergo significant oxidation only in the presence of catalytic metals, such as copper or iron.

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ABBREVIATIONS

DETAPAC—Diethylene triamine pentaacetic acid

EDTA—Ethylene diamine tetraacetic acid

HEPES—4(2-Hydroxyethyl) 1-piperazine ethane sulfonic acid

SOD—Superoxide dismutase

PQ—Paraquat