TRANSITION METALS AS CATALYSTS OF "AUTOXIDATION" REACTIONS

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Abstract—Superoxide (O2⁻), hydrogen peroxide (H2O2), and hydroxyl radical (·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 (·OH) initiate oxidation of lipids, protein, or DNA (reaction (1)). Subsequent propagation reactions (reactions (2,3)) further oxidize these molecules via numerous reaction pathways.

\[
\text{RH} + \cdot\text{OH} \rightarrow \text{R}^- + \text{H}_2\text{O} \quad (1)
\]

\[
\text{R}^- + \text{O}_2 \rightarrow \text{ROO}^- \quad (2)
\]

\[
\text{ROO}^- + \text{RH} \rightarrow \text{ROOH} + \text{R}^- \quad (3)
\]

The formation of ·OH for reaction (1) requires the sequential one electron reduction of dioxygen to superoxide (O2⁻), hydrogen peroxide (H2O2), and finally to ·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)).
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 $10^{-15}$ 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.

$$\text{RH}_2 + O_2 \rightarrow \text{RH}^+ + O_2^- + H^+ \quad (5)$$
$$\text{RH}_2 + O_2 \rightarrow \text{R} + H_2O_2 \quad (6)$$

These types of reactions are termed "autoxidations," defined as the "...apparently uncatalyzed oxidation of a substance exposed to the oxygen of the air...". 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.

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.$^4$

```
\[ \text{ENERGY} \]
\[ \uparrow \uparrow \quad \uparrow \uparrow \quad \uparrow \uparrow \quad \uparrow \uparrow \]
\[ \sigma_{p_z} \quad \pi_{p_x} \quad \pi_{p_y} \quad \pi_{p_z} \]
```

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 ($O\equiv O\cdot$), 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 $\pi^*$ orbitals, probably accounting for the unfavorable free energy change of this reaction. The addition of two electrons to dioxygen, forming hydrogen peroxide ($H_2O_2$), results in significant electronic rearrangement yielding two sets of $\sigma$ 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 $H_2O_2$, 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:

$$\Delta G' = -nFE'$$
$$\Delta G'' = -RT\ln K$$
$$\ln K = \frac{-nFE'}{RT}$$

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>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbate</td>
<td><img src="image" alt="Ascorbate Structure" /></td>
<td>4,5</td>
</tr>
<tr>
<td>Cysteine</td>
<td><img src="image" alt="Cysteine Structure" /></td>
<td>6</td>
</tr>
<tr>
<td>Dopamine</td>
<td><img src="image" alt="Dopamine Structure" /></td>
<td>7,8</td>
</tr>
<tr>
<td>Epinephrine</td>
<td><img src="image" alt="Epinephrine Structure" /></td>
<td>9</td>
</tr>
<tr>
<td>Flavins</td>
<td><img src="image" alt="Flavins Structure" /></td>
<td>10</td>
</tr>
<tr>
<td>Glutathione</td>
<td><img src="image" alt="Glutathione Structure" /></td>
<td>11</td>
</tr>
<tr>
<td>Homogenesic acid</td>
<td><img src="image" alt="Homogenesic acid Structure" /></td>
<td>12</td>
</tr>
<tr>
<td>Cysteamine*</td>
<td>HSC\textsubscript{2}CH\textsubscript{2}NH\textsubscript{2}</td>
<td>13</td>
</tr>
</tbody>
</table>
Table 1. (Continued)

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dialuric Acid*</td>
<td><img src="image" alt="Structure" /></td>
<td>14</td>
</tr>
<tr>
<td>Dihydroxy fumarate*</td>
<td><img src="image" alt="Structure" /></td>
<td>15,16</td>
</tr>
<tr>
<td>Dithiothreitol*</td>
<td><img src="image" alt="Structure" /></td>
<td>17</td>
</tr>
<tr>
<td>6-Hydroxydopamine*</td>
<td><img src="image" alt="Structure" /></td>
<td>14</td>
</tr>
<tr>
<td>Pyrogallol*</td>
<td><img src="image" alt="Structure" /></td>
<td>18</td>
</tr>
</tbody>
</table>

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

RH₂ + O₂ → RH⁺ + O₂⁻ + H⁺  
(1 electron transfer) (5)

RH₂ + O₂ → R + H₂O₂  
(2 electron transfer) (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°' indicates that the half reaction is thermodynamically unfavored (ΔG°' > 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 O₂⁻ production favored), the reactant other than dioxygen (i.e., RH₂) must have an E°' < -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 O₂⁻ is produced

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

<table>
<thead>
<tr>
<th>Reaction</th>
<th>E°'/Volts†</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>O₂⁻ + e⁻ → O₂⁻</td>
<td>-0.33</td>
<td>21</td>
</tr>
<tr>
<td>O₂(a) + e⁻ → O₂⁻</td>
<td>-0.161</td>
<td>22</td>
</tr>
<tr>
<td>O₂⁻ + 2 e⁻ + 2H⁺ → H₂O₂</td>
<td>0.33</td>
<td>23</td>
</tr>
<tr>
<td>O₂⁻ + 3 e⁻ + 3H⁺ → H₂O₂⁻ + -OH</td>
<td>0.36</td>
<td>21</td>
</tr>
<tr>
<td>O₂⁻ + 4 e⁻ + 4H⁺ → 2H₂O</td>
<td>0.82</td>
<td>21</td>
</tr>
<tr>
<td>O₂⁻ + e⁻ + 2H⁺ → H₂O₂</td>
<td>0.94</td>
<td>21</td>
</tr>
<tr>
<td>H₂O₂⁻ + e⁻ + H⁺ → H₂O + -OH</td>
<td>0.33</td>
<td>21</td>
</tr>
<tr>
<td>-OH + e⁻ + H⁺ → H₂O</td>
<td>2.31</td>
<td>23</td>
</tr>
</tbody>
</table>

*These values of E°' are for aqueous solutions with O₂ 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 O₂ instead of 1 atm.
from ascorbate autoxidation, yet it has been shown that the aerobic oxidation of ascorbate strictly requires a metal catalyst. Based on the redox potentials listed in Tables 2 and 3, O₂⁻ production from ascorbate is not thermodynamically favored, whereas H₂O₂ formation is:

\[
AH^- + O_2 \rightarrow \text{Ascorbyl radical (AH·)} + O_2^\cdot \quad \Delta G^\circ = +20.3 \text{ kcal/mole} \ (7)
\]

or

\[
AH^- + O_2 \rightarrow \text{Ascorbyl radical (A^-·)} + O_2 + H^+ \quad \Delta G^\circ = +10.1 \text{ kcal/mole} \ (8)
\]

\[
AH^- + O_2 + H^+ \rightarrow \text{dehydroascorbate (A)} + H_2O_2 \quad \Delta G^\circ = -12.6 \text{ kcal/mole} \ (9)
\]

Because a direct oxidation of ascorbate by dioxygen does not occur, even though H₂O₂ production does not occur, even though H₂O₂ production from ascorbate and oxygen 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. The molecular orbitals of dioxygen have the appropriate symmetry to overlap with most biomolecules. The occupancy of dioxygen's antibonding orbitals (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 H₂O₂ 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 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 to be 6 x 10⁻⁷ s⁻¹, which results in an observed second order rate constant of approximately 2 x 10⁻⁷ 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

\[k = A \exp(-E_a/RT)\]

where A is the frequency factor, and E_a is the activation energy.

This approach has been successfully attempted to explain rates of reactions of oxygen and superoxide. (See Reference 97).
Table 4. Standard Reduction Potentials of Metal Complexes at pH 7

<table>
<thead>
<tr>
<th>Reactions</th>
<th>(E^{°}/\text{Volts}^*)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{Fe}^{3+} + e^- \rightarrow \text{Fe}^{2+})</td>
<td>0.11</td>
<td>33</td>
</tr>
<tr>
<td>(\text{EDTA--Fe(II)} + e^- \rightarrow \text{EDTA--Fe(II)})</td>
<td>0.12</td>
<td>34</td>
</tr>
<tr>
<td>(\text{DETAPAC--Fe(III)} + e^- \rightarrow \text{DETAPAC--Fe(II)})</td>
<td>0.03</td>
<td>21</td>
</tr>
<tr>
<td>(\text{Ferrioxamine--Fe(III)} + e^- \rightarrow \text{Ferrioxamine--Fe(II)})</td>
<td>-0.45</td>
<td>35</td>
</tr>
<tr>
<td>((1,10\text{-phen})_3--\text{Fe(III)} + e^- \rightarrow (1,10\text{-phen})_3--\text{Fe(II)})</td>
<td>+1.1</td>
<td>33</td>
</tr>
<tr>
<td>(\text{Cytochrome c--Fe(III)} + e^- \rightarrow \text{Cytochrome c--Fe(II)})</td>
<td>0.27</td>
<td>36</td>
</tr>
<tr>
<td>(\text{Ferritin--Fe(III)} + e^- \rightarrow \text{Ferritin--Fe(II)})</td>
<td>-0.19</td>
<td>37</td>
</tr>
<tr>
<td>(\text{Cu}^{2+} + e^- \rightarrow \text{Cu})</td>
<td>0.15</td>
<td>38</td>
</tr>
<tr>
<td>((1,10\text{-phen})_3--\text{Cu(II)} + e^- \rightarrow (1,10\text{-phen})_3--\text{Cu(I)})</td>
<td>0.17</td>
<td>38</td>
</tr>
<tr>
<td>(\text{SOD--Cu(II)} + e^- + H^+ \rightarrow \text{SOD--Cu(I)} + H)</td>
<td>0.42</td>
<td>39</td>
</tr>
<tr>
<td>(\text{Transferrin--Fe(III)} + e^- \rightarrow \text{Transferrin--Fe(II)})</td>
<td>-0.40^t</td>
<td>40</td>
</tr>
</tbody>
</table>

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

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):

\[
\text{O} \quad \text{C} \quad \text{C} \quad \text{H} + \text{Cu(II)} \rightarrow \\
\text{O} \quad \text{H}^+ \quad \text{NH}_2^+ 
\]

\[
\text{O} \quad \text{C} \quad \text{C} \quad \text{H} + \text{Cu(II)} \rightarrow \\
\text{O} \quad \text{C} \quad \text{CH}_2 \quad \text{NH}_2 \quad \text{H}^+ 
\]
For aromatic compounds, or chelators containing double bonds, six-membered rings tend to be the most stable. For example, salicylate chelated Fe(III):

\[
\begin{align*}
\text{C}_6\text{H}_5\text{OH}^- + \text{Fe(III)} & \rightarrow \\
\text{C}_6\text{H}_5\text{O}^- + \text{Fe(III)} + \text{H}^+ \quad (11)
\end{align*}
\]

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:

\[
\begin{align*}
\text{R} - \text{OH} + \text{Fe(III)} & \rightarrow \\
\text{R} - \text{OH} + \text{Fe(III)} + 2\text{H}^+ \quad (12)
\end{align*}
\]

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. 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.

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.

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.

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. From the standard reduction potential for the Fe(III)/Fe(II) couple \( E^\circ = 0.77 \) volts, a log \( K \) of 13.0 for reaction (16) is obtained:

\[
\begin{align*}
\text{EDTA:Fe(III)} & \rightarrow \text{EDTA} + \text{Fe (III)} \\
\log K &= -25.0 \quad (14) \\
\text{EDTA} + \text{Fe(II)} & \rightarrow \text{EDTA: Fe(II)} \\
\log K &= 14.3 \quad (15) \\
\text{Fe(III)} + e^- & \rightarrow \text{Fe(II)} \\
\log K &= 13.0 \quad (16)
\end{align*}
\]

An \( E^\circ \) 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. 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 rate constants for the reactions. Again using EDTA can be obtained by examining the equilibria and the charges can be calculated.

\[
\text{EDTA:Fe(II)} + O_2 \rightarrow \text{EDTA:Fe(III)} + O_2^-
\]

\[\Delta G^{\circ} = +6.2 \text{ kcal/mole} \ (18)\]

\[2\text{EDTA:Fe(II)} + O_2 + 2H^+ \rightarrow 2\text{EDTA:Fe(III)} + H_2O_2\]

\[\Delta G^{\circ} = -8.8 \text{ kcal/mole} \ (19)\]

\[\text{EDTA:Fe(II)} + O_2^+ + 2H^+ \rightarrow \text{EDTA:Fe(III)}\]

\[+ H_2O_2; \Delta G^{\circ} = -19 \text{ kcal/mole} \ (20)\]

Although it is well known that EDTA:Fe(II) rapidly autoxidizes, it is unlikely that significant O$_2^-$ is produced, because O$_2^-$ production is thermodynamically unfavorable. Any O$_2^-$ that may be produced would be rapidly converted to H$_2$O$_2$, either by O$_2^-$ dismutation or by reaction with EDTA:Fe(II) or EDTA:Fe(III) \((k \sim 10^8 \text{ M}^{-1}\text{s}^{-1} \text{ for either complex})\).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.60 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, similar to the EDTA:Fe(II) autoxidation rate expression.60 Therefore autoxidation of unchelated Fe$^{2+}$ by dioxygen will likely produce H$_2$O$_2$ rather than O$_2^-$ 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 unfavorable 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 hindrance 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 $\sim 10^{-5} \text{ M}^{-1}\text{s}^{-1}$). 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 \([10^{-9}\text{ M for Cu(II)}; 10^{-18}\text{ M for Fe(III)}]\), 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. 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 \(\text{O}_2^{-}\), \(\text{H}_2\text{O}_2\) 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, if at all, thereby preventing \(\text{O}_2^{-}\) or \(\text{H}_2\text{O}_2\) 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 I 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. 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, and in vivo. 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 saturable, 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 concentrations 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 "oxidative stress." As stated above, the toxicities of these compounds may arise from altered iron or copper metabolism. 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 resulting from "oxidative stress" is lipid peroxidation. Although the role of copper in the catalysis of lipid peroxidation is not clear,

Fe(II) oxidation by O$_2$, O$_2^-$, H$_2$O$_2$, or other oxidants can far exceed the cellular capacity to reduce Fe(III), again releasing from ferritin it may be kept in the reduced state or be oxidized by O$_2^-$ or H$_2$O$_2$ produced by the dismutation of O$_2^-$.

The rapid and indiscriminate manner with which ·OH reacts has led several investigators to question the role ·OH in the development of "oxidative stress." Specifically, it has been argued that the lifetime of ·OH is too short to cause significant damage to lipids, unless ·OH is generated in very close proximity to the lipid molecules. It is possible that ·OH could oxidize DNA or proteins if iron is bound to these molecules. As stated above, an Fe(II):Fe(III) complex has been postulated as the initiator of lipid peroxidation. In this proposed model, the role of O$_2^-$ as a ferritin iron reductant is still valid. Once the iron is released from ferritin, however, the importance of O$_2^-$ and H$_2$O$_2$ no longer reside with ·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 peroxidation 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 peroxidation does not occur. Similarly, if the rates of Fe(II) oxidation by O$_2$, O$_2^-$, H$_2$O$_2$, or other oxidants far exceed the cellular capacity to reduce Fe(III), again an Fe(II):Fe(III) complex will not form. Partial oxidation of the Fe(II) pool may produce the Fe(II):Fe(III) complex.
complex and result in the initiation of lipid peroxidation. Oxidation of Fe(II) by H$_2$O$_2$ would also produce •OH. A comparison of the rates of Fe(II) oxidation by O$_2$, O$_2$~*, or H$_2$O$_2$ 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$_2$O$_2$. 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$_2$O$_2$ production from these molecules. However, as discussed earlier, production of O$_2$~* and H$_2$O$_2$ 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$_2$O$_2$.. 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 and cysteine 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. However, if the reagent possesses significant metal catalysis, 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. 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.

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 I-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. 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. 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. 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. 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
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\(^{63}\) (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 \(O_2^\cdot\) 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 a fatty acid, Tris had no effect on the rate of lipid peroxidation.\(^{64}\) In addition Tris is also an effective \(\cdotOH\) 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 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 apo-ferritin.\(^{91,92}\) From these studies, it has been asserted that apo-ferritin possesses a ferroxidase activity. Other studies have shown that apo-ferritin 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 apo-ferritin was due to a HEPES—Fe(II) or HEPES—Fe(II)—apo-ferritin 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.,\(^{45}\) may assist in the interpretation of observed results.

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

<table>
<thead>
<tr>
<th>Buffer</th>
<th>30 Sec</th>
<th>5 Min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate</td>
<td>87</td>
<td>98</td>
</tr>
<tr>
<td>HEPES</td>
<td>6</td>
<td>99</td>
</tr>
<tr>
<td>Tris</td>
<td>6</td>
<td>28</td>
</tr>
<tr>
<td>NaCl</td>
<td>0</td>
<td>5</td>
</tr>
</tbody>
</table>

Note. Reaction mixtures (3 ml) contained the indicated buffer or salt solution at concentrations of 50 mM NaCl. Ferrous chloride or 1 mM ADP and 200 \(\mu\)M 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 complex (2 ml total volume) and the absorbance at 564 nm due to the tris ferrozine:Fe(II) complex was measured.

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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