Role of Alpha-Tocopherol, Ascorbic Acid, Citric Acid and EDTA as Oxidants in Model Systems

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ABSTRACT

The effects of four widely employed "antioxidants" and iron-mediated hydroxyl radical formation and lipid peroxidation were studied in aqueous model systems. Iron and copper served as catalysts for the reaction which oxidized ascorbic acid and alpha-tocopherol and reduced oxygen. Ferrous ion spontaneously reduced oxygen to $\text{O}_2$ (superoxide anion radical) which led to $\cdot \text{OH}$ (hydroxyl radical) and $\text{H}_2\text{O}_2$ generation and lipid peroxidation. Precipitation or sequestration of iron greatly depressed these oxidative events. Complexation by EDTA and citric acid, however, formed catalytically active iron chelates. The concomitant increase in iron solubility explained the substantial enhancement of iron-driven redox reactions by EDTA and citric acid.

INTRODUCTION

THE UBIQUITY of both iron and oxygen in plants and animals has been well-established. These elements are essential to the survival of most organisms, yet they also pose a constant menace which necessitates the presence of an active protective mechanism in living tissues. In food the defensive mechanisms against oxidative damage are lacking or greatly diminished, leading to discoloration, putrescence and textural changes. The shelf-life of foods can be extended by limiting microbiological contamination, the chemical or physical removal of oxygen or the addition of antioxidants.

Two classes of antioxidants are radical scavengers and metal sequestrants. The former class includes butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylated hydroquinone (TBHQ), propyl gallate, tocopherols, and ascorbic acid. These compounds fail to block the initial generation of radicals—they merely react with them to form less reactive radicals. Therefore, they are being consumed and shelf-life is proportional to the concentration of antioxidants. However, the initial concentration is often limited by solubility, federal regulations, sensory changes, and by the bimodal effects of antioxidants such as vitamin E. High concentrations of alpha-tocopherol have been shown to exert a prooxidant effect on the autoxidation of linoleic acid (Cillard et al., 1980a, b; Koskas et al., 1984). Similarly, in the presence of transition metal ions ascorbic acid exhibited a prooxidant effect on the linoleate-mediated oxidation of beta-carotene (Kanner and Mendel, 1977).

The second category of antioxidants consists of metal sequestrants, i.e., chelating agents that either precipitate the metal or suppress its reactivity by occupying all coordination sites. Precipitation of transition metals by phosphate and pyrophosphate results in greatly diminished oxidative damage by lowering the amount of soluble reactive metal. Phospholipids, such as lecithin, may act both as antioxidants and prooxidants depending on the solubility of the iron chelates formed (Brandt et al., 1973). Complexation of iron by soluble chelating agents such as ethylenediamine tetraacetic acid (EDTA) and citrate, however, does not preclude iron from participating in the Haber-Weiss cycle (Graf et al., 1984):

\[
\begin{align*}
\text{Fe}^{3+} + \text{O}_2^- & \rightleftharpoons \text{Fe}^{2+} + \text{O}_2 \\
2\text{O}_2^- + 2\text{H}^+ & \rightleftharpoons \text{H}_2\text{O}_2 + \text{O}_2 \\
\text{Fe}^{2+} + \text{H}_2\text{O}_2 & \rightleftharpoons \cdot \text{OH} + \text{OH}^- + \text{Fe}^{3+}
\end{align*}
\]

This Haber-Weiss cycle may be initiated by numerous reducing substances, including ascorbic acid, alpha-tocopherol, dopamine, catechols and reduced glutathione. Ferrous ions will then reduce oxygen to $\text{O}_2$ which spontaneously disproportionate into $\text{H}_2\text{O}_2$ and $\text{O}_2$ (Halliwell and Gutteridge, 1984). The resulting combination of $\text{Fe}^{2+}$ and $\text{H}_2\text{O}_2$, known as the Fenton reagent (Wallig, 1975), produces highly reactive $\cdot \text{OH}$ (hydroxyl radical) which indiscriminately oxidizes most food constituents. The above metal-catalyzed production of $\cdot \text{OH}$ from $\text{H}_2\text{O}_2$ requires iron containing at least one free coordination site, a condition met by Fe(III)-citrate and Fe(III)-EDTA (Graf et al., 1984). Iron chelated by EDTA has seven coordination sites one of which is occupied by $\text{H}_2\text{O}$ (Lind et al., 1964) and is therefore available for redox reactions. This characteristic together with the high solubility of Fe(III)-EDTA renders this chelate an efficacious oxidation catalyst. For example, Fe(III)-EDTA is a widely employed bleaching agent in photographic film processing, and it has been used for the oxidation of benzoic acid and salicylic acid to their respective hydroxy derivatives (Grinstead, 1962), for the hydroxylation of anisole to guaiacol (Julia and Bost, 1973), and for the oxidation of phenol to hydroquinone and pyrocatechol (Ube Industries, Ltd., 1974).

Despite these well-established chemical principles, EDTA, citrate, alpha-tocopherol, and ascorbic acid continue to be hailed as excellent antioxidants (Andres, 1985). The objective of the present paper was to illustrate the potential deleterious effects of these common "antioxidants" in model systems.

MATERIALS & METHODS

MATERIALS were purchased from the following sources: acetylacetone, adenine diphosphate (ADP), adenosine triphosphate (ATP), arachidonic acid, bovine serum albumin (BSA), citric acid, dimethyl sulfoxide (DMSO), diethylenetriamine pentaacetic acid (DTPA), ethylenediamine tetraacetic acid (EDTA), ethylene glycol bis(beta-aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA), ethylenediame

tido-hydroxyphenylacetic acid) (EHPG), hypoxanthine, salicylic acid, sodium ascorbate, sodium azide, sodium phosphate, thiorbarbituric acid (TBA), trichloroacetic acid (TCA), and xanthine oxidase from Sigma Chemical Company (St. Louis, MO); copper sulfate (CuSO$_4 \cdot 5\text{H}_2\text{O}$) from Fisher Scientific Company (Fairlawn, NJ); FeSO$_4$ and FeCl$_3 \cdot 6\text{H}_2\text{O}$ from J.T. Baker Chemical Company (Phillipsburg, NJ); dihydroxybenzoic acid (DHB) and nitritoterephthalic acid (NTA) from Aldrich (Milwaukee, WI); desferrioxamine B (Desferal) from Ciba Pharmaceuticals Company (Summit, NJ); all other chemicals were analytical grade.

The concentration of phytic acid was determined by phosphate analysis after dry-ashing variable moisture-containing powder from Sigma Chemical Company. The level of copper and iron contamination of phytate was ascertained by atomic absorption by Medallion Laboratories. The sample contained 0.06 nmol Cu/mmol phytate and 0.21 nmol Fe/mmol phytate.

Hydroxyl radical formation catalyzed by iron and its chelates was measured by determining formaldehyde produced from DMSO as pre

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PROXIDANT EFFECTS OF ANTIOXIDANTS

Fig. 1—Oxidation of ascorbate in 50 mM Tris, pH 7.4 was followed by monitoring the absorbance at 265 nm. A: 0 (-----), 0.25 μM (----), 0.5 μM (-----), and 1.0 μM (-----) Cu²⁺. B: 0 (-----), 15 μM (----), and 49 μM (-) Fe³⁺. The reaction was initiated by the addition of ascorbate.

RESULTS & DISCUSSION

FERRIC ION ALONE does not facilitate the generation of oxy-radicals in the presence of dioxygen (O₂) nor does it promote lipid peroxidation. Ferrous ion in solution results in the production of oxy-radicals, including the ·OH, by reactions I, II, and III. Ferrous ion will also initiate a single cycle of lipid peroxidation (Graf et al., 1984). However, the inclusion of a reducing agent (O₂, catechols, dopamine, reduced glutathione, alpha-tocopherol, ascorbic acid) with iron provides a continuous source of Fe²⁺ which in the presence of dioxygen leads to oxidative damage.

Ascorbic acid chelates most polyvalent cations which allows a direct electron transfer from ascorbate to transition metals (Martell, 1982). The autoxidation of ascorbic acid was effectively catalyzed by iron or copper. Figure 1A illustrates the increased rate of ascorbate oxidation in the presence of varying concentrations of Cu²⁺. Most foods contain 0.2 to 2 ppm Cu²⁺ which in the presence of dioxygen leads to oxidative damage.

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Figure 2 shows the effect of iron and ascorbate on malondialdehyde production and hydroxyl radical generation. Ferric ion alone did not generate MDA or ·OH, while ascorbate alone produced a small amount of MDA and ·OH, presumably as a result of trace iron contamination. Desferal (50 μM) blocked ascorbate-mediated ·OH production by forming an inactive chelate with iron (Graf et al., 1984). The combination of iron, ascorbate and oxygen, however, resulted in the production of substantial amounts of MDA and ·OH.

Ascorbic acid is generally regarded as an antioxidant and food preservative. However, there are conditions when ascorbate can contribute to oxidative damage by reducing Fe³⁺ to Fe²⁺. Figure 3 shows the dependence of Cu²⁺-mediated ·OH generation on ascorbate concentration. Ascorbate enhanced the formation of hydroxyl radical at low concentrations, whereas at high concentrations it exerted a prooxidant effect by scavenging oxy-radicals. In addition to its role as a radical scavenger, high concentrations of ascorbate might form Cu²⁺-ascorbate complexes that are less reactive than Cu²⁺ alone with O₂. Based on the previously published formation constant of 39.8 M⁻¹ (Martell, 1982), 28% (0.7 μM) and 80% (2.0 μM) of the total copper is chelated at 10 mM and 100 mM ascorbate. This plot indicated that there was a minimum concentration above which ascorbate delayed the onset of oxidative damage. Below this concentration a prooxidant effect was exerted. Since ascorbate is consumed in its role as an antioxidant, the concentration of ascorbate will eventually fall into the prooxidant range, i.e., a concentration where ascorbate produces more radicals than it is able to scavenge. The shape of the curve in
Iron and copper are important catalysts of the reactions that generate oxy-radicals. Removal of these metals by either sequestration or precipitation may be an effective method for preventing oxidative damage. The ability of iron chelates to participate in the Fenton reaction was recently examined (Graf et al., 1984). The coordination chemistry of the chelates was found to be crucial in their ability to produce hydroxyl radicals. Table 1 compares \( \cdot \text{OH} \) formation and lipid peroxidation catalyzed by a number of iron complexes. Chelates capable of participating in the Fenton reaction supported equally well MDA production. These results indicated that EDTA, which is widely used as a preservative, might not be the ideal choice for an iron chelator to prevent oxidative damage under certain conditions. In fact, W. R. Grace & Co. markets Fe(III)-EDTA as a catalyst for oxidation and hydroxylation reactions. Both the solubility and the oxidation-reduction potential of Fe(III)-EDTA are greater than those of iron alone (+0.177 vs -0.771). Since phytic acid is effective in preventing both \( \cdot \text{OH} \) and MDA oxidative damage. At the latter concentration, alpha-tocopherol functioned as a prooxidant by providing reducing equivalents for trace amounts of transition metals which catalyzed this oxidation reaction.

Fig. 3 depends on the temperature and concentration of \( \text{O}_2 \), \( \text{H}^+ \), transition metal catalysts, chelating agents and other reducing substances. The role of ascorbate in whole food systems is more complex, yet this model provides a basis for the understanding of the oxidant bimodality of ascorbic acid.

Alpha-tocopherol functions as an efficacious radical scavenger and thereby terminates the propagation of radical chain reactions. In this capacity alpha-tocopherol is an important antioxidant, especially in fat systems due to its strong hydrophobicity. However, alpha-tocopherol in high concentrations will reduce transition metals and function as a prooxidant. This redox phenomenon of alpha-tocopherol accounts for the previously reported bimodal effect shown in Fig. 4 (Cillard et al., 1980b). This figure shows that 0.025 mM alpha-tocopherol protected against linoleic acid oxidation (the rearrangement of double bonds to form conjugated dienes was measured at 234 nm), while 50 mM alpha-tocopherol promoted an increase in oxidative damage. At the latter concentration, alpha-tocopherol functioned as a prooxidant by providing reducing equivalents for trace amounts of transition metals which catalyzed this oxidation reaction.
PROXIDANT EFFECTS OF ANTIOXIDANTS...

Table 1—Availability of iron coordination site and catalytic activity of various iron chelates

<table>
<thead>
<tr>
<th>Chelator</th>
<th>Aquo site*</th>
<th>OH Formation* (nmol HCHO/30 min)</th>
<th>Lipid peroxidation* (nmol MDA/60 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>+</td>
<td>31.1 ± 1.4</td>
<td>7.9 ± 0.1</td>
</tr>
<tr>
<td>Citrate</td>
<td>+</td>
<td>28.3 ± 1.2</td>
<td>4.8 ± 0.2</td>
</tr>
<tr>
<td>Salicylate</td>
<td>+</td>
<td>37.4 ± 1.5</td>
<td>8.1 ± 0.1</td>
</tr>
<tr>
<td>EDTA</td>
<td>+</td>
<td>16.1 ± 0.3</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>EGTA</td>
<td>+</td>
<td>10.4 ± 0.5</td>
<td>0.57 ± 0.11</td>
</tr>
<tr>
<td>GTA</td>
<td>+</td>
<td>23.8 ± 0.3</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>CDTA</td>
<td>+</td>
<td>10.4 ± 0.1</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>ADP</td>
<td>+</td>
<td>63.2 ± 1.0</td>
<td>7.9 ± 0.2</td>
</tr>
<tr>
<td>ATP</td>
<td>+</td>
<td>11.9 ± 0.8</td>
<td>9.3 ± 0.2</td>
</tr>
<tr>
<td>DHDA</td>
<td>+</td>
<td>69.5 ± 1.9</td>
<td>4.3 ± 0.2</td>
</tr>
<tr>
<td>Phytate</td>
<td>-</td>
<td>0.0</td>
<td>0.38 ± 0.05</td>
</tr>
<tr>
<td>EHPP</td>
<td>-</td>
<td>0.0</td>
<td>0.03 ± 0.04</td>
</tr>
<tr>
<td>DTPA</td>
<td>-</td>
<td>0.0</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Deferal</td>
<td>-</td>
<td>0.0</td>
<td>-0.02 ± 0.06</td>
</tr>
</tbody>
</table>

* The presence of an available iron coordination site was determined by UV-visible spectroscopy as described previously (Graf et al., 1984).

The formation of hydroxyl radical catalyzed by 50 μM Fe^{2+} and 500 μM chelating agent was previously measured (Graf et al., 1984).

* The peroxidation of arachidonic acid was determined by the thiobarbituric acid method as described under Materials and Methods. Each value represents the mean and standard deviation of three determinations.

Fig. 5—Effect of various chelators on iron-catalyzed ascorbate oxidation. The oxidation of 60 μM ascorbate in 50 mM Tris, pH 7.4 was followed photometrically in the presence of 20 μM iron and 100 μM chelating agent. The blank contained no iron.

generation, it appears to be superior to EDTA in preventing oxidative damage (Graf, 1986).

The coordination chemistry that applies to hydroxyl radical formation and lipid peroxidation also pertains to metal-catalyzed oxidation of ascorbate. The effect of various iron chelates on ascorbate oxidation is represented by Fig. 5. The ability of these chelators to promote or inhibit ascorbic oxidation correlated well with their similar role in 'OH generation and lipid peroxidation. Iron chelated by citrate and EDTA was available to participate in the oxidation of ascorbate while iron sequestered by Desferal or DTPA was catalytically inactive. In fact, metal-free Desferal when added to ascorbate solution slowed the rate of autoxidation, suggesting that trace amounts of iron contaminated the system.

Transition metals, such as iron and copper, are efficacious catalysts of oxidative reactions leading to discoloration, rancidity and undesirable textural changes in food. Since the formation of oxy-radicals is initiated by transition metal ions in their lower oxidation state, reducing equivalents will greatly augment metal-mediated oxidative damage. Indeed, the results from the present paper demonstrated that under different model conditions both ascorbic acid and alpha-tocopherol might act as an antioxidant or a prooxidant due to their ability to both scavenge oxy-radicals and reduce iron and copper.

The results of hydroxyl radical production and lipid peroxidation by iron chelates clearly demonstrated that not all iron chelators were equally effective at preventing oxidation reactions. In fact, several chelators including EDTA and citrate actually promoted oxidative damage by increasing both the solubility and the oxidation-reduction potential of iron. These effects presumably would be especially pronounced in frozen foods, a hypothesis currently under investigation in our laboratories. These investigations point out the need for careful consideration of the effects of ascorbic acid, alpha-tocopherol, and metal chelators as preservatives for products containing oxidizable biomolecules.

REFERENCES

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