Original Contribution

FENTON REAGENTS MAY NOT INITIATE LIPID PEROXIDATION IN AN EMULSIFIED LINOLEIC ACID MODEL SYSTEM

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Abstract—This study includes two parts. First, the Fe^{2+} autooxidation and chelation processes in the presence of the chelators ethylenediaminetetraacetic acid (EDTA) and diethylenetriamine pentaacetic acid (DTPA) were studied by measuring UV light absorbance alterations. Competition for Fe^{3+} between chelators and water or phosphate buffer (PB) ions was confirmed. The addition of EDTA or DTPA to Fe^{3+} in water or PB only slowly turned the water/PB-Fe^{3+} complexes to EDTA-Fe^{3+} or DTPA-Fe^{3+} complexes. In the second part of this study, the initiation mechanisms of Tween 20 emulsified linoleic acid peroxidation under stimulation by chelator-Fe-O_{2} complexes were studied by measuring changes in UV light absorbance following diene conjugation. Fe^{3+} in the presence of EDTA or DTPA did not stimulate diene conjugation. Fe^{2+} (0.10 mM) and EDTA (0.11 mM) stimulated diene conjugation of the linoleic acid emulsion, but only after apparent Fe^{2+} autoxidation. Fe^{2+} and DTPA, as well as premixed DTPA-Fe^{2+} complex, resulted in very fast diene conjugation in a wide range of concentrations. A nonlinear, mainly square root relation between Fe^{2+} concentration and peroxidation rate was noted. Superoxide dismutase (SOD), catalase, and mannitol did not prevent the lipid peroxidation. H_{2}O_{2} substantially decreased the DTPA-Fe^{2+} stimulated, otherwise rapid, diene conjugation but slightly enhanced the slower one stimulated by EDTA-Fe^{2+}. Without ambient oxygen, Fenton reagents did not result in 'H abstraction-related diene conjugation. The findings suggest that 'OH resulting from Fenton reagents may not be the main cause for the initiation of peroxidation in this model system. Furthermore, a study with different combinations of Fe^{2+} and Fe^{3+} did not support the Fe^{2+}/Fe^{3+} (1:1) optimum ratio hypothesis. We therefore conclude that perferryl ions or chelator-Fe-O_{2} complexes may be responsible for the first-chain initiation of lipid peroxidation, at least in this model system.

Keywords—Diene conjugation, DTPA, Fenton reaction, First-chain initiation, Iron autoxidation, Linoleic acid, Lipid peroxidation, Perferryl, Free radicals

INTRODUCTION

The initiation mechanisms of lipid peroxidation, which may result in damage to a variety of organic components in living cells as well as induce rancidification of foods, have recently been found to be extremely complex. Fenton chemistry, leading to the formation of 'OH radicals, was long considered the most likely mechanism for producing highly oxidizing species in biological systems. However, when simple iron salts, in the absence of chelators such as EDTA, were added to reaction mixtures containing H_{2}O_{2}, most 'OH scavengers failed to protect significantly against peroxidative damage. To explain this, Gutteridge proposed that ionic iron may bind weakly to the "detector molecules" (equivalent to chelators, or targets in other studies) resulting in "site-specific" 'OH radical reactions in direct relation to the bound metal. An alternative proposal suggests that peroxidation induced by simple iron salts is mediated by the ferryl ion Fe^{2+O_{2}^+} rather than by 'OH. Moreover, a perferryl(Fe^{2+O_{2}^+}) hypothesis for the initiation of peroxidation has been suggested by Ernster, Aust, and their colleagues, although Koppenol calculated only weak 'H abstraction capability by perferryl ions. Aust and his co-workers also found that both Fe^{2+} and Fe^{3+} (optimum ratio 1:1) were necessary to stimulate peroxidation, suggesting that an Fe^{2+O_{2}^{-}}Fe^{3+} complex could be of importance.

The further investigation of the initiation mecha-
nisms of lipid peroxidation thus needs precise methods to detect the first-chain initiation processes.\textsuperscript{2,13} To improve understanding with current techniques, the measuring of diene conjugation in a simple and pure model system (without disturbance of biological material) is of great interest, especially when considering that once the initiation reaction of unsaturated fatty acids is started (\textsuperscript{.}H abstraction), the consequent diene conjugation (to stabilize the radical) could be observed before oxygen consumption.\textsuperscript{14}

EDTA and DTPA are widely used transition metal chelators in oxygen radical research because they can drastically alter the efficiency of iron as a catalyst for the Haber-Weiss reaction.\textsuperscript{15} The effects of EDTA and DTPA on iron autooxidation and related lipid peroxidation have been well documented.\textsuperscript{16-18} EDTA, in contrast to DTPA, can stimulate the rapid oxidation of Fe\textsuperscript{2+} at certain pH.\textsuperscript{16,18,19} Fe\textsuperscript{2+} autooxidation has been measured in many different ways.\textsuperscript{19,20} Also, a technique for the direct determination of EDTA-Fe\textsuperscript{3+} and DTPA-Fe\textsuperscript{3+} concentrations by measuring UV absorbance at 258 nm has been reported.\textsuperscript{21} Fe\textsuperscript{2+} autooxidation, facilitated by EDTA chelation, has been shown to parallel lipid peroxidation when the lipid is detergent dispersed.\textsuperscript{22}

DTPA has previously been found to block the iron-catalyzed Haber-Weiss reaction (e.g., the superoxide-drive Fenton reaction) by preventing Fe\textsuperscript{3+} $\rightarrow$ Fe\textsuperscript{2+} reduction.\textsuperscript{23,24} In a DTPA-Fe\textsuperscript{2+} complex, however, Fe\textsuperscript{2+} can still react with H\textsubscript{2}O\textsubscript{2} to form \textsuperscript{.}OH,\textsuperscript{25,26} and the DTPA-Fe\textsuperscript{2+} complex is reported to be even more powerful than the EDTA-Fe\textsuperscript{2+} complex in inducing the Fenton reaction.\textsuperscript{15,27}

The present study, using a Tween 20 emulsified linoleic acid model system, strongly suggests that the early stage of lipid peroxidation, under the influence of DTPA-Fe\textsuperscript{2+} complexes, may not start from a methylene \textsuperscript{.}H abstraction due to Fenton reaction produced \textsuperscript{.}OH radicals, but rather be initiated by a DTPA-Fe\textsuperscript{2+}-O\textsubscript{2} complex, a perferryl-type radical.

**MATERIALS AND METHODS**

**Chemicals**

DTPA, bovine erythrocyte SOD, catalase (from bovine liver, c-10), and mannitol were purchased from Sigma Chemical Co. (St. Louis, MO). EDTA disodium salt, hydrogen peroxide, iron(II) sulfate (FeSO\textsubscript{4} $\cdot$ 7H\textsubscript{2}O) and iron(III) chloride (FeCl\textsubscript{3} $\cdot$ 6H\textsubscript{2}O) were products of E. Merck AG (Darmstadt, Germany). Linoleic acid (\textgreater{} 99% pure) was obtained from Nu Chek-Prep. Inc. (Elysian, MN) and polyoxyethylene sorbitan monolaurate (Twee 20) from KEBO AB (Stockholm, Sweden). All other chemicals used were of analytical grade. Deionized water, redistilled through a Büchi Fontavapor 285 (Flawil, Switzerland), was used for all solutions.

**Ionic iron stock solutions, chelator stock solutions, and premixed iron-chelator complexes**

Fresh stock solutions (10 mM) of Fe\textsuperscript{2+} (sulfate) and Fe\textsuperscript{3+} (chloride) were prepared daily in redistilled water. The pH of the Fe\textsuperscript{2+} and Fe\textsuperscript{3+} stock solutions were about 4.2 and 2.3, respectively (original water pH was about 7). Precipitations were not observed when stock solutions were kept for a few hours in air or after being diluted 100 times in phosphate buffer (PB), pH 6.5.

EDTA salt or DTPA were dissolved in redistilled water by moderate heating to obtain 11 mM stock solutions.

Equal volumes of ionic iron and chelator stock solutions were mixed just before experiments to obtain freshly mixed iron-chelator (5.0/5.5 mM) stem solutions. In this study, premixed iron-chelator complexes are expressed as DTPA-Fe\textsuperscript{2+} or EDTA-Fe\textsuperscript{3+}. The terms DTPA + Fe\textsuperscript{2+}, or EDTA + Fe\textsuperscript{3+}, are used to indicate that these chemicals (stock solutions) were added separately to a solution.

To study autooxidation and chelation processes, 30 \textmu L each of 10 mM ionic iron (Fe\textsuperscript{2+} or Fe\textsuperscript{3+}) and 11 mM chelators (EDTA or DTPA) were added successively to 2.94 mL redistilled water or 0.05 M PB (pH 6.5) in quartz cuvettes. The absorption spectra or absorption alterations at different wavelengths (e.g., EDTA + Fe, 258 nm; DTPA + Fe, 258 nm; PB + Fe, 275 nm) were recorded with a Perkin-Elmer (Norwalk, CT) Spectrophotometer 200.

**Linoleic acid emulsion**

Emulsified linoleic acid was used as substrate for the initiation peroxidation studies. The emulsion was prepared daily and kept in an ice bath (freezing can destroy micelles), purged with N\textsubscript{2} to suppress emulsion autooxidation, and covered with aluminum foil to prevent photooxidation. The emulsion was prepared by a technique based on methods previously used in research on peroxidation of dietary lipids.\textsuperscript{28} Thus, 0.50 mg Twee 20 was dissolved in 8 mL PB (1 M), and 0.25 mL linoleic acid was added drop-wise during stirring. After a thorough magnet mixing (5–10 min), two or three pieces of KOH (about 0.4 g) were added and a thick emulsion was obtained after further stirring. This emulsion (pH 10–11) was then slowly diluted to 160 mL with redistilled water. The final emulsion pH was adjusted to 6.5 with concentrated HCl. A nearly transparent emulsion containing 5 mM linoleic acid and 0.3% (w/v) Twee 20 in 0.05
M PB was thus obtained. Possible ionic iron contamination (mainly from PB) could range up to 10 \( \mu \text{M} \). Control experiments, however, showed that adventitious iron in the ferric state does not induce diene conjugation in this model system.

High concentration (> 0.25%) of Tween 20 may cause inhibition on linoleate peroxidation. Thus the concentration of Tween 20 (final concentration [conc] 0.03% during reaction) was chosen at the lowest optimum condition. Control experiments, using up to three times the actual Tween 20 concentration, did not show noticeable effects on the peroxidation rate in this system.

**Measurement of early lipid peroxidation processes**

For measuring the initiation (dienyl radicals, see Discussion, Scheme 1) or peroxidation processes (hydroperoxide radicals, see Scheme 1), the linoleic acid emulsion was 10-fold diluted in quartz cuvettes with 0.05 M phosphate buffer, pH 6.5. A low 233-nm absorbance (0.1-0.2, see Fig. 4C) of the diluted emulsion indicated a well-emulsified linoleic acid substrate. All reactions were carried out at room temperature (23°C) in air. Diene formation was measured at 233 nm with a Perkin-Elmer Spectrophotometer 200. Spectrum scanning was often carried out to confirm the diene conjugation of the reactions.

To establish a pure Fenton reaction system, oxygen-free linoleic acid emulsion was carefully created by bubbling argon directly in the cuvettes for 2-3 min before the addition of further reagents. Fe\(^{2+}\) as well as \( \text{H}_2\text{O}_2 \) were transferred to the oxygen-free reaction system promptly from argon-bubbled stock solutions. The reaction system was then sealed to prevent the entry of air.

**RESULTS**

**Iron autooxidation and chelation in water**

When freshly made Fe\(^{2+}\) or Fe\(^{3+}\) stock solutions (10 mM) were added in small volumes (0.030 mL) to redistilled water (2.97 mL) in quartz cuvettes, different absorption spectra were recorded. As shown in Fig. 1, 0.10 mM Fe\(^{2+}\) (final concn) has almost no absorbance between 200 and 350 nm (line a), while Fe\(^{3+}\) shows substantial UV absorption with a plateau from 260 to 290 nm (line b). These spectra were stable for several hours but were changed by the further addition of 0.030 mL EDTA stock solution (11 mM) to the ionic iron in water solutions (final EDTA concn 0.11 mM), finally showing absorption peaks at 258 nm. The Fe\(^{2+}\) and Fe\(^{3+}\) spectra changed, however, with different velocities. When EDTA was added to the water-Fe\(^{2+}\) solution, the 258-nm absorption peak reached a maximum within 2 min (compare lines a and e). In contrast, when EDTA was added to the water-Fe\(^{3+}\) solution, the 258 nm EDTA-Fe\(^{3+}\) peak rose only slowly (compare lines b, c, and d).

For the DTPA-Fe\(^{3+}\) complex in water, a 260-nm absorption peak was observed. Typical absorption spectra of EDTA-Fe\(^{3+}\) and DTPA-Fe\(^{3+}\) complexes are given for comparison (inserted in Fig. 1). The absorption spectra of EDTA (0.11 mM) and DTPA (0.11 mM) are also shown in the same inset.

The Fe\(^{2+}\) autooxidation and the chelation of iron to EDTA or DTPA in water (measured at 258 nm) are demonstrated in Fig. 2. The autooxidation/chelation processes between ionic iron (0.10 mM) and chelators (0.11 mM) were significantly affected by the addition sequences of the components in concentrated form to the water. In the EDTA case (Fig. 2A) chelation and the chelation-induced Fe\(^{2+} \rightarrow \text{Fe}^{3+}\) oxidation were essentially instantaneous both when Fe\(^{2+}\) was added after EDTA or EDTA added after Fe\(^{2+}\). Also, when Fe\(^{3+}\) was added after EDTA, the chelation process was immediate. In contrast, the addition of concentrated EDTA to diluted Fe\(^{3+}\) showed a very slow EDTA chelation processes. In the DTPA case (Fig. 2B), independent of the mixing order, the oxidation/chelation of Fe\(^{2+}\) did not result in an absorption spectrum typical for the DTPA-Fe\(^{3+}\) complex until after about 20 min. When DTPA was added before Fe\(^{3+}\), the chelation product formed immediately, whereas the chelation between Fe\(^{3+}\) and DTPA, similar to the EDTA experiments, took 1 d to complete when DTPA was added after Fe\(^{3+}\).

**Iron autooxidation and chelation in phosphate buffer**

The addition of Fe\(^{2+}\) to 0.05 M pH 6.5 phosphate buffer (final Fe\(^{2+}\) concn 0.10 mM) resulted in increasing 275-nm absorption (Fig. 3, inset), being rather fast within the first few minutes and leveling off after 6 min. The rate was pH dependent (not shown). This change in absorbance represented Fe\(^{2+}\) autooxidation, since the scanning spectrum of an "aged" PB-Fe\(^{2+}\) solution (Fig. 3, line b) was analogous to that of a freshly mixed PB-Fe\(^{3+}\) solution (Fig. 3, line c).

Similar to the process of EDTA chelation of Fe\(^{3+}\) in water, once the PB-Fe\(^{3+}\) complex (275 nm) was formed (no matter if Fe\(^{2+}\) or Fe\(^{3+}\) was added to PB), the further addition of EDTA did not immediately create the typical (258 nm) EDTA-Fe\(^{3+}\) absorption peak. On the contrary, the gradual formation of a typical EDTA-Fe\(^{3+}\) peak (Fig. 3, line d) took about 1 d to complete.
Chelated iron-induced diene conjugation of linoleic acid

Stable absorbance values of the emulsion were obtained before the addition of ferrous iron (see Fig. 4C). When ionic iron and chelators were added, either separately or as complexes to linoleic acid emulsion, diene conjugation (always confirmed by spectral scanning) was observed at very different velocities (Fig. 4 and Table 1).

The addition of Fe\(^{2+}\) without chelators to linoleic acid induced an unstable increase in absorbance at 233 nm after a 5-10 min lag period (Fig. 4A, line: Fe(II) with linoleic acid), which corresponded to Fe\(^{2+}\) autooxidation in PB buffer (Fig. 4A, line: Fe(II) without linoleic acid). When EDTA was involved (Fig. 4B), the appearance of the 233-nm absorbance contributed by oxidized Fe\(^{2+}\) was noted early and could be easily discriminated from the 233-nm absorbance due to diene conjugation. Thus, the diene conjugation rates (average 3 min) were calculated subsequent to the initial fast increase in absorbance, which indicated the apparent Fe\(^{2+}\) chelation/autooxidation. In the DTPA-related peroxidation reactions, the Fe\(^{2+}\) (0.10 mM) autooxidation gave an 0.045 ΔA/min increase at 233 nm (Fig. 4C, line: DTPA + Fe(II) without linoleic acid), which was only about 6% of the total early rate of DTPA-Fe\(^{2+}\) induced diene conjugation (Fig. 4C, line: DTPA + Fe(II) or DTPA-Fe(II) with linoleic acid). This low value of Fe\(^{2+}\) autooxidation in DTPA case was not deduced in Tables 1, 2, and 3. A typical absorption spectrum of DTPA-Fe\(^{2+}\) induced diene products is shown in Fig. 4C, inset.

As shown in Table 1, no Fe\(^{3+}\) combinations induced diene conjugation of the linoleic acid emulsion (no further increase in absorbance after the Fe\(^{3+}\) dependent change). The addition of EDTA and Fe\(^{2+}\) to the reaction system (final concentrations 0.11 mM and 0.10 mM, respectively) resulted in diene conjugation at a rate of about 0.050 ΔA/min. Addition of DTPA and Fe\(^{2+}\), added either separately or premixed (see Materials and Methods), stimulated an extremely fast absorption increase (about 0.72 ΔA/min) at 233 nm. This rate was about 15 times faster than the diene conjugation stimulated by EDTA and Fe\(^{2+}\). This great pro-oxidative potential of the DTPA-Fe\(^{2+}\) complex continued for quite a long time (about 10-15 min for 100 μM Fe\(^{2+}\)) until most Fe\(^{2+}\) ions were oxidized.

Also surprisingly, the pro-oxidative potential of premixed DTPA-Fe\(^{2+}\) and EDTA-Fe\(^{2+}\) stem solutions lasted for a long period. Although the potential decreased or altered differently, it had not completely disappeared even after 10 d (Table 1).

The rate of the DTPA and Fe\(^{2+}\) (ratio 1.1:1.0) in-
Fe-induced diene conjugation increased with Fe$^{2+}$ concentration. The relationship between Fe$^{2+}$ concentration and diene conjugation rate is demonstrated in Fig. 5. When the Fe$^{2+}$ concentration was as low as 4 \( \mu \text{M} \) (DTPA 4.4 \( \mu \text{M} \)), the absorbance increase at 233 nm was about 0.13 \( \Delta A/\text{min} \) (the slight absorbance in-
are shown in Table 2. Cu,Zn-SOD in a concentration of 50 units/mL decreased the peroxidation rate by 8.3%, but heat-inactivated SOD (same amount) did the same. Neither catalase (200 units/mL) nor mannitol (0.1 mM) effectively prevented the reaction. Catalase by itself induced substantial diene conjugation in this system (due to its hemoprotein subunits) after a 1-2 min lag phase. Catalase did not prevent the DTPA + Fe^{2+} induced diene conjugation during this lag period, which indicated that catalase did not prevent the first-chain initiation in the system.

Effect of H$_2$O$_2$ on Fe$^{2+}$ and Fe$^{2+}$-chelator induced diene conjugation

The effects of H$_2$O$_2$ on linoleic acid diene conjugation are shown in Table 3. H$_2$O$_2$ suppressed the Fe$^{2+}$ induced diene conjugation, whereas it slightly enhanced the diene conjugation induced by Fe$^{2+}$ and EDTA. Equivalent amounts of H$_2$O$_2$ (50 µM) and DTPA-Fe$^{2+}$ complex resulted in an apparent Fe$^{2+}$ → Fe$^{3+}$ autooxidation within 1 min (absorbance change at 233 nm is similar, as in Fig. 4B). The diene conjugation rate observed afterward was only 0.066 ΔA/min. An H$_2$O$_2$ concentration (500 µM), typically used in Fenton reaction experiments, almost completely prevented the DTPA + Fe$^{2+}$ or DTPA-Fe$^{2+}$ complex initiated diene conjugation (500 µM H$_2$O$_2$ induced an absorbance about 0.04 at the working wavelength of 233 nm, and this value did not interfere with the observed diene conjugation).

Effects of Fenton reagents on diene conjugation in the absence of oxygen

Results from a pure Fenton reaction system after careful argon deoxygenation (see Materials and Methods) are demonstrated in Figs. 6 and 7. The addition of Fe$^{2+}$ and H$_2$O$_2$ to a linoleic acid emulsion in the presence of DTPA resulted in some chelation-related stepwise increase of background absorbance, but did not initiate diene conjugation (see Fig. 7). Immediately after the addition of Fe$^{2+}$ or H$_2$O$_2$ to the reaction system, a minute decrease of 233-nm absorbance could be observed, probably indicating decomposition of tiny amounts of hydroperoxides formed due to incomplete deoxygenation. The linoleic acid emulsion with Fenton reagents could stay stable for hours if the reaction system was successfully separated from ambient air. When the solution was then stirred in air, diene conjugation immediately started (Fig. 6).
Table 1. Diene Conjugation (233 nm) of Linoleic Acid Emulsion in 0.05 M PB Induced by Chelated Iron

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>Diene Conjugation (ΔA/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No catalyst</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fe²⁺</td>
<td>0.045* (velocity calculated after a lag period of 5-10 min)</td>
</tr>
</tbody>
</table>

Chelators and Iron added separately

<table>
<thead>
<tr>
<th>Chelator</th>
<th>Fe²⁺</th>
<th>DTPA</th>
<th>Fe³⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA + Fe²⁺</td>
<td>0.054 ± 0.006* (difficult to distinguish from Fe³⁺ autooxidation)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDTA + Fe³⁺</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DTPA + Fe²⁺</td>
<td>0.73 ± 0.03 (0.045 ΔA/min depending on Fe³⁺ autooxidation not deduced)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DTPA + Fe³⁺</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Chelator and iron added as a complex (either freshly mixed or 10 d old)

<table>
<thead>
<tr>
<th>Complex</th>
<th>Freshly mixed complex</th>
<th>10-d-old complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA-Fe²⁺</td>
<td>0.044 ± 0.003</td>
<td>0.07*</td>
</tr>
<tr>
<td>EDTA-Fe³⁺</td>
<td>&lt;0.001</td>
<td>0.002</td>
</tr>
<tr>
<td>DTPA-Fe²⁺</td>
<td>0.71 ± 0.02</td>
<td>0.18*</td>
</tr>
<tr>
<td>DTPA-Fe³⁺</td>
<td>&lt;0.001</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Reaction mixture: 0.5 mM linoleic acid, 0.03% Tween 20, 0.10 mM ionic iron (Fe²⁺ or Fe³⁺), and 0.11 mM EDTA or DTPA in 0.05 M phosphate buffer, pH 6.5. The reactions were carried out in quartz cuvettes at 23°C in air. The Fe²⁺ or chelator-Fe²⁺ complexes were added finally. Statistical data are from three duplicates; means ± SD are given.

* Unstable reaction average velocity was calculated during 3 min subsequent to absorbance increase resulting from iron autooxidation/chelation.

Effects of various Fe²⁺ and Fe³⁺ combinations on the linoleic acid model system

The slow Fe²⁺ autooxidation in the presence of DTPA suggests that this is a useful model to obtain a relatively accurate Fe²⁺/Fe³⁺ ratio in order to test the Fe²⁺-O₂-Fe³⁺ hypothesis for the initiation of lipid peroxidation. The results of diene conjugation stimulated by combinations of DTPA-Fe²⁺/Fe³⁺ complexes are given in Fig. 8. The total concentration of ionic iron (Fe²⁺ + Fe³⁺) was 100 μM, and that of DTPA was 110 μM. The highest diene conjugation rate was obtained when no Fe³⁺ but only 100 μM Fe²⁺ was added to emulsion in the presence of DTPA. Fe²⁺ (50 μM) added after 50 μM Fe³⁺ in the presence of DTPA caused slower (about 80% of the former) diene conjugation. Results in Fig. 8 clearly indicate that the lower the Fe²⁺/Fe³⁺ ratio, the slower the diene conjugation. Thus, Fe³⁺ in the presence of DTPA did not accelerate Fe²⁺ induced diene conjugation in this system.

DISCUSSION

The linoleic acid emulsion model system, general remarks

In the early 1970s, detergent-dispersed linoleic acid, or linoleate, was found to form a monomolecular film at an oil-water interface, which could be used to estimate lipid peroxidation in aqueous solution. In these early studies, the peroxidation process was mainly monitored by oxygen consumption because UV spectrophotometry methods to measure diene conjugation during the early stage of lipid peroxidation require optically clear solutions. This may be achieved either by adjusting the pH to 9, where linoleate forms a true solution, or by diluting with ethanol. Unfortunately, neither of these “true solution” systems can be used to study physiological Fenton-type reactions. In the present study, we have developed a method to obtain a transparent, stable linoleate emulsion which can conveniently be used for spectrophotometric detection of lipid diene conjugation. (Lubrol and octyl sulfate have also been used for the same purpose.) Using this transparent linoleic acid emulsion model system, the early initiation processes of lipid peroxidation may be directly investigated. The mechanisms of early stage peroxidation of linoleic acid are generally considered as shown in Scheme 1. Three steps are given by the mechanisms:

Table 2. Antioxidative Effects on Diene Conjugation (233 nm) Induced by DTPA + Fe²⁺

<table>
<thead>
<tr>
<th>Antioxidants</th>
<th>Peroxidation Rate (ΔA/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No antioxidants</td>
<td>0.72 ± 0.02</td>
</tr>
<tr>
<td>SOD (50 units)</td>
<td>0.66 ± 0.02</td>
</tr>
<tr>
<td>SOD (heat denatured)</td>
<td>0.66 ± 0.02</td>
</tr>
<tr>
<td>Catalase (200 units)</td>
<td>0.74 ± 0.04*</td>
</tr>
<tr>
<td>Mannitol (100 μM)</td>
<td>0.64 ± 0.03</td>
</tr>
</tbody>
</table>

Reaction mixture: 0.5 mM linoleic acid, 0.03% Tween 20, 0.11 mM DTPA, and 0.10 mM Fe²⁺ in 0.05 M phosphate buffer, pH 6.5. Ionic iron was added finally.

Means ± SD of three experiments.

* Catalase itself can stimulate diene conjugation after a lag phase of 1–2 min.
Table 3. Effects of \( \text{H}_2\text{O}_2 \) on Diene Conjugation (233 nm) Induced by Ionic Iron or Iron-Chelator Complex

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>Without ( \text{H}_2\text{O}_2 )</th>
<th>50 ( \mu\text{M} ) ( \text{H}_2\text{O}_2 )</th>
<th>500 ( \mu\text{M} ) ( \text{H}_2\text{O}_2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>No catalyst</td>
<td>&lt;0.001</td>
<td>—</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>0.10 mM Fe(^{2+})</td>
<td>0.042 ± 0.008</td>
<td>—</td>
<td>0.012 ± 0.003</td>
</tr>
<tr>
<td>*EDTA-Fe(^{2+})</td>
<td>0.052 ± 0.004</td>
<td>0.066 ± 0.003</td>
<td>0.059 ± 0.004</td>
</tr>
<tr>
<td>DTPA-Fe(^{2+})</td>
<td>0.71 ± 0.02</td>
<td>0.066 ± 0.003</td>
<td>0.014 ± 0.002</td>
</tr>
</tbody>
</table>

Reaction conditions same as given in Table 1. Means ± SD of three experiments.

* Premixed EDTA-Fe\(^{2+}\) complexes were chosen because stable increase of 233 nm absorbance was obtained subsequent to iron autooxidation (see Fig. 4).

The effects of EDTA and DTPA chelation on iron autooxidation

As shown in Figs. 1 and 2, Fe\(^{2+}\) has no UV absorption and is stable in redistilled water. The addition of slight overequivalent amounts of EDTA and DTPA induced a 258-nm absorbance increase, indicating Fe\(^{2+}\) autooxidation. In the presence of EDTA, Fe\(^{2+}\) autooxidation was very fast, as indicated by increase of absorbance at 258 nm. In the presence of DTPA, however, Fe\(^{2+}\) autooxidation induced increase of absorbance at 258 nm was slow (<0.05 \( \Delta \text{A}/\text{min} \)). The time required for 90% autooxidation/chelation (up to absorbance about 0.9, see Figs. 1 and 2) was about 0.4 min for EDTA-Fe\(^{2+}\) and about 15 min for DTPA-Fe\(^{2+}\) at the experimental conditions (in water or in 0.05 M neutral phosphate buffer), which was in agreement with reports using other methods.\(^{\text{19,22}}\) The absorbance increase at 233 nm was very similar to what was observed at 258 nm (compare Fig. 2 and Fig. 4). Therefore, the absorption change due to Fe\(^{2+}\) autooxidation can be easily distinguished from lipid peroxidation.

The relationship between Fe\(^{2+}\) concentration and diene conjugation rate (measured at 233 nm). Reaction mixture contained 0.5 mM linoleic acid, 0.03% Tween 20, in 0.05 M phosphate buffer, pH 6.5. DTPA and Fe\(^{2+}\) ratio were always 1.1:1.0. Fe\(^{2+}\) was added finally, and its concentration is shown in the figure. Reactions were carried out at 23°C in air.

\[ y = 5.4660 \cdot 50.332x + 253.81x^2 \quad R^2 = 0.997 \]
Fe-induced diene conjugation

It is well known that the ground state of dioxygen \( ^3\Sigma_g^+ \text{O}_2 \) is triplet due to two unpaired antibonding \((\pi^*)\) orbital electrons. Thus, reactions between oxygen and biomolecules which are mostly in singlet state are impossible due to spin restriction. This restriction seems to be retained also in Fe\(^{2+}\) water solution (although Fe\(^{2+}\) by definition is a free radical)—that is, water-dissolved oxygen (about 0.25 mM at 23°C) cannot interact directly with Fe\(^{2+}\) to cause Fe\(^{2+}\) autooxidation (see Fig. 1, line a). The addition of EDTA or DTPA, however, must result in some significant energy rearrangement to "3d" or "4s" orbitals of Fe\(^{2+}\), causing free energy alterations. In other words, the barrier of spin restriction is removed by the formation of chelator-Fe\(^{2+}\) complexes. After the removal of spin restriction, EDTA apparently initiates a very fast Fe\(^{2+}\) autooxidation while DTPA does it fairly slowly (Fig. 2), although there are similarities in stability constants when EDTA and DTPA chelate with ionic iron (Scheme 2).\(^{36}\) However, these chelation stability con-

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**Fig. 6.** Effect of Fenton reagents on initiation of lipid peroxidation (measured at 233 nm). Reaction materials are indicated in the figure. Their concentrations were: linoleic acid, 0.50 mM; Tween 20, 0.03%; phosphate buffer, 0.05 M (pH 6.5); DTPA, 0.11 mM; Fe\(^{2+}\), 0.10 mM; H\(_2\)O\(_2\), 0.05 mM. Air was strictly excluded from the reaction solution.

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**Fig. 7.** Scanning spectra of Fenton reagents related to lipid peroxidation initiation. Reaction conditions were the same as in Fig. 6. Scannings were conducted a few min after the addition of chemicals.
Fig. 8. Relationship between Fe$^{2+}$/Fe$^{3+}$ ratio and diene conjugation rate (measured at 233 nm). Reaction conditions were the same as in Fig. 5. The DTPA (110 μM), ionic iron (Fe$^{2+}$ + Fe$^{3+}$ = 100 μM) ratio was 1:1:1.0.

stants of EDTA and DTPA with ionic iron do not parallel the oxidation reaction rate constants.$^{21}$ When considering redox potentials of the Fe$^{2+}$ $\rightarrow$ Fe$^{3+}$ autoxidation, the reaction between oxygen (O$_2$/O$_2^-$, $-0.33$ v) and ionic iron (Fe$^{2+}$/Fe$^{3+}$, $-0.77$ v) is thermodynamically not favored.$^{18}$ EDTA chelation shifts Fe$^{2+}$/Fe$^{3+}$ half-reaction potential to $-0.12$ v, making the reactions favorable.$^{18}$ Notwithstanding that the DTPA chelation may shift the Fe$^{2+}$/Fe$^{3+}$ half-reaction potential from $-0.77$ v to $-0.13$ v (similar to EDTA chelation), the Fe$^{2+}$ autooxidation in a DTPA solution behaves very differently as compared with an EDTA solution.

According to current knowledge, the Fe$^{2+}$ autooxidation seems to be dominated by the geometrical structure of the chelator molecules.$^{16,21}$ Significant stereochemical differences between EDTA-Fe and DTPA-Fe complexes have been reported.$^{16,36}$ EDTA may occupy six coordination sites for a coordination number of 7 for iron, leaving one coordination position to a labile water molecule. This open coordination position reasonably facilitates a fast Fe$^{2+}$ autooxidation. In contrast, the DTPA ligand is octadentate, such that the DTPA-Fe$^{2+}$ complex is not expected to have any coordinated water molecule (all positions are saturated). O$_2$ may first interact with the "3d" orbitals of iron, participating an outer-sphere electron-transfer process$^{16,37}$ in order to compete for a coordination position. Thus a stringent requirement for an open coordination site of DTPA has been suggested by Graf et al.$^{36}$

On the other hand, it is necessary to reiterate the significance of chelation competitions in solutions. From the autooxidation figures given by Cohen,$^{19}$ one may notice that Fe$^{2+}$ autooxidation may not be much beyond 90% because several factors may affect the Fe$^{2+}$ autooxidation/chelation estimation. Competition for ionic iron in a multichelator solution is intense. For a metal ion M$_{2}^{n+}$ in reaction with EDTA$^{4-}$ in an X buffered solution, we get the general equilibrium shown in Scheme 3.$^{38}$ Apparently, many factors may interfere with the EDTA-metal ion chelation, such as the dissociation constant of MeOH, the H$^{+}$ concentration (affecting the EDTA$^{4-}$ concentration), and the stability constants for all Me-chelator complexes involved (including phosphate).

The complicated chelation equilibrium (Scheme 3) indicates complex EDTA chelation of Fe$^{3+}$ in water and PB due to competition for Fe$^{3+}$ by water, H$_2$PO$_4$ and HPO$_4^{2-}$, respectively. Also, the OH$^{-}$ chelation (a simplified description for Fe$^{3+}$($\text{H}_2\text{O})_{6-n}$(OH$_{-n}$)$_{n}$) of ionic iron explains the pH decrease when Fe$^{2+}$ and Fe$^{3+}$ are dissolved in redistilled water, as indicated in Materials and Methods. Thus the slow Fe$^{3+}$ chelation by EDTA or DTPA in a water or a PB solution should be interpreted as due to the competition process for Fe$^{3+}$ between water and other chelators. In the present study, thus, ionic iron was always added finally to reaction mixtures in order to get rid of the chelation competition-related interferences in detecting absorbance change during diene conjugation. This chelation competition may also affect the Fe$^{2+}$ or Fe$^{3+}$ measurements when another chelator is added to the reaction mixture. Therefore, the direct measurement of 233-nm absorption (similar to 258 nm) of EDTA- and DTPA-iron complex during chelation/autooxi-
Fe-induced diene conjugation

Linoleic acid

\[ \text{Linoleic acid} \]

\[ \begin{array}{c}
\text{H} & \text{O} \\
\text{(Hydrogen abstraction)}
\end{array} \]

\[ \begin{array}{c}
\text{O} \\
\text{(Diene conjugation)}
\end{array} \]

\[ \begin{array}{c}
\text{H} \\
\text{(Oxygen uptake)}
\end{array} \]

Scheme 1. Mechanisms of early stage linoleic acid peroxidation.

Lipid peroxidation induced by a chelator-Fe-O₂ complex

The current interpretation of the mechanisms of iron-introduced oxygen radicals is as follows: In an Fe²⁺ solution, superoxide and Fe³⁺ may be produced through the intermediate formation of perferryl species with a structure intermediate between an Fe²⁺-oxygen complex and an Fe³⁺-superoxide complex:

\[ \text{Fe}^{2+} + \text{O}_2 \rightarrow (\text{Fe}^{2+}-\text{O}_2) \leftrightarrow \text{Fe}^{3+} + \text{O}_2 \quad (1) \]

The sum of reaction 1 and a Fenton reaction (reaction 2) gives a general reaction (reaction 3):

\[ \text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \cdot \text{OH} + \text{OH}^- \quad (2) \]

\[ \text{Fe} \cdot \text{O}_2 + \text{H}_2\text{O}_2 \rightarrow \cdot \text{OH} + \text{OH}^- + \text{O}_2 \quad (3) \]

Reaction 3 is the iron-catalyzed Haber-Weiss reaction, or the superoxide-driven Fenton reaction.

Complications arise when ionic iron binds to synthetic or biologic chelating agents such as EDTA,

\[
\begin{array}{c}
\text{MeOH}^{-} + \text{OH}^- + \text{Me}^{2+} + \text{EDTA}^{3-} = \text{MeEDTA}^{4-} \\
\text{Me(OH)}_2^{n-} + \text{H}^+ + \text{X} + \text{HEDTA}^{3-} \\
\text{MeX} + \text{H}_2\text{EDTA}^{2-}
\end{array}
\]

Scheme 3. Chelation competition for metal ions in a buffered EDTA solution.
DTPA, ADP, citrate, or phosphate. The present study shows that the weak potential for initiating diene conjugation by EDTA and Fe\(^{2+}\) must be due to the fast Fe\(^{3+}\) autooxidation (Fig. 4B). After Fe\(^{3+}\) autooxidation in the presence of EDTA, this reaction system seems to be nearly redox balanced (Fe\(^{2+}\) may not be completely oxidized). The consequent stimulation of diene conjugation thus may represent a redox-cycle-related lipid peroxidation. Such post-Fe\(^{2+}\)-autooxidation provoxidation protective effect, which does not occur in Fe\(^{3+}\) solutions, is also observed in Fe\(^{2+}\) (oxidized due to PB chelation) induced diene conjugation (Fig. 4A). Again it is possible that the chelation-competition retains some prooxidative ability of oxidized Fe\(^{2+}\) or chelator-Fe\(^{2+}\) in the reaction systems.

The emphasis of this study was on the very fast diene conjugation stimulated by DTPA and Fe\(^{2+}\), which seemed to be a first-chain initiation. The slow Fe\(^{2+}\) autooxidation of this system seemed to create an excellent oxygen radical source for the initiation of lipid peroxidation. The diene conjugation rate increased with the increase of Fe\(^{2+}\) concentration, and the possible reaction kinetics may be described as

\[-d[\text{Diene}] / dt = k_c [\text{DTPA-Fe}^{3+}-\text{O}_2^•]^{1/2} \cdot [\text{RH}] / \cdots\]

indicating an exponential consumption of Fe\(^{2+}\) complexes for an increase of peroxidation rates.

The addition of H\(_2\)O\(_2\) did not enhance but strongly suppressed the DTPA and Fe\(^{2+}\) induced diene conjugation. These findings strongly contradict the current opinion that DTPA-Fe\(^{2+}\) complexes can react with H\(_2\)O\(_2\) to form \(\cdot\)OH\(^{15,27}\) resulting in \(\cdot\)H abstraction-related peroxidation. The H\(_2\)O\(_2\) depression of the diene conjugation rate may suggest a competition for \(\cdot\)OH between Fe\(^{2+}\) and linoleic acid. In light of recent findings on the formation of an oxidized iron-DTPA or EDTA intermediate\(^{39}\), a profound study on this matter is necessary.

The addition of SOD, catalase, or the \(\cdot\)OH scavenger mannitol did not prevent the initiation, suggesting that O\(_2^•\) or \(\cdot\)OH were either not involved or that the site-specific effect dominated. To obtain direct observations for judging whether Fenton chemistry is initiation responsible, a Fenton model system (Figs. 6 and 7) was studied. In the presence of DTPA, \(\cdot\)H abstraction-related diene conjugation was not induced by H\(_2\)O\(_2\) and Fe\(^{2+}\) for hours as long as conditions were anaerobic.

These findings suggest that diene conjugation in this model system may occur through a perferryl mechanism rather than through Fenton chemistry. However, previous studies have shown that more hydroxyl radicals (\(\cdot\)OH) form in DTPA-Fe\(^{2+}\) solutions than in EDTA-Fe\(^{2+}\) solutions\(^{15,27}\). It is therefore necessary to reconsider the possible reaction mechanisms between \(\cdot\)OH and linoleic acid. In the literature, hydroxyl radicals are often related to two types of reactions: hydrogen abstraction and addition reactions. The hydrogen abstraction reactions mainly concern saturated organic compounds or some carbonyl compounds,\(^{40}\) whereas addition reactions of \(\cdot\)OH usually occur on double bond (\(\pi\) electron) related compounds resulting in hydroxylation products (e.g., aromatic hydroxylation, and many spin trap reactions)\(^{41,42}\).

The occurrence of \(\cdot\)OH addition reactions, which compete overwhelmingly with hydrogen abstraction for unsaturated organic compounds, seems to be overlooked in lipid peroxidation studies. A lack of discrimination methods may be responsible for current ambiguity in understanding initiation mechanisms of lipid peroxidation. The diene conjugation method developed in this study may help to solve the problem. When the electrophilic characteristic of \(\cdot\)OH is considered, the present results may be easily explained: The DTPA-Fe\(^{2+}\) induced \(\cdot\)OH may add to double bondings of linoleic acid rather than abstracting the allylic methylene hydrogens. (Besides these, \(\cdot\)OH is also able to react with other parts of the linoleic acid. Such random attack should not be mixed up with well-defined lipid peroxidation.)

If the initiation of diene conjugation is not, as described earlier, due to \(\cdot\)H abstraction by \(\cdot\)OH radicals, the possible peroxidation initiator then would be the Fe\(^{2+}\)-O\(_2\)-Fe\(^{3+}\) complexes as suggested by Aust et al.\(^{11,12}\) However, when the Fe\(^{2+}/Fe^{3+}\) ratio was 1:1, a slower diene conjugation rate was found in this study. Considering that the Fe-O\(_2\)-Fe complex is often related to Fe\(^{3+}\) \(\rightarrow\) Fe\(^{3+}\) autooxidation processes,\(^{16,43,44}\) a free site of chelator-Fe-O\(_2\) complex seems to be important for the complex to attack lipid molecules forming a chelator-Fe-O\(_2\)-lipid intermediate during initiation reactions.

Following all these discussions, the perferryl ions, or more precisely DTPA-Fe\(^{3+}\)-O\(_2\) complexes, seem to be the most possible initiator of lipid peroxidation, although direct proof for this mechanism has been developed slowly\(^{45,46}\). Still, there are two possible sites for oxidative attack: (a) the double bond, and (b) the active methylene group. According to Bolland and Gee\(^{47}\) (thermodynamic calculations with O\(_2\) bond energy), the estimated heats of these two types of oxidative attack (a and b) are \(\Delta H_a = 14\) kcal, and \(\Delta H_b = -4\) kcal. The latter reaction, \(\cdot\)H abstraction, is favored. Such \(\cdot\)H abstraction mechanisms are strongly supported by analysis of peroxidation products (hydroperoxides)\(^{48}\). The other thermodynamic calculation on the \(\cdot\)H-abstraction capability of perferryl ions was performed by Koppenol\(^{10}\) according to a myoglobin-oxygen binding approximation (assuming ferrous...
chelate has an oxygen affinity of -4 kcal, or 0.17 v), which suggested a quite weak 'H-abstraction potential of perferryl ions. It seems, according to findings in this study, that the thermodynamic estimation should be reconsidered.

Diene conjugation induced by Fenton reagents has been studied before in detergent-emulsified linoleate and liposome systems. The experimental details of emulsification and deoxygenation processes, however, have not been described. The present study suggests that the deoxygenation should be carried out very carefully for all solutions involved through every mixing step until the final sealing of the reaction cuvette. In our experiments, whenever the deoxygenation was incomplete, sharp absorbance increased at the start, which could be observed at 233 nm when the Fenton reagents were added.*

The existence of minute amounts of hydroperoxides in most commercial lipids has been a great problem in the study of initiation mechanisms of lipid peroxidation. Fortunately, the increase or decrease (due to decomposition) of the contaminated hydroperoxide species in our emulsion system is under direct observation. After addition of Fe²⁺ to a deoxygenated solution, the stable absorbance at 233 nm (Fig. 6) without any 233-nm peak (Fig. 7) may indicate that there is neither a sign of Fe²⁺-hydroperoxide-dependent diene conjugation nor evidence of hydroperoxide decomposition in the anoxic environment in this model system.

In summary, Fe²⁺ autooxidation and chelation are complicated processes. The importance of chelation equilibrium in iron autooxidation and associated lipid peroxidation must be more carefully considered. Very fast diene conjugation initiated by a DTPA-Fe²⁺-O₂ complex cannot be prevented by SOD, catalase, or mannitol but rather can be inhibited by H₂O₂. Fenton reagents in the absence of oxygen cannot initiate diene conjugation, which suggests that reactions between unsaturated fatty acids and 'OH radicals produced by Fenton chemistry may be addition reactions rather than 'H abstraction reactions. The optimum Fe²⁺/Fe³⁺ ratio (1:1) has been disproved in this system. Our findings thus strongly suggest that DTPA-Fe²⁺-O₂ complexes are the key initiators of diene conjugation in the Tween 20 emulsified linoleic acid model system.

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* The strong absorption decreased immediately, probably representing hydroperoxide decomposition in this situation.

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**ABBREVIATIONS**

ΔA—Absorbance  
DTPA—diethylenetriamine pentaacetic acid  
EDTA—ethylenediaminetetraacetic acid  
PB—phosphate buffer  
SOD—superoxide dismutase  
TBA—thiobarbituric acid  
TBA—thiobarbituric acid  
TBA—thiobarbituric acid  
UV—ultra violet