The Involvement of Iron in Lipid Peroxidation

IMPOR TANCE OF FERRIC TO FERROUS RATIOS IN INITIATION*

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Intense lipid peroxidation of brain synaptosomes initiated with Fenton's reagent \( \text{H}_2\text{O}_2 + \text{Fe}^{3+} \) began instantly upon addition of \( \text{Fe}^{2+} \) and preceded detectable \( \text{OH}^- \) formation. Although mannitol or Tris partially blocked peroxidation, concentrations required were \( 10^2 \)-fold in excess of \( \text{OH}^- \) actually formed, and inhibition by Tris was pH dependent. Lipid peroxidation also was initiated by either \( \text{Fe}^{3+} \) or \( \text{Fe}^{2+} \) alone, although significant lag phases (minutes) and slowed reaction rates were observed. Lag phases were dramatically reduced or nearly eliminated, and reaction rates were increased by a combination of \( \text{Fe}^{3+} \) and \( \text{Fe}^{2+} \). In this instance, lipid peroxidation initiated by optimal concentrations of \( \text{H}_2\text{O}_2 \) and \( \text{Fe}^{2+} \) could be mimicked or even surpassed by providing optimal ratios of \( \text{Fe}^{3+} \) to \( \text{Fe}^{2+} \). Peroxidation observed with \( \text{Fe}^{3+} \) alone was dependent upon trace amounts of contaminating \( \text{Fe}^{2+} \) in \( \text{Fe}^{3+} \) preparations. Optimal ratios of \( \text{Fe}^{3+}:\text{Fe}^{2+} \) for the rapid initiation of lipid peroxidation were on order of 1:1 to 7:1. No \( \text{OH}^- \) formation could be detected with this system.

Although low concentrations of \( \text{H}_2\text{O}_2 \) or ascorbate increased lipid peroxidation by \( \text{Fe}^{2+} \) or \( \text{Fe}^{3+} \), respectively, high concentrations of \( \text{H}_2\text{O}_2 \) or ascorbate (in excess of iron) inhibited lipid peroxidation due to oxidative or reductive maintenance of iron exclusively in \( \text{Fe}^{3+} \) or \( \text{Fe}^{2+} \) form. Stimulation of lipid peroxidation by low concentrations of \( \text{H}_2\text{O}_2 \) or ascorbate was due to the oxidative or reductive creation of \( \text{Fe}^{3+}:\text{Fe}^{2+} \) ratios. The data suggest that the absolute ratio of \( \text{Fe}^{3+} \) to \( \text{Fe}^{2+} \) was the primary determining factor for the initiation of lipid peroxidation reactions.

The role of iron in initiating lipid peroxidation reactions within biological membranes has been examined in a variety of systems. Ferric iron \( \text{Fe}^{3+} \) or ferric chelates can initiate lipid peroxidation reactions provided that a reducing agent is present to reduce \( \text{Fe}^{3+} \) to \( \text{Fe}^{2+} \). In many cases, superoxide \( \text{O}_2^- \) generated by the reaction of xanthine or hypoxanthine with xanthine oxidase may act as the reductant (Tien et al., 1982). However, other cellular reducing agents such as ascorbate may also reduce \( \text{Fe}^{3+} \) via a presumably \( \text{O}_2^- \)-independent mechanism (Buchler et al., 1983a). In the former case, initiation of lipid peroxidation both \textit{in vitro} and \textit{in vivo} by \( \text{Fe}^{3+} \) and \( \text{O}_2^- \) has been hypothesized to occur via production of \( \text{OH}^- \) arising through the iron-catalyzed Haber-Weiss reaction

\[
\begin{align*}
\text{Fe}^{3+} + \text{O}_2^- & \rightarrow \text{Fe}^{2+} + \text{O}_2 \quad (1) \\
\text{O}_2^- + \text{H}_2\text{O}_2 & \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \quad (2) \\
\text{H}_2\text{O}_2 + \text{Fe}^{3+} & \rightarrow \text{OH}^- + \text{OH}^- + \text{Fe}^{2+} \quad (3)
\end{align*}
\]

Certain iron chelates, particularly those with ADP, are known to accelerate the Haber-Weiss reaction and the formation of \( \text{OH}^- \). Although some investigators have provided evidence for the role of Haber-Weiss-generated \( \text{OH}^- \) in lipid peroxidation reactions (Lai and Piette, 1977), others question its involvement (Tien et al., 1982). Ferrrous iron \( \text{Fe}^{2+} \) may also initiate lipid peroxidation reactions provided an oxidant is present to oxidize \( \text{Fe}^{2+} \) to \( \text{Fe}^{3+} \). In most cases, the oxidant is either molecular \( \text{O}_2 \),

\[
\text{Fe}^{2+} + \text{O}_2 \rightarrow \text{Fe}^{3+} + \text{O}_2^- \quad (4)
\]

or \( \text{H}_2\text{O}_2 \)

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

The combination of \( \text{Fe}^{2+} \) and \( \text{H}_2\text{O}_2 \), known as Fenton's reagent, is often used to initiate lipid peroxidation reactions. While Fenton's reagent yields large amounts of the very reactive \( \text{OH}^- \), the role of \( \text{OH}^- \) in initiating lipid peroxidation with Fenton's reagent has been questioned (Koppenol and Liebman, 1984; Bors et al., 1979) in that an iron-oxygen complex \( \text{Fe}^{3+}\text{O}^\text{2+} \) or \( \text{FeO(OH)}^{2+} \) derived from \( \text{OH}^- \) and \( \text{Fe}^{3+} \) or \( \text{Fe}^{2+} \) and \( \text{H}_2\text{O}_2 \) could be the oxidizing species. Alternatively, the autoxidation of \( \text{Fe}^{2+} \) by \( \text{O}_2 \) which is accelerated by iron chelation and can result in \( \text{OH}^- \) formation (via Reactions 1–3) has been proposed by Weiss (1965) to result in the formation of a reactive iron-oxygen complex

\[
\text{Fe}^{2+} + \text{O}_2 \Rightarrow \text{Fe}^{3+} + \text{O}_2^- \quad (5)
\]

which is stabilized in the presence of certain ligands such as phosphate or chelators (Michelson, 1977).

Regardless of the radical generating system used or the nature of the lipid substrate, a consistent observation in studies involving iron has been that, without exception, oxidation of \( \text{Fe}^{2+} \) or reduction of \( \text{Fe}^{3+} \) is necessary for the initiation of lipid peroxidation. Although conflicting data on the involvement of \( \text{OH}^- \) or \( \text{O}_2^- \) in lipid peroxidation is widespread, \( \text{Fe}^{2+} \leftrightarrow \text{Fe}^{3+} \) interconversion is obligatorily required.

Buchler et al. (1983b) originally reported on the requirement of \( \text{Fe}^{2+} \) chelates in the initiation of lipid peroxidation by \( \text{Fe}^{2+} \) chelates. Their work suggested that ratios of \( \text{Fe}^{2+} \) chelate:Fe\textsuperscript{3+} chelate were involved in the initiation of lipid peroxidation reactions, perhaps through the formation of an \( \text{Fe}^{3+} \)-di-oxygen-Fe\textsuperscript{2+} chelate complex. The importance of iron ratios in supporting lipid peroxidation also has been alluded to in a report by Ernst et al. (1982), although the concept was not tested directly. We have conducted a series of studies concerning the

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involvement of nonchelated iron in peroxidation of brain synaptosomal lipids. The results of this work extend the findings of Bucher et al. (1983b) and are in excellent agreement with their observations. Our findings support their original hypothesis that optimal Fe³⁺:Fe²⁺ ratios are required for the initiation of lipid peroxidation reactions. In this regard, various free radical generating systems used to initiate lipid peroxidation serve chiefly to create an Fe³⁺:Fe²⁺ ratio within the system. This hypothesis explicitly explains observed paradoxical differences in the function of oxidizing or reducing agents in iron-dependent lipid peroxidation.

MATERIALS AND METHODS

Rat brain synaptosomes were freshly prepared as described (Braughler, 1985), except that synaptosomes were washed and suspended in 0.9% NaCl, pH 7.0. All incubations were carried out at 37 °C in 0.9% NaCl. Since most buffers can trap OH⁻ radicals or interfere with iron conversions (as described in Fig. 5), reactions were unbuffered, and the pH of 0.9% NaCl used in these experiments was carefully adjusted to 7.0 immediately prior to use. Fe²⁺ solutions were prepared as either Fe(NH₄)₂(SO₄)₂ or FeCl₃ in degassed H₂O purged with either argon or nitrogen. Fe³⁺ was prepared as FeCl₃ in H₂O. All iron solutions were prepared fresh and used immediately. Under these circumstances, precipitation of Fe³⁺ as Fe(OH)₃ was not a problem.

Lipi¬peroxidation was followed by monitoring the rate of O₂ consumption in incubations using a Clark-style O₂ electrode. Incubations containing 0.5 mg of synaptosomal protein/ml were continuously stirred at 37°C in a 600-μl sealed chamber in which the O₂ electrode was immersed via a sealed port. Additions to the incubation were made via a resealable port on top of the chamber. The O₂ amplifier was interfaced with an Apple II computer, and the O₂ content of the chamber and the instantaneous O₂ consumption rate were continuously monitored using "Micro 2" O₂ analysis software developed by Instech Laboratories, Horsham, PA. Overall O₂ consumption rates for various reactions were calculated through a point-by-point analysis.

Conjugated diene formation in detergent-dispersed synaptosomes (0.01 mg of synaptosomal protein/ml of 0.9% NaCl containing 1% Lubrol) was monitored continuously at 232 nm using a Gilford response spectrophotometer. Reference samples contained synaptosomes and all other reactants except iron. Conjugated diene formation was corrected for absorbance changes in 232 nm arising from Fe²⁺ to Fe³⁺ conversions (Graf et al., 1984) by subtracting the absorbance of incubations containing all reactants except synaptosomes.

The formation of thiobarbituric acid-reactive oxidation products (TBAR) during incubations was determined as described by Buege and Aust (1978) with some modification. One hundred-μl reactions containing 0.1 mg of synaptosomal protein/ml were stopped by the addition of 500 μl of ice-cold 0.8 M HCl containing 12.5% trichloroacetic acid. One hundred μl of H₂O₂ was then added containing 50 μM desferrioxamine to prevent further iron-catalyzed TBAR formation. Thiobarbituric acid (final concentration 0.67%) was added, and samples were boiled in the presence or absence of 0.05% butylated hydroxytoluene (BHT) for 20 min. Following boiling, samples were cooled, centrifuged at 1500 × g for 15 min, and the absorbance of the supernatant read at 532 nm. Quantitation was based upon a molar extinction coefficient of 1.56 × 10⁵.

Lipid hydroperoxide was assayed by the iodometric procedure described by Buege and Aust (1978). All reagents were prepared fresh daily, degassed, and purged with argon. One-ml reactions containing 0.1 mg of synaptosomal protein/ml were stopped with the addition of 5 ml of ice-cold chloroform:methanol (2:1). Following centrifugation, 3 ml of the organic phase was removed and taken to dryness in a preweighed glass tube under argon at 25°C. 1 ml of acid chloroform (2:1) was added, followed by 50 ml of 0.18 M KI in H₂O. The tube was stopped and incubated in the dark at 25°C for exactly 5 min, after which 3 ml of 0.5% Ca(OH)₂ was added and the sample was centrifuged at 1500 × g for 10 min. The absorbance of the upper layer was read at 535 nm. A molar extinction coefficient of 2.8 × 10⁵ was used for quantitation.

The abbreviations used are: TBAR, thiobarbituric acid-reactive oxidation products; BHT, butylated hydroxytoluene.

The formation of OH⁻ radicals or iron-catalyzed lipid peroxidation products of salicylate in the absence of added synaptosomes as described by Halliwell (1978). Two-ml incubations containing 20 mM salicylate and no synaptosomes were stopped by the addition of 80 μl of 12 M HCl, 0.5 g of NaCl, and 4 ml of ice-cold diethyl ether. The sample was mixed, and the ether layer was removed and evaporated to dryness. The residue was dissolved in 0.25 ml of cold distilled H₂O, and the following additions were made in order: 0.125 ml of 10% trichloroacetic acid dissolved in 0.5 M HCl; 0.25 ml of 10% sodium tungstate; and 0.25 ml of 0.5% sodium nitrite (prepared fresh). After 5 min at 25°C, 0.5 ml of 0.5 M KOH was added and the absorbance was read at 510 nm. A molar extinction coefficient of 3.26 × 10⁴ was derived from solutions of 2,3-dihydroxybenzoate carried through the same extraction and assay procedure was used for quantitation.

H₂O₂ was assayed by the oxidation of phenol red. One hundred-μl reactions were stopped by the addition of 800 μl of phenol red solution containing 0.56 mM phenol red, horseradish peroxidase (19 units/ml), 5.5 mM dextrose, 140 mM NaCl, and 10 mM phosphate buffer, pH 7.4. The sample was incubated for 10 min at 25°C, and 100 μl of 1 N NaOH was added. The absorbance was read at 610 nm. A standard curve was based upon known amounts of H₂O₂.

O₂ consumption curve data were from representative experiments, and O₂ consumption rates were based upon mean rate calculations from data accumulation rates of 1 point/s. Other data in figures and tables are the means of triplicate determinations from representative experiments.

RESULTS

The addition of 200 μM Fe²⁺ to an incubation of rat brain synaptosomes containing 100 μM H₂O₂ resulted in the formation of OH⁻, conjugated dienes, and lipid hydroperoxide, as well as a rapid consumption of O₂ (Fig. 1). Careful analysis of the reaction time course indicates a burst in O₂ consumption as well as conjugated diene and lipid hydroperoxide formation within the first 5 s after Fe²⁺ addition that was associated with minimal detected OH⁻ formation. Despite continuous O₂ consumption and conjugated diene formation, lipid hydroperoxide concentrations fell acutely between 5 s and 1 min. The acute fall in lipid hydroperoxide was presum-
ably due to iron-catalyzed degradation of lipid hydroperoxide to either alkoxy or peroxy radicals. This would be expected to result in lipid peroxidation chain propagation and branching reactions initiated by alkoxy and peroxy radicals. Since conjugated diene formation continued to increase between 5 s and 1 min before reaching a steady state at 1 min, chain branching was likely. OH· formation increased sharply during this time period. After 1 min, lipid hydroperoxide formation again increased in the presence of a continued O₂ consumption. Although the O₂ concentration was near zero by 4 min, some lipid hydroperoxide formation continued despite the presence of only trace amounts of O₂ dissolved in the reaction media.

The formation of TBAR in brain synaptosomes was also examined in incubations containing Fe²⁺ and H₂O₂ (Table I). TBAR actually formed during Fe²⁺ + H₂O₂-induced peroxidation at 37 °C was measured by including 0.05% BHT during sample boiling to prevent further formation of TBAR presumably from the breakdown of lipid hydroperoxide (Buege and Aust, 1978).Alternatively, boiling samples in the absence of BHT yielded large amounts of TBAR that represented both TBAR formed during the original incubation as well as TBAR produced during the boiling step. Thus, the difference between TBAR assays boiled in the absence and presence of BHT yielded information concerning the production of potential TBAR-forming material that do not produce TBAR under standard incubation conditions at 37 °C but do decompose to TBAR under conditions of the thiobarbituric acid assay (100 °C at acidic pH). As can be seen in Table I, within 5 s after the addition of Fe²⁺ to an incubation of brain synaptosomes containing H₂O₂ little TBAR was formed in the sample itself; however, a significant amount of TBAR-forming material was produced. After 10 min at 37 °C, there was an increase in both TBAR formed during the incubation as well as in the production of TBAR-forming material. No detectable TBAR-forming material or lipid hydroperoxide was present in synaptosomes not exposed to Fe²⁺ (not shown).

Contrasting observations to those observed with Fe²⁺ and H₂O₂ were obtained using either Fe³⁺ or Fe⁵⁺ alone. The overall peroxidation rate induced by 200 μM Fe⁵⁺ was quite slow during the first 30 min as evidenced by a slow consum-

| TABLE I |
| TBAR formation in brain synaptosomes with Fe²⁺ and H₂O₂, Fe³⁺, or Fe⁵⁺ |

The production of TBAR (+BHT) and TBAR-forming material (-BHT (+BHT) values) is presumed to represent products of lipid peroxidation that do not produce TBAR under conditions of the thiobarbituric acid assay (100 °C at acidic pH). As can be seen in Table I, within 5 s after the addition of Fe²⁺ to an incubation of brain synaptosomes containing H₂O₂ little TBAR was formed in the sample itself; however, a significant amount of TBAR-forming material was produced. After 10 min at 37 °C, there was an increase in both TBAR formed during the incubation as well as in the production of TBAR-forming material. No detectable TBAR-forming material or lipid hydroperoxide was present in synaptosomes not exposed to Fe²⁺ (not shown).

Contrasting observations to those observed with Fe²⁺ and H₂O₂ were obtained using either Fe³⁺ or Fe⁵⁺ alone. The overall peroxidation rate induced by 200 μM Fe⁵⁺ was quite slow during the first 30 min as evidenced by a slow consum-

Fe²⁺ plus 100 μM

| Time | +BHT | BHT | BHT (-BHT) |
|------|------|-----|------------|
| 5 s  | 0.1  | 2.6 | 2.5        |
| 10 min | 2.1  | 7.9 | 4.8        |

Fe⁵⁺

| Time | +BHT | BHT | BHT (-BHT) |
|------|------|-----|------------|
| 5 s  | ND*  | 1.0 | 1.0        |
| 10 min | 0.8  | 3.3 | 2.5        |
| 30 min | 8.6  | 12.6 | 4.0       |
| 60 min | 16.4 | 19.8 | 4.4       |

Fe⁵⁺

| Time | +BHT | BHT | BHT (-BHT) |
|------|------|-----|------------|
| 5 s  | 0.8  | 3.5 | 2.7        |
| 10 min | 8.4  | 12.5 | 4.1       |
| 30 min | 8.0  | 12.9 | 4.9       |
| 60 min | 10.0 | 14.4 | 4.4       |

*None detected.
Based on the results in Fig. 1 with Fe\(^{3+}\) and H\(_2\)O\(_2\), the involvement of OH\(^-\) in peroxidation of brain synaptosomes induced by Fenton's reagent must be questioned. An analysis of rates of H\(_2\)O\(_2\) reduction by Fe\(^{3+}\) and the formation of OH\(^-\) during an incubation in the absence of synaptosomes (Fig. 4) reveals marked differences in their kinetics. Greater than 90% of the H\(_2\)O\(_2\) was consumed within 5 s following the addition of Fe\(^{3+}\), while OH\(^-\) production during this time was minimal.

Under these circumstances either: 1) OH\(^-\) formation is not stoichiometric with H\(_2\)O\(_2\) reduction, or 2) the high reactivity and short \(t_{1/2}\) of OH\(^-\) prohibited its reaction with the trap used for its detection (salicylate); thus only a fraction of OH\(^-\) formed was detected. In view of this second possibility, it is difficult to perceive how OH\(^-\) formed through Fenton's reaction could attack an allylic hydrogen buried within the phospholipid structure unless it was generated at that site.

Further studies into the involvement of OH\(^-\) in lipid peroxidation induced with Fenton's reagent are shown in Table II where the OH\(^-\) traps, Tris and mannitol, were included in reactions with Fe\(^{3+}\) and H\(_2\)O\(_2\). Mannitol at a concentration 10\(^{-3}\)-fold that of OH\(^-\) actually detected caused only a modest reduction in H\(_2\)O\(_2\)/Fe\(^{3+}\)-induced O\(_2\) consumption and early conjugated diene formation. Tris has been reported to be an excellent OH\(^-\) (Tien et al., 1982), and at pH 7.4, 10 mM Tris caused a large reduction in both O\(_2\) consumption and conjugated diene formation. At pH 6.5, however, Tris was far less effective. Small effects of each trap were observed on TBAR formation during the 10-min assay.

When Fe\(^{3+}\) is oxidized either by autoxidation or by H\(_2\)O\(_2\) to Fe\(^{3+}\), the Fe\(^{3+}\) species formed absorbs light strongly in the UV range, and the absolute absorbance of the UV spectrum is indicative of the concentration of Fe\(^{3+}\) present in solution (Graf et al., 1984). Some insight into the actions of mannitol and Tris on Fe\(^{3+}\)/H\(_2\)O\(_2\)-induced peroxidation can be gained by examining their effects on the UV iron spectrum (Fig. 5). As shown in Fig. 5A, the absorbance of a reaction containing 200 \(\mu\)M Fe\(^{3+}\) and 100 \(\mu\)M H\(_2\)O\(_2\) increased rapidly with time. The initial absorbance scan of 200 \(\mu\)M Fe\(^{3+}\) and 100 \(\mu\)M H\(_2\)O\(_2\) at 5 s after the addition of Fe\(^{3+}\) was identical to an initial scan of a combination of 100 \(\mu\)M Fe\(^{3+}\) and 100 \(\mu\)M Fe\(^{2+}\) (Fig. 7A), indicating that the reaction of Fe\(^{3+}\) with H\(_2\)O\(_2\) was very rapid and essentially complete within 5 s, in agreement with data shown in Fig. 4. With time, the absorbance of a solution of Fe\(^{2+}\) and H\(_2\)O\(_2\) continues to increase rapidly, and by 10 min the spectrum is similar to that of a solution containing almost exclusively Fe\(^{3+}\). Mannitol, which caused only a slight reduction in the peroxidation rate, only very slightly slowed the formation of Fe\(^{3+}\) (Fig. 5B). In contrast, 10 mM Tris at pH 7.4, which inhibited peroxidation, caused a striking reduction in Fe\(^{3+}\) formation with time and altered the shape of the UV spectrum as well (Fig. 5C). Interestingly, the initial 5-s spectra with Tris, pH 7.4, suggested that rapid oxidation of Fe\(^{3+}\) by H\(_2\)O\(_2\) to an Fe\(^{2+}\) species had occurred. Quite different results were obtained with 10 mM Tris at pH 6.5, as Fe\(^{3+}\) conversion was not inhibited, but its rate of formation was reduced (Fig. 5D). These findings are consistent with the effects of mannitol and Tris on Fe\(^{3+}\)/H\(_2\)O\(_2\)-induced peroxidation of brain synaptosomes shown in Table II.

Taken together, these results suggest that it is perhaps not OH\(^-\) formation which is important for the initiation of lipid peroxidation by Fe\(^{3+}\) and H\(_2\)O\(_2\), but rather it is the conversion of Fe\(^{2+}\) to Fe\(^{3+}\) that is in some way responsible. As demonstrated in Fig. 2 and Table I, peroxidation of brain synaptosomes did occur in the presence of Fe\(^{3+}\) alone. However, the rate was much slower than for the combination of Fe\(^{3+}\) and H\(_2\)O\(_2\). With Fe\(^{3+}\) alone, a considerable lag phase was observed before O\(_2\) consumption (peroxy radical formation) achieved a rapid rate.

As demonstrated in Fig. 6, the lag phase associated with Fe\(^{3+}\)-induced peroxidation could be dramatically reduced by adding a combination of Fe\(^{3+}\) and Fe\(^{2+}\). Higher Fe\(^{3+}:Fe^{2+}\) ratios were associated and shorter lag phases and more rapid rates of lipid peroxidation as assessed by more rapid O\(_2\) consump-

![FIG. 4. H\(_2\)O\(_2\) consumption and OH\(^-\) formation with H\(_2\)O\(_2\) and Fe\(^{3+}\). OH\(^-\) (O) and H\(_2\)O\(_2\) (●) were determined as described under *Materials and Methods* in the absence of synaptosomes. Incubations contained 100 \(\mu\)M H\(_2\)O\(_2\), and reactions were initiated by the rapid addition of 200 \(\mu\)M Fe\(^{3+}\).*](image-url)

### Table II

| Addition       | O\(_2\) consumption | \(A_{520nm}\) 5 min | \(A_{520nm}\) 10 min | +BHT | -BHT | -BHT - (+BHT) |
|----------------|---------------------|---------------------|---------------------|------|------|---------------|
| None           | 49.6                | 0.615               | 0.740               | 3.2  | 9.6  | 6.4           |
| Mannitol (20 mm) | 38.7                | 0.527               | 0.756               | 2.7  | 9.0  | 7.7           |
| Tris, pH 7.4 (10 mm) | 7.3                | 0.270               | 0.456               | 2.5  | 8.9  | 6.5           |
| Tris, pH 6.5 (10 mm) | 21.6               | 0.386               | 0.728               | 2.3  | 8.1  | 5.8           |
Bations contained 0.1 mg of synaptosomal protein/ml, and reactions were initiated by the rapid addition of iron. Micromolar concentrations of Fe3+ and Fe2+ were: 175 and 25. Each reaction contained 200 μM Fe2+ and 100 μM H2O2. TBAR formation was determined as described under “Materials and Methods.” Incubations (100 μl) contained 0.1 mg of synaptosomal protein/ml and were initiated by rapid addition of the concentrations of Fe3+ and Fe2+ indicated. Reactions were terminated after 10 min.

| Fe3+ (μM) | Fe2+ (μM) | (OH‘) | TBAR formation (nmol TBAR/ml) |
|----------|----------|-------|-----------------------------|
| 0        | 200      | 0.9   | 4.5                         |
| 25       | 175      | 1.6   | 7.2                         |
| 50       | 150      | 1.5   | 5.6                         |
| 100      | 200      | 3.2   | 8.3                         |
| 150      | 50       | 14.6  | 21.5                        |
| 175      | 25       | 13.9  | 19.0                        |
| 200      | 0        | 9.3   | 15.1                        |
| 100 μM H2O2 plus 200 μM Fe3+ | 3.5 | 8.1 | 4.6 |

**Fig. 6.** O2 consumption by brain synaptosomal membrane with various combinations of Fe2+ and Fe3+. Media O2 content was determined as described under “Materials and Methods.” Incubations contained 0.1 mg of synaptosomal protein/ml, and reactions were initiated by the rapid addition of iron. Micromolar concentrations of Fe2+ and Fe3+ were: 175 and 25 (---); 150 and 50 (-----); 100 and 100 (--); 50 and 150 (--); 25 and 175 (----). One reaction contained 200 μM Fe3+ and 100 μM H2O2 (-----).

**Fig. 6.** O2 consumption by brain synaptosomal membrane with various combinations of Fe2+ and Fe3+. Media O2 content was determined as described under “Materials and Methods.” Incubations contained 0.1 mg of synaptosomal protein/ml, and reactions were initiated by the rapid addition of iron. Micromolar concentrations of Fe2+ and Fe3+ were: 175 and 25 (---); 150 and 50 (-----); 100 and 100 (--); 50 and 150 (--); 25 and 175 (----). One reaction contained 200 μM Fe3+ and 100 μM H2O2 (-----).

**TABLE III**

TBAR formation in brain synaptosomes with different Fe3+ : Fe2+ combinations

| Fe3+ (μM) | Fe2+ (μM) | (OH‘) | TBAR formation (nmol TBAR/ml) |
|----------|----------|-------|-----------------------------|
| 0        | 200      | 0.9   | 4.5                         |
| 25       | 175      | 1.6   | 7.2                         |
| 50       | 150      | 1.5   | 5.6                         |
| 100      | 200      | 3.2   | 8.3                         |
| 150      | 50       | 14.6  | 21.5                        |
| 175      | 25       | 13.9  | 19.0                        |
| 200      | 0        | 9.3   | 15.1                        |
| 100 μM H2O2 plus 200 μM Fe3+ | 3.5 | 8.1 | 4.6 |

**TABLE IV**

Effects of Tris and mannitol on O2 consumption by brain synaptosomal membranes with different Fe2+ : Fe3+ combinations

Overall O2 consumption rates were determined as described under “Materials and Methods.” Incubations contained 0.1 mg of synaptosomal protein/ml and the concentrations of Fe2+, Fe3+, Tris, mannitol, superoxide dismutase, or catalase indicated. Reactions were initiated by the rapid addition of iron.

| Fe3+ (μM) | Fe2+ (μM) | Addition | O2 consumption (nmol/min) in 6 min |
|----------|----------|----------|----------------------------------|
| 175      | 25       | None     | 45.4                             |
| 175      | 25       | Tris, pH 7.4(10 mM) | 16.7                             |
| 175      | 25       | Tris, pH 6.5(10 mM) | 46.4                             |
| 175      | 25       | Mannitol (20 mM) | 46.8                             |
| 150      | 50       | None     | 40.4                             |
| 150      | 50       | Tris, pH 7.4(10 mM) | 16.5                             |
| 150      | 50       | Tris, pH 6.5(10 mM) | 45.9                             |
| 150      | 50       | Mannitol (20 mM) | 42.1                             |
| 175      | 25       | Superoxide dismutase (150 units/ml) | 45.9                             |
| 175      | 25       | Catalase (100 units/ml) | 48.7                             |
| 175      | 25       | Superoxide dismutase plus catalase | 50.0                             |

The OH‘ assay during incubations containing various ratios of Fe2+ : Fe3+ (not shown). Nevertheless, Tris at pH 7.4 inhibited O2 consumption caused by combinations of Fe2+ and Fe3+ just as it inhibited O2 consumption by Fe2+ and H2O2 (Table IV). Neither Tris, pH 6.5, nor mannitol affected peroxidation caused by combinations of Fe2+ and Fe3+. Peroxidation by Fe3+ : Fe2+ combinations was also not affected by superoxide dismutase, catalase, or their combination.

These observations suggested that the absolute ratio of Fe2+ : Fe3+ was more important than OH‘ in determining the rate or extent of lipid peroxidation induced by Fenton’s reagent (Fe3+/H2O2). This hypothesis would predict that the primary function of H2O2 in such a system would be to create a pool of Fe3+ from Fe2+. Furthermore, this hypothesis also predicts that generation of a small amount of Fe2+ from Fe3+ should accelerate Fe2+ -induced peroxidation and that trapping or oxidative-reductive maintenance of either Fe3+ or Fe2+ in their respective forms should inhibit lipid peroxidation. Such was the case. As demonstrated in Table V, O2 consumption in the presence of Fe3+ was markedly accelerated in the presence of 25 μM ascorbate. Ascorbate reduces Fe3+ to Fe2+, and low concentrations have been shown by others to stimulate iron-dependent lipid peroxidation (Buchert et al., 1983a).
The rate of O$_2$ consumption in the presence of 200 µM Fe$^{3+}$ and 25 µM ascorbate was somewhat faster than for a combination of 175 µM Fe$^{3+}$ and 25 µM Fe$^{2+}$. High concentrations of ascorbate were found to inhibit lipid peroxidation, probably by direct antioxidant properties (Bucher et al., 1983a), but also by maintaining iron in its reduced form. Similarly, H$_2$O$_2$ could either stimulate or inhibit Fe$^{2+}$-dependent lipid peroxidation. Indeed, O$_2$ consumption rates in the presence of 100 µM H$_2$O$_2$ and 200 µM Fe$^{2+}$ were found to be different than in the presence of the combination of 100 µM Fe$^{2+}$ and 100 µM Fe$^{3+}$. H$_2$O$_2$ in excess of Fe$^{2+}$, however, which was expected to affect complete conversion of Fe$^{2+}$ to Fe$^{3+}$, inhibited peroxidation. Such an inhibitory effect of H$_2$O$_2$ has been reported by others (Thomas et al., 1986; Tien et al., 1982).

Low concentrations of ascorbate also increased TBAR formation in brain synaptosomes by Fe$^{2+}$, whereas higher concentrations were inhibitory (Table VI). As with O$_2$ consumption, the combination of Fe$^{2+}$ and a low concentration of ascorbate caused slightly more peroxidation as assessed by TBAR formation than did the iron-equivalent combination of Fe$^{3+}$ and Fe$^{2+}$. Considerably more TBAR-forming material was formed in the absence of ascorbate, however. TBAR formation with the combination of 175 µM Fe$^{3+}$ and 25 µM Fe$^{2+}$ was inhibited by 100 µM H$_2$O$_2$, probably due to complete conversion of Fe$^{2+}$ to Fe$^{3+}$. In fact, TBAR formation in the presence of the combination of 175 µM Fe$^{3+}$, 25 µM Fe$^{2+}$, and 100 µM H$_2$O$_2$ was similar to that observed with 200 µM Fe$^{3+}$ alone.

The O$_2$ consumption and TBAR formation observed with Fe$^{2+}$ alone (Fig. 3, Tables I, V, and VI) resulted from contamination of the Fe$^{3+}$ preparation with small amounts of Fe$^{2+}$, thus providing a ratio of Fe$^{3+}$:Fe$^{2+}$. As demonstrated in Tables V and VI, preincubation of Fe$^{3+}$ preparations for 5 min with H$_2$O$_2$ or excess EDTA to assure complete Fe$^{2+}$ oxidation prior to addition of synaptosomal membranes rendered Fe$^{2+}$ incapable of initiating peroxidation. Furthermore, preincubation of Fe$^{2+}$ with EDTA was associated with a small burst in O$_2$ consumption consistent with autoxidation of contaminating Fe$^{2+}$. Finally, freshly prepared solutions of FeC$_3$O were found to contain about 1.0% Fe$^{2+}$ using 2',2',2'-tripyridine as an indicator of ferrous iron (Schade et al., 1954). These findings confirm that freshly prepared FeC$_3$O solutions contained some iron in the Fe$^{3+}$ form.

TBAR formation by Fe$^{2+}$ during the incubation was stimulated by both high and low concentrations of H$_2$O$_2$ (Table VI). While TBAR formed in the presence of 200 µM Fe$^{3+}$ and 100 µM H$_2$O$_2$ was similar to that with a combination of 100 µM Fe$^{2+}$ and 100 µM Fe$^{3+}$, much more TBAR and TBAR-forming material was produced in incubations with 200 µM Fe$^{3+}$ containing 400 µM H$_2$O$_2$. Conversely, if Fe$^{2+}$ and H$_2$O$_2$ were allowed to react prior to the introduction of synaptosomes, more TBAR was formed in a reaction that had contained the low concentration of H$_2$O$_2$ compared with the higher concentration. Thus, under certain circumstances, excess H$_2$O$_2$ could directly inhibit lipid peroxidation reactions. H$_2$O$_2$ at both high or low concentrations inhibited TBAR formation by Fe$^{2+}$.

These results are consistent with the idea that the ratio of Fe$^{3+}$:Fe$^{2+}$ is the important determinant for lipid peroxidation reactions. The observations with Fe$^{2+}$ and H$_2$O$_2$ also suggest that the rapidity with which conversions of Fe$^{3+}$ to Fe$^{2+}$ take place plays a role in lipid peroxidation. An examination of iron spectral changes reveals that although H$_2$O$_2$ accelerates the early conversion of Fe$^{3+}$ to Fe$^{2+}$, it reduces subsequent Fe$^{2+}$ to Fe$^{3+}$ conversions (Fig. 7, A and B). Similarly, while Fe$^{2+}$ displays some spectral changes with time, indicative of Fe$^{3+}$ to Fe$^{2+}$ to Fe$^{3+}$ interconversions, H$_2$O$_2$ greatly reduced these. Addition of small amounts of ascorbate to Fe$^{2+}$ to create a pool of Fe$^{3+}$, on the other hand, allows for more extensive Fe$^{2+}$ to Fe$^{3+}$ conversion associated with more extensive lipid peroxidation. Such changes in the rates of Fe$^{3+}$ to Fe$^{2+}$ conversion can have profound effects on lipid peroxidation, as evidenced by the data in Tables V and VI.

**DISCUSSION**

Lipid peroxidation of brain synaptosomes induced with Fenton’s reagent (Fe$^{2+}$ and H$_2$O$_2$) was associated with an
FIG. 7. Spectral changes with time of Fe$^{2+}$ and Fe$^{3+}$ solutions containing ascorbate or H$_2$O$_2$. Solutions of Fe$^{3+}$ in 0.9% NaCl were scanned at 40 nm/s from 450 to 200 nm. Bottom-most scans in each panel were at 5 s after addition of iron. Subsequent scans were at 1-min intervals. Reactions contained: A, 100 μM Fe$^{3+}$, 100 μM Fe$^{2+}$; B, 400 μM H$_2$O$_2$, 100 μM Fe$^{3+}$, 100 μM Fe$^{2+}$; C, 200 μM Fe$^{3+}$, 100 μM H$_2$O$_2$, 200 μM Fe$^{3+}$, 25 μM ascorbate, 200 μM Fe$^{3+}$.  

instantaneous (within 5 s) and intense production of conjugated dienes, lipid hydroperoxide, and TBAR-forming material upon the addition of Fe$^{2+}$. The burst in lipid hydroperoxide and conjugated diene formation was coincident with a rapid loss of O$_2$ and H$_2$O$_2$ from the media. In parallel incubations containing no synaptosomes, little or no OH$^-$ was detected during the first seconds following the addition of Fe$^{3+}$, despite the consumption of 90% of the H$_2$O$_2$. Thus, the kinetics of OH$^-$ formation by Fenton's reagent did not follow the kinetics of lipid peroxidation.

The direct involvement of OH$^-$ in the initiation of lipid peroxidation was further challenged by results obtained with OH$^-$ traps. Both Tris and, to a lesser extent, mannitol were found to interact with iron in a manner which slowed or blocked the formation of Fe$^{2+}$. These findings suggested that under conditions employed in these studies, OH$^-$ traps were not affecting the interaction of OH$^-$ with lipids by scavenging OH$^-$. per se, but rather were interfering with iron in some manner.

Peroxidation of brain synaptosomes could be induced by Fe$^{2+}$ alone, although peroxidation was preceded by a significant lag phase. This is in agreement with studies by Bucher et al., (1983b) who demonstrated that Fe$^{2+}$ chelates could initiate lipid peroxidation following a short lag phase. Lag phases with Fe$^{2+}$ chelates (Bucher et al., 1983b) were considerably shorter (5–6 min) than those reported here for free Fe$^{2+}$ (30 min). The lag phase associated with Fe$^{2+}$-induced peroxidation was significantly shortened by adding a combination of Fe$^{2+}$ and Fe$^{3+}$ to brain synaptosomal incubations. Again, similar observations have been reported by Bucher et al., (1983b) using iron chelates. In the present study, even in the absence of chelators, lipid peroxidation rates could be achieved that were similar or greater than those obtained with Fenton's reagent (Fe$^{3+}$ + H$_2$O$_2$) simply by using ratios of Fe$^{3+}$ to Fe$^{2+}$. Although lag phases may be explained by the presence of endogenous antioxidants in membrane preparations this is not likely since lag periods in the present studies correspond primarily to the time required for autoxidation of Fe$^{3+}$ to yield a suitable ratio of Fe$^{3+}$:Fe$^{2+}$.

The findings with ascorbate and H$_2$O$_2$ were consistent with the hypothesis that Fe$^{3+}$:Fe$^{2+}$ ratios are important determinants for lipid peroxidation. Reduction of small amounts of Fe$^{3+}$ by low concentrations of ascorbate markedly enhanced peroxidation by Fe$^{3+}$, while oxidation of some Fe$^{2+}$ by H$_2$O$_2$ enhanced peroxidation. On the other hand, high concentrations of ascorbate or H$_2$O$_2$ in excess of Fe$^{3+}$ or Fe$^{2+}$, respectively, inhibited lipid peroxidation. In view of the fact that significant lipid peroxidation could be initiated in vitro by the addition of various molar ratios of Fe$^{3+}$:Fe$^{2+}$, it is apparent that a major portion of the peroxidation induced in systems utilizing either Fenton's reagent or Fe$^{2+}$ reduction may be directly attributed to the oxidative or reductive generation of an Fe$^{3+}$:Fe$^{2+}$ ratio.

No detectable OH$^-$ was formed by various combinations of Fe$^{3+}$ and Fe$^{2+}$ nor was peroxidation caused by Fe$^{3+}$:Fe$^{2+}$ combinations inhibited by superoxide dismutase, catalase, their combination, or mannitol. These data argue against the involvement of OH$^-$ in Fe$^{3+}$:Fe$^{2+}$ initiation. Nevertheless, it is possible that OH$^-$ generated within the phospholipid environment of the membrane or reacting rapidly with Fe$^{3+}$ to form some ferry ion species (Sheldon and Kochi, 1981; Aust et al., 1985; Halliwell and Gutteridge, 1984) could escape traps and participate in Fe$^{3+}$:Fe$^{2+}$-induced lipid peroxidation. Girotti and Thomas (1984a, 1984b) have in fact proposed that it is OH$^-$ formed at or on the membrane and not in the media that is responsible for the initiation of lipid peroxidation by iron. Their conclusion is based upon studies demonstrating that while OH$^-$ traps were unable to inhibit lipid peroxidation in a membrane system containing xanthine, xanthine oxidase, and Fe$^{3+}$, peroxidation was inhibited by iron chelators, catalase, and superoxide dismutase. These findings are not in disagreement with those reported here as they are consistent with chelation of iron or enzymatic elimination of oxidant (H$_2$O$_2$) or reductant (O$_2^-$) preventing formation of an iron ratio. Our findings that catalase and superoxide dismutase do not block peroxidation with an Fe$^{3+}$:Fe$^{2+}$ ratio support this contention.

It is unlikely that the initiation of lipid peroxidation by combinations of Fe$^{3+}$ and Fe$^{2+}$ may be through iron-catalyzed decomposition of lipid hydroperoxide pre-existing in synaptosomal membranes. In that regard, free transition metals are relatively weak compared with chelated metals at decomposing lipid hydroperoxide, and Fe$^{2+}$ is at least an order of magnitude more effective than Fe$^{3+}$ (O'Brien, 1969; Halliwell and Gutteridge, 1984). Assays of freshly prepared brain synaptosomes used in the present studies revealed no detectable conjugated dienes, lipid hydroperoxide, or TBAR-forming material within the detection limits of the assays. Although this does not rule out the presence of pre-existing lipid hydroperoxide in the synaptosomal membranes used in these studies, it is unlikely that lipid peroxidation initiated by Fe$^{3+}$:Fe$^{2+}$ ratios can be attributed to iron-catalyzed lipid hydroperoxide decomposition since reaction rates and lag periods were slower when Fe$^{3+}$ was in predominance than when Fe$^{2+}$ was in excess. An absence of preexisting lipid hydroperoxide was also supported by studies in which Fe$^{3+}$ was preincubated with excess EDTA prior to addition of synaptosomes to elminate contam-
In those studies, Fe$^{3+}$-EDTA chelate (which did not contain Fe$^{3+}$) was incapable of initiating lipid peroxidation.

In summary, the rapid and extensive peroxidation of rat brain synaptosomal membranes could be initiated upon the addition of various combinations of Fe$^{3+}$ and Fe$^{2+}$ without need for added chelators, oxidizing or reducing agents. In other preliminary unpublished studies, similar results have been obtained using either phosphotidylcholine liposomes or lipid micelles prepared from purified linoleic, linolenic, or arachidonic acids as the lipid substrate. Thus, it is unlikely that the results presented here are artifacts due to the presence of endogenous metal chelators, oxidizing or reducing substances provided by the synaptosomal preparation. Peroxidation initiated by Fe$^{3+}$:Fe$^{2+}$ ratios did not rely upon detectable production of OH$^-$ radicals, although OH$^-$ radical involvement cannot be completely ruled out at this time. The findings suggest that lipid peroxidation initiated by combinations of iron with oxidizing or reducing agents such as Fenton’s reagent or Fe$^{3+}$ and ascorbate may largely result from the formation of optimal Fe$^{3+}$:Fe$^{2+}$ ratios.

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REFERENCES
Aust, S. D., Morehouse, L. A., and Thomas, C. E. (1985) J. Free Rad. Biol. Med. 1, 3–25
Braughler, J. M. (1985) J. Neurochem. 44, 1282–1288
Bucher, J. R., Tien, M., Morehouse, L. A., and Aust, S. D. (1983a) Fund. Appl. Tox. 3, 222–226
Bucher, J. R., Tien, M., and Aust, S. D. (1983b) Biochem. Biophys. Res. Commun. 111, 777–784
Buege, J. A., and Aust, S. D. (1978) Methods Enzymol. 52, 302–310
Ernster, L., and Nordenbrand, K. (1982) in Lipid Peroxides in Biology and Medicine (Yagi, K., ed) pp. 55–78, Academic Press, Orlando, FL
Girotti, A. W., and Thomas, J. P. (1984a) J. Biol. Chem. 259, 1744–1752
Girotti, A. W., and Thomas, J. P. (1984b) Biochem. Biophys. Res. Commun. 118, 474–480
Graf, E., Mahoney, J. R., Bryant, R. G., and Eaton, J. W. (1984) J. Biol. Chem. 259, 3620–3624
Halliwell, B. (1978) FEBS Lett. 92, 321–326
Halliwell, B., and Gutteridge, J. M. C. (1984) Biochem. J. 219, 1–14
Lai, C. S., and Piette, L. H. (1978) Arch. Biochem. Biophys. 190, 27–38
Michelson, A. M. (1977) in Superoxide and Superoxide Dismutase (McCord, J. M., and Fridovich, I., eds) pp. 77–86, Academic Press, Orlando, FL
O’Brien, P. J. (1969) Can. J. Biochem. 47, 485–492
Schade, A. L., Oyama, J., Reinhart, R. W., and Miller, J. R. (1954) Proc. Soc. Exp. Biol. Med. 87, 443–448
Sheldon, R. A., and Kochi, J. K. (1981) Metal-Catalyzed Oxidations of Organic Compounds, pp. 33–70, Academic Press, Orlando, FL
Thomas, C. E., Morehouse, L. A., and Aust, S. D. (1985) J. Biol. Chem. 260, 3275–3280
Tien, M., Svingen, B. A., and Aust, S. D. (1982) Arch. Biochem. Biophys. 216, 142–151