NADPH-dependent lipid peroxidation occurs in two distinct sequential radical steps. The first step, initiation, is the ADP-perferryl ion-catalyzed formation of low levels of lipid hydroperoxides. The second step, propagation, is the iron-catalyzed breakdown of lipid hydroperoxides formed during initiation generating reactive intermediates and products characteristic of lipid peroxidation. Propagation results in the rapid formation of thiobarbituric acid-reactive material and lipid hydroperoxides. Propagation can be catalyzed by ethylenediamine tetraacetate-chelated ferrous ion, diethylenetriamine pentaaetic acid-chelated ferrous ion, or by ferric cytochrome P-450. However, cytochrome P-450 is destroyed during propagation.

The mechanism of NADPH-dependent lipid peroxidation has been the subject of intense research in recent years. Several years ago it was demonstrated that NADPH-dependent microsomal lipid peroxidation required ADP, or pyrophosphate-chelated iron for maximal activity (1-9).

Only recently, however, has it been shown that NADPH-dependent lipid peroxidation is catalyzed by NADPH-cytochrome P-450 reductase. The role of NADPH-cytochrome P-450 reductase in lipid peroxidation was established, in part, by the ability of antibody to the reductase to inhibit NADPH-dependent microsomal lipid peroxidation (10). The use of purified NADPH-cytochrome P-450 reductase allowed the reconstitution of ADP-iron-requiring, NADPH-dependent lipid peroxidation in liposomes (10) and a more detailed investigation into the mechanism of peroxidation.

Several mechanisms by which NADPH-dependent lipid peroxidation is catalyzed have been proposed. Pederson et al. (10) and others (4), have proposed that initiation occurs via an ADP-perferryl ion (ADP-Fe[3+] - O2 ↔ ADP-Fe[2+] - O2-) catalyzed abstraction of methylene hydrogen from polyunsaturated fatty acids. The ADP-perferryl ion was proposed to be formed by the direct reduction of ADP-Fe[3+], catalyzed by NADPH-cytochrome P-450 reductase, and the subsequent reaction of ADP-Fe[2+] with molecular oxygen.

Other investigators have proposed the participation of several different reduced forms of oxygen in the initiation of NADPH-dependent lipid peroxidation. Fong et al. (11), McCav et al. (12), and King et al. (13) proposed that NADPH-dependent microsomal lipid peroxidation occurred via an ADP-ferrous ion-facilitated Haber-Weiss reaction (14). They found that NADPH-dependent lipid peroxidation was dependent on ADP-Fe[3+], superoxide (O2•-), H2O2, and the hydroxyl radical (OH•). The actual initiating species was proposed to be OH•. The authors therefore suggested the following scheme for NADPH-dependent lipid peroxidation involving O2•- produced by NADPH-cytochrome P-450 reductase and OH•:

\[2O2•- + 2 H+ → H2O2 + O2 (1)\]
\[H2O2 + O2•- → O2 + OH− + OH• (2)\]

The flux of OH• produced via Reactions 1, 3, and 4 was proposed to be much greater than by Reactions 1 and 2, thus, the requirement for catalytic amounts of ADP-Fe[3+] for enzymatic NADPH-dependent lipid peroxidation.

Several objections have been raised by other investigators as to the viability of the proposed scheme for O2•- dependent lipid peroxidation. First, the participation of OH• in initiation of O2•- dependent lipid peroxidation has not been confirmed (15-17). Second, since the proposed mechanism is dependent upon H2O2 catalase would be expected to inhibit O2•- dependent lipid peroxidation. However, others have found that catalase enhances O2•- dependent lipid peroxidation (15, 18). Third, it is unlikely that OH• would diffuse from its site of formation before reacting (19) as would be required by the proposed mechanism. Fourth, the rate of nonenzymatic dismutation of O2•- at physiological pH may be minor in comparison to other reactions of O2•- (20, 21). Finally, the concentration of H2O2 obtained during microsomal NADPH-oxidation in the absence of azide may be too low to account for the rate of enzymatic lipid peroxidation observed (22). It must also be realized that microsomes are contaminated with catalase.

Pederson et al. (10) showed that the reconstitution of NADPH-dependent lipid peroxidation in liposomes containing purified protease solubilized NADPH-cytochrome P-450 reductase required ADP-iron (10, 23). Lipid peroxidation was greatly enhanced by the addition of EDTA-iron. A mechanism involving initiation of peroxidation by the ADP-perferryl ion was proposed. The role of EDTA-iron in lipid peroxidation was undefined, although it was suggested that it replaced an endogenous microsomal component that participated in lipid peroxidation.

Recently, using similar techniques, it was demonstrated that lipid peroxidation occurs in two sequential steps, initiation and propagation (24). The role of ADP-Fe[3+] in the initiation of NADPH-dependent lipid peroxidation was more clearly defined. The ADP-perferryl ion was proposed to catalyze the rapid initial formation of lipid hydroperoxides. The
Initiation did not occur via singlet oxygen ('O_2) since ~O_2-
initiation was found to be superoxide dismutase sensitive. The ADP-perferryl ion-dependent reaction was termed initiation. Specific iron chelates, other than ADP-Fe^{3+}, propagate lipid peroxidation at the expense of initially formed lipid hydroperoxides. The Fenton reaction can replace EDTA-Fe^{3+} in catalysis of propagation. In microsomes, where the EDTA-Fe^{3+} propagation agent is not required, ferriyochrome P-450 is shown to be an endogenous propagating agent. Cytochrome P-450 is destroyed during the propagation of lipid peroxidation.

MATERIALS AND METHODS

Chemicals—The materials used in these studies were obtained from the following sources: 2,5-diphenylfuran, Eastman Organic Co.; bovine serum albumin, Pentex Chemical Co.; 2,5-diphenylfuran, Eastman Organic Co.; cytochrome c (type IV), NADPH, and diethylenetriamine pentaacetic acid-Fe^{3+} can replace EDTA-Fe^{3+} in catalysis of propagation. In microsomes, where the EDTA-Fe^{3+} propagation agent is not required, ferriyochrome P-450 is shown to be an endogenous propagating agent. Cytochrome P-450 is destroyed during the propagation of lipid peroxidation.

The buffers used in preparation of the ferrous chelates were saturated with argon before use to prevent autoxidation of the ferrous ion. All solvents were purified with argon and all operations were performed under argon.

Enzyme Sources—Bovine erythrocyte superoxide dismutase (EC 1.15.1.1) and soybean lipoygenase (type I) (EC 1.13.11.13) were obtained from Sigma Chemical Co. Superoxide dismutase activity was measured by the method of McCord and Fridovich (27). NADPH-cytochrome P-450 reductase (EC 1.6.4.6) was isolated from freshly prepared microsomes by the method of Folch et al. (25). All solvents were purified with argon and all operations were performed under argon.

Protein was determined by the method of Lowry et al. (30). Total lipid phosphate was assayed by the method of Bartlett (31).

RESULTS

It has been previously proposed that lipid peroxidation can be divided into two distinct sequential reactions, initiation and propagation (24). Initiation was defined as the formation of lipid hydroperoxides accompanied by the minimal formation of MDA. Initiation was proposed to be catalyzed by the ADP-perferryl ion in both NADPH-dependent and O_2-dependent lipid peroxidation. Propagation was suggested to involve the iron-catalyzed breakdown of the initially formed lipid hydroperoxides generating reactive intermediates and products of lipid peroxidation. Propagation was proposed to result in the rapid formation of MDA and additional lipid hydroperoxides. Initial results (24) indicated that the participation of EDTA-iron in propagation was of major significance while participation by ADP iron was minimal.

It would be difficult to assess lipid hydroperoxide breakdown in a matrix where additional lipid hydroperoxides could be formed as in liposomes. Thus, cumene hydroperoxide (CHP) was used to assess the ability of different possible propagators to catalyze hydroperoxide breakdown. The choice of CHP was based on its ability to support NADPH-independent microsomal lipid peroxidation (32). Cumene hydroperoxide apparently replaces the initially formed lipid hydroperoxides in the sequence of reactions. In NADPH-dependent liposomal peroxidation, ADP Fe^{3+} and EDTA Fe^{3+} are required for maximal activity (10). To assess the participation of the ferric and ferrous forms of these iron chelates in propagation, these agents were incubated with CHP and their affect on the breakdown of CHP was determined (Fig. 1). The ferrous ion chelates were formed by enzymatic reduction with NADPH-cytochrome P-450 reductase. As shown in Fig. 1, neither ADP-Fe^{3+}, EDTA-Fe^{3+}, ADP-Fe^{2+}, nor the action of NADPH-cytochrome P-450 reductase alone can catalyze the breakdown of CHP. Only EDTA-Fe^{3+} can catalyze the rapid breakdown of CHP.

The abbreviations used are: TBA, thiobarbituric acid; MDA, malondialdehyde; BHT, butylated hydroxytoluene; DTPA, diethylenetriamine pentaacetic acid; CHP, cumene hydroperoxide; DPF, 2,5-diphenylfuran.
The propagation of lipid peroxidation by iron chelates

**TABLE I**
The propagation of lipid peroxidation by iron chelates

<table>
<thead>
<tr>
<th></th>
<th>MDA</th>
<th>Lipid hydroperoxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no additions)</td>
<td>0.01</td>
<td>0.7</td>
</tr>
<tr>
<td>ADP-Fe²⁺</td>
<td>0.02</td>
<td>0.6</td>
</tr>
<tr>
<td>EDTA-Fe²⁺</td>
<td>0.01</td>
<td>1.1</td>
</tr>
<tr>
<td>ADP-Fe³⁺</td>
<td>0.60</td>
<td>1.4</td>
</tr>
<tr>
<td>EDTA-Fe³⁺</td>
<td>1.16</td>
<td>9.3</td>
</tr>
</tbody>
</table>

**TABLE II**
The propagation of lipid peroxidation by EDTA Fe²⁺

<table>
<thead>
<tr>
<th></th>
<th>MDA</th>
<th>Lipid hydroperoxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no additions)</td>
<td>0.01</td>
<td>0.7</td>
</tr>
<tr>
<td>EDTA-Fe²⁺</td>
<td>0.16</td>
<td>1.3</td>
</tr>
<tr>
<td>EDTA-Fe²⁺ + superoxide dismutase</td>
<td>1.28</td>
<td>7.1</td>
</tr>
<tr>
<td>EDTA-Fe²⁺ + DPF</td>
<td>1.16</td>
<td>7.2</td>
</tr>
<tr>
<td>EDTA-Fe²⁺ + BHT</td>
<td>0.16</td>
<td>1.2</td>
</tr>
<tr>
<td>EDTA-Fe²⁺ + benzene</td>
<td>1.29</td>
<td>10.3</td>
</tr>
</tbody>
</table>
The addition of NADPH gives rise to the enzymatic formation of EDTA-Fe++. The ability of NADPH-cytochrome P-450 reductase to reduce EDTA-Fe++ was demonstrated in a reaction mixture containing the reductase, NADPH, and EDTA-Fe++. NADPH oxidation occurred only in the presence of all three components. These results were substantiated by ferrous ion chromatophore formation when an excess of bathophenanthroline was added to an anaerobic reaction mixture after a 5-min reaction time. It is apparent that the function of EDTA-Fe++ in NADPH-dependent lipid peroxidation is to catalyze, in its reduced form, the propagation of lipid peroxidation.

Investigation into the nature of the reactive intermediates formed during enzymatic propagation (Table III) yielded results equivalent to those obtained in nonenzymatic propagation (Table II). The addition of superoxide dismutase inhibited MDA formation by 8% and lipid hydroperoxide formation by 15%. The addition of DPF inhibited MDA and lipid hydroperoxide formation by 13% and 28%, respectively. Again, BHT showed essentially complete inhibition of propagation. Benzoate did not inhibit NADPH-dependent propagation, but actually enhanced propagation as detected by lipid hydroperoxide formation. It appears that the enzyme-catalyzed propagation reaction gives rise to radical intermediates of lipid peroxidation, however, OH• is not among them. In addition, O2• is apparently formed during the propagation of lipid peroxidation.

The propagation reaction accounts for a significant portion of the MDA and lipid hydroperoxides formed during NADPH-dependent liposomal peroxidation (Table IV). In the presence of ADP-iron alone, the rate of MDA and lipid hydroperoxide formation in NADPH-dependent lipid peroxidation is 0.3 nmol/min/ml and 1.8 nmol/min/ml, respectively. When the propagating agent, EDTA-Fe+, is included, MDA and lipid hydroperoxide formation is increased 11-fold.

NADPH-dependent lipid peroxidation in the reconstituted system, shown in Table IV, is characterised by the simultaneous occurrence of both initiation and propagation reactions. Superoxide dismutase inhibits both MDA and lipid hydroperoxide formation by approximately 85% in a reconstituted reaction mixture catalyzing both initiation and propagation reactions. In contrast, in a similar system catalyzing only the propagation reaction (Table III), superoxide dismutase inhibits MDA and lipid hydroperoxide formation by only 8% and 15%, respectively. Such a difference between the two reaction mixtures serves to emphasize the dependence of propagation on initiation. The addition of DPF to an NADPH-dependent reaction mixture catalyzing both initiation and propagation (Table IV) inhibits MDA and lipid hydroperoxide formation by 9% and 16%, respectively. The inhibition observed is equivalent to that observed during the NADPH-dependent catalysis of propagation (Table III). These results show that O2 participates in only propagation and not in initiation. The addition of BHT completely inhibits both MDA and lipid hydroperoxide formation demonstrating the radical nature of initiation. The radical nature of the propagation reaction was demonstrated in Table III. Similar to the results in Table III, no effect on the rate of lipid peroxidation upon the addition of benzoate was observed (Table IV) indicating that OH• does not participate in either initiation or propagation of NADPH-dependent liposomal peroxidation.

The iron chelate diethylenetriamine pentaaetate acid (DPTA) has been used by some investigators (34) to inhibit metal-catalyzed lipid peroxidation. Considering that the structure of DPTA is similar to that of a dimer of EDTA, the ability of DPTA-Fe++ to replace EDTA-Fe++ in NADPH-dependent lipid peroxidation was examined (Table V). The addition of DPTA to an NADPH-dependent reaction mixture catalyzing only the propagation reaction accounts for a significant portion of the MDA and lipid hydroperoxides formed during NADPH-dependent liposomal peroxidation. Reaction mixtures were performed as described under “Materials and Methods.”

### Table III

**Propagation of NADPH-dependent liposomal peroxidation**

Reaction mixtures contained 1.0 μmol of lipid phosphate/ml, 0.1 unit of NADPH-cytochrome P-450 reductase/ml, 1.7 mM ADP, 0.1 mM FeCl3, and 0.1 mM NADPH in 0.05 M Tris-HCl, pH 7.5 at 37°C. The following additions were made as indicated: 0.11 mM EDTA, 0.1 mM FeCl3, and 0.1 mM NADPH in 0.05 M Tris-HCl, pH 7.5 at 37°C. The following additions were made as indicated: 1.7 mM ADP, 0.1 mM FeCl3, and 0.1 mM NADPH in 0.05 M Tris-HCl, pH 7.5 at 37°C. The following additions were made as indicated: 0.11 mM EDTA, 0.1 mM FeCl3, and 0.1 mM NADPH in 0.05 M Tris-HCl, pH 7.5 at 37°C.

<table>
<thead>
<tr>
<th>MDA</th>
<th>Lipid hydroperoxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>nmol/min/ml</td>
<td>nmol/min/ml</td>
</tr>
</tbody>
</table>

Control (-NADPH) | 0.03 | 0.2
+NADPH | 0.30 | 0.1
+EDTA-Fe+++ | 0.02 | 0.2
+NADPH + EDTA-Fe+++ | 3.40 | 10.5
+NADPH + EDTA-Fe+++ + superoxide dismutase | 0.50 | 3.5
+NADPH + EDTA-Fe+++ + DPF | 3.10 | 10.3
+NADPH + EDTA-Fe+++ + BHT | 0.00 | 0.0
+NADPH + EDTA-Fe+++ + benzoate | 3.45 | 20.1

### Table IV

**NADPH-dependent liposomal peroxidation**

Reaction mixtures contained 1.0 μmol of lipid phosphate/ml, 0.1 unit of NADPH-cytochrome P-450 reductase/ml, and 0.1 mM NADPH in 0.05 M Tris-HCl, pH 7.5 at 37°C. The following additions were made as indicated: 0.11 mM EDTA, 0.1 mM FeCl3, 1.7 mM ADP, 0.1 mM FeCl3, and 0.1 mM NADPH in 0.05 M Tris-HCl, pH 7.5 at 37°C. The following additions were made as indicated: 0.11 mM EDTA, 0.1 mM FeCl3, 1.7 mM ADP, and 0.1 mM FeCl3. Reactions were initiated by the addition of NADPH. Incubations and assays were performed as described under “Materials and Methods.”

<table>
<thead>
<tr>
<th>MDA</th>
<th>Lipid hydroperoxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>nmol/min/ml</td>
<td>nmol/min/ml</td>
</tr>
</tbody>
</table>

Control (-NADPH) | 0.01 | 0.2
+NADPH | 0.01 | 0.3
+NADPH + EDTA-Fe+++ | 0.01 | 0.2
+NADPH + DTPA-Fe+++ | 0.02 | 0.4
+NADPH + EDTA-Fe+++ | 0.01 | 0.2
+NADPH + DTPA-Fe+++ | 0.01 | 0.2
+NADPH + ADP-Fe+++ | 0.35 | 1.9
+NADPH + ADP-Fe+++ | 3.46 | 19.6
+NADPH + ADP-Fe+++ | 3.19 | 18.9

### Table V

**Propagation of NADPH-dependent lipid peroxidation by EDTA-Fe+++ and DTPA-Fe+++**

Reaction mixtures contained 1.0 μmol of lipid phosphate/ml, 0.1 unit of NADPH-cytochrome P-450 reductase/ml, and 0.1 mM NADPH in 0.05 M Tris-HCl, pH 7.5 at 37°C. The following additions were made as indicated: 0.11 mM EDTA, 0.1 mM FeCl3, 1.7 mM ADP, and 0.1 mM FeCl3. Reactions were initiated by the addition of NADPH. Incubations and assays were performed as described under “Materials and Methods.”

<table>
<thead>
<tr>
<th>MDA</th>
<th>Lipid hydroperoxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>nmol/min/ml</td>
<td>nmol/min/ml</td>
</tr>
</tbody>
</table>

Control (-NADPH) | 0.01 | 0.2
+NADPH | 0.01 | 0.3
+NADPH + EDTA-Fe+++ | 0.01 | 0.2
+NADPH + DTPA-Fe+++ | 0.02 | 0.4
+NADPH + EDTA-Fe+++ | 0.01 | 0.2
+NADPH + DTPA-Fe+++ | 0.01 | 0.2
+NADPH + ADP-Fe+++ | 0.35 | 1.9
+NADPH + ADP-Fe+++ | 3.46 | 19.6
+NADPH + ADP-Fe+++ | 3.19 | 18.9
The Propagation of NADPH-dependent Lipid Peroxidation

results demonstrated that 1,10-phenanthroline (1,10-P) could efficiently replace EDTA-Fe³⁺. Addition of DTPA-Fe³⁺, in the absence of ADP-Fe³⁺, showed that DTPA-Fe³⁺ could not function in the initiation of NADPH-dependent lipid peroxidation. The results indicate DTPA-Fe³⁺ functions as a propagating agent in lipid peroxidation catalyzing the breakdown of lipid hydroperoxides to reactive intermediates of lipid peroxidation. These findings may explain the kinetic findings of Thomas et al. (34) since they used 1,10-P to chelate contaminating iron in a system that is capable of reducing DTPA-Fe³⁺ to DTPA-Fe²⁺ via O₂⁻.

The involvement of cytochrome P-450 in microsomal lipid peroxidation was investigated utilizing aminopyrine and SKF 525-A to inhibit NADPH-dependent microsomal lipid peroxidation. Neither compound nor their metabolic products were found to be antioxidants at the concentration used as determined by their inability to inhibit ascorbate-dependent lipid peroxidation.

The addition of 100 μM SKF 525-A to microsomes inhibited NADPH-dependent lipid peroxidation by 67% (Table VI). SKF 525-A inhibits cytochrome P-450-catalyzed reactions by preferentially binding to cytochrome P-450 displacing other substrates (35). The addition of EDTA-Fe³⁺, which has been shown to enhance NADPH-dependent microsomal lipid peroxidation (10), completely reversed the SKF 525-A inhibition of NADPH-dependent microsomal lipid peroxidation, indicating that SKF 525-A is specifically inhibiting an endogenous microsomal propagating agent. The specificity of SKF 525-A indicates that an endogenous propagating agent in microsomes is cytochrome P-450.

Aminopyrine was proposed to inhibit NADPH-dependent microsomal lipid peroxidation by competing for reducing equivalents (36, 37). The addition of 5 mM aminopyrine to a microsomal reaction mixture inhibited NADPH-dependent lipid peroxidation by 57% (Table VII). The addition of EDTA-Fe³⁺ completely reversed aminopyrine inhibition. Since EDTA-Fe³⁺ must be reduced to be active in propagation, drug substrate inhibition of NADPH-dependent microsomal lipid peroxidation apparently does not occur by competition for reducing equivalents. It would appear that drug substrates inhibit NADPH-dependent lipid peroxidation by interaction with cytochrome P-450. Inhibition may be the result of cytochrome P-450 peroxidase activity utilizing lipid hydroperoxides and oxidizable drugs as substrates in a manner analogous to the CHP-dependent drug metabolism observed by others (38-40). These data suggest that the endogenous propagating agent is cytochrome P-450 as previously indicated by experiments with SKF 525-A.

The ability of ferric cytochrome P-450 to promote propagation of lipid peroxidation was investigated by addition of cytochrome P-450 to a reaction mixture in which lipid hydroperoxides were generated in situ by soybean lipoxygenase (Table VIII). In detergent-treated liposomes, lipoxygenase catalyzed initial rates of formation of 0.08 nmol of MDA/min/ml and 0.5 nmol of lipid hydroperoxide/min/ml. The addition of 0.3 nmol/ml of ferric cytochrome P-450 to the reaction mixture resulted in a 11-fold increase in the rate of MDA formation and a 3-fold increase in the rate of lipid hydroperoxide formation. From these data it appears that cytochrome P-450 is an excellent propagating agent. The data indicate that on a mol basis cytochrome P-450 is a better propagating agent than is EDTA-Fe³⁺. However, as others have previously shown, cytochrome P-450 is degraded during lipid peroxidation thereby limiting its propagating abilities (41, 42).

The complete reconstitution of NADPH-dependent microsomal lipid peroxidation in liposomes utilizing ferric cytochrome P-450 as the propagating agent is shown in Table IX. In the absence of cytochrome P-450, the ability of ADP-Fe³⁺ to initiate lipid peroxidation was essentially eliminated by the addition of EDTA-Fe³⁺.

### Table VI
**The effect of EDTA-Fe³⁺ on the SKF 525-A inhibition of NADPH-dependent microsomal lipid peroxidation**

<table>
<thead>
<tr>
<th>Reaction Mixture</th>
<th>Lipid Hydroperoxide</th>
<th>nmol/min/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (-NADPH)</td>
<td>+EDTA-Fe³⁺</td>
<td>0.20</td>
</tr>
<tr>
<td>+NADPH</td>
<td>2.81</td>
<td></td>
</tr>
<tr>
<td>+NADPH + Aminopyrine</td>
<td>2.81</td>
<td></td>
</tr>
<tr>
<td>+NADPH + Aminopyrine + EDTA-Fe³⁺</td>
<td>2.81</td>
<td></td>
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</table>

### Table VII
**The effect of EDTA-Fe³⁺ on the aminopyrine inhibition of NADPH-dependent microsomal lipid peroxidation**

<table>
<thead>
<tr>
<th>Reaction Mixture</th>
<th>Lipid Hydroperoxide</th>
<th>nmol/min/ml</th>
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</thead>
<tbody>
<tr>
<td>Control (-NADPH)</td>
<td>+Aminopyrine</td>
<td>0.12</td>
</tr>
<tr>
<td>+NADPH</td>
<td>0.30</td>
<td></td>
</tr>
<tr>
<td>+NADPH + Aminopyrine</td>
<td>0.85</td>
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</table>

### Table VIII
**Propagation of lipid peroxidation by ferric cytochrome P-450**

<table>
<thead>
<tr>
<th>Reaction Mixture</th>
<th>Lipid Hydroperoxide</th>
<th>nmol/min/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (-NADPH)</td>
<td>+Ferric cytochrome P-450</td>
<td>0.02</td>
</tr>
<tr>
<td>+Ferric cytochrome P-450</td>
<td>Lipoxygenase</td>
<td>0.08</td>
</tr>
<tr>
<td>+Lipoxygenase</td>
<td>+Ferric cytochrome P-450</td>
<td>0.88</td>
</tr>
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</table>

### Table IX
**Propagation of NADPH-dependent liposomal peroxidation by ferric cytochrome P-450**

<table>
<thead>
<tr>
<th>Reaction Mixture</th>
<th>Lipid Hydroperoxide</th>
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</thead>
<tbody>
<tr>
<td>Control (-NADPH)</td>
<td>+Ferric cytochrome P-450</td>
<td>0.01</td>
</tr>
<tr>
<td>+NADPH</td>
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<td></td>
</tr>
<tr>
<td>+NADPH + ferric cytochrome P-450</td>
<td>1.51</td>
<td></td>
</tr>
</tbody>
</table>

The involvement of cytochrome P-450 in microsomal lipid peroxidation was investigated utilizing aminopyrine and SKF 525-A to inhibit NADPH-dependent microsomal lipid peroxidation. Neither compound nor their metabolic products were found to be antioxidants at the concentration used as determined by their inability to inhibit ascorbate-dependent lipid peroxidation.

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The Propagation of NADPH-dependent Lipid Peroxidation 5897

Addition of superoxide dismutase would then shift the reaction equilibrium to the right, effectively decreasing the concentration of propagation agent. Table II also suggests that minor amounts of $O_2^-$ may be produced during the propagation reaction, perhaps via the reaction of two lipid hydroperoxide radicals (24, 45). The minor inhibition exhibited by DPF indicates that $O_2^-$ is a product of perhaps only some of several possible propagerative reactions. The inhibition of peroxidation upon the addition of BHT to the reaction mixture indicates the radical nature of the propagation reaction. The lack of inhibition upon the addition of benzoyl peroxide to the reaction mixture indicates that $OH^-$ is not one of the radicals that mediate propagation of lipid peroxidation.

Using NADPH-cytochrome P-450 reductase to catalyze the formation of EDTA-Fe$^{3+}$ in situ (Table III), it was demonstrated that the enzymatic reaction was identical with the nonenzymatic reaction (Table I). Enzymatically catalyzed propagation was superoxide dismutase-insensitive, showed some inhibition by DPF, showed no inhibition by benzoyl peroxide, and was completely inhibited by BHT. Thus, the overall enzyme-catalyzed reaction, mediated by EDTA-Fe$^{3+}$ was radical in nature, however, minor amounts of $O_2^-$ may be formed and participate in propagation.

The effect of various inhibitors of lipid peroxidation on the total reconstitution of NADPH-dependent lipid peroxidation (Table IV) are consistent with the mechanism of initiation previously suggested (24) and the mechanism of propagation being proposed here. Superoxide dismutase almost completely inhibits lipid peroxidation because it inhibits the ADP-perferryl ion-catalyzed initiation, as previously shown (24). We have previously shown that superoxide dismutase has minimal effect in the propagation reaction alone (Tables II and III). The slight inhibition of peroxidation by the addition of DPF, indicates that DPF does not inhibit initiation (24), but does inhibit propagation to a minor extent (Tables II and III) indicating that $O_2^-$ is a minor product of propagation. Butylated hydroxytoluene completely inhibits NADPH-dependent lipid peroxidation indicating that initiation is radical in nature. The radical nature of propagation was shown in Tables II and III. The lack of benzoyl peroxide inhibition in NADPH-dependent lipid peroxidation indicates that $OH^-$ does not participate in either the initiation or propagation of peroxidation. These data clearly show that NADPH-dependent lipid peroxidation occurs in two successive radical steps dependent upon metal catalysis.

The significant contribution of EDTA-Fe$^{3+}$-catalyzed propagation to the total quantity of product formed during NADPH-dependent lipid peroxidation has been shown previously (10, 24) and is demonstrated by the data presented in Table IV. The addition of EDTA-Fe$^{3+}$ to a NADPH-dependent liposomal peroxidation mixture increased the rates of MDA and lipid hydroperoxide formation by 11-fold over the rates in the presence of ADP-Fe$^{3+}$ and NADPH alone. Thus, it appears that propagation is a key reaction in lipid peroxidation accounting for more than 90% of total product formation.

Table V demonstrates that EDTA is not unique among the chelators of iron in its ability to facilitate iron-catalyzed propagation. EDTA-Fe$^{3+}$ can replace EDTA-Fe$^{2+}$ in NADPH-dependent lipid peroxidation in liposomes. ADP-Fe$^{3+}$ is an efficient initiator of lipid peroxidation but a very poor catalyst for the breakdown of organic hydroperoxides (Fig. 1) or the propagation of lipid peroxidation (Table I). Chelation of iron by EDTA greatly stabilizes the ferric complex, lowering the reduction potential of the complex to 0.254 V (46, 47) making the ferric complex a relatively strong reducing agent. Che-
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ulation of iron by ADP does not stabilize the ferric complex to as great a degree as does EDTA. Such an increase in the reducing ability of iron upon complexation by EDTA enhances the catalysis of lipid hydroperoxide breakdown. The effect of iron chelation on the breakdown of H$_2$O$_2$ by Fenton-type reagents has been previously demonstrated (48). If propagation is a Fenton-type reaction, efficient reaction with EDTA-Fe$^{3+}$ as compared to free iron would be predicted and is consistent with the experimental data. This proposed function of iron chelation is supported by the ability of DTPA-Fe$^{3+}$ to replace EDTA-Fe$^{3+}$. DTPA chelation of iron is similar to that of EDTA (49). The data suggest that the key to the formation of an efficient propagating agent may be the lowering of the iron reduction potential upon chelation.

The ability of DTPA-Fe$^{3+}$ to catalyze propagation may offer an alternative explanation to the experimental data reported recently by Thomas et al. (34). Thomas et al. (34) proposed that O$_2^-$-dependent lipid peroxidation occurred via the reaction of O$_2^-$ with lipid hydroperoxides as follows:

$$\text{O}_2^- + \text{LOOH} \rightarrow \text{LO}^- + \text{O}_2 + \text{OH}^-$$

(6)

The authors found that peroxidation was dependent on lipid hydroperoxides formed by autoxidation. The reaction constant for Reaction 6 was found to be $7 \times 10^{10}$ M$^{-1}$ s$^{-1}$. However, the rate of self-dismutation of O$_2^-$ at the pH of the investigation, 7.4, is at least 2 orders of magnitude greater than the rate constant for Reaction 6. Thus, the importance of Reaction 6 in O$_2^-$-dependent lipid peroxidation may be minimal. The observation that peroxidation was dependent upon preformed lipid hydroperoxides, correlates well with the mechanism of propagation of lipid peroxidation proposed here. The kinetic data of the observed reaction (34) also agree with the kinetics of EDTA-Fe$^{3+}$- or DTPA-Fe$^{3+}$-catalyzed propagation reported here. That is, the rate of lipid hydroperoxide formation during propagation, as defined here, is approximately equal to the rates of peroxidation observed by Thomas et al. (34). These two observations, together with the use by Thomas et al. (34) of DTPA to chelate metals (most likely iron) in the phosphate buffer used in their reaction mixture, suggest that they may have observed a metal chelate-catalyzed propagation of lipid peroxidation. The chelate involved likely would be DTPA-Fe$^{3+}$ which is reduced by O$_2^-$ to DTPA-Fe$^{2+}$, an active propagating agent as demonstrated in this paper.

Pederson et al. (10, 23) proposed that EDTA-Fe$^{3+}$ replaced an endogenous microsomal agent that participated in NADPH-dependent microsomal lipid peroxidation. Table VI shows the ability of EDTA-Fe$^{3+}$ to reverse the SKF 525-A inhibition of NADPH-dependent microsomal lipid peroxidation. At the concentration used, SKF 525-A acts by biuncing the cytochrome P-450 and not by disruption of microsomal electron transport (35). The reversal of SKF 525-A inhibition by addition of EDTA-Fe$^{3+}$ indicates that cytochrome P-450 may be a microsomal entity responsible for propagation in NADPH-dependent microsomal lipid peroxidation.

The proposed role of cytochrome P-450 in microsomal lipid peroxidation is supported by the EDTA-Fe$^{3+}$ reversal of amidopyrine inhibition of NADPH-dependent microsomal lipid peroxidation (Table VII). The data indicate that drug substrates of the microsomal mixed function oxidase system do not inhibit NADPH-dependent lipid peroxidation by competing for reducing equivalents as others have proposed (36, 37). If the inhibitory effect was a result of the competition for electrons, addition of EDTA-Fe$^{3+}$ which must be reduced to its active form, should not completely reverse the observed inhibition. The observations that hydrogen donors such as tetramethyl-p-phenylenediamine are oxidized in the presence of P-450 and lipid hydroperoxides (50, 51) and that CHP can support enzymatic oxidation of microsomal mixed function oxidase drug substrates (38-40), indicate that drug substrates may inhibit lipid peroxidation by reducing lipid hydroperoxides via a cytochrome P-450 peroxidase-type mechanism. Such a peroxidase reaction would inhibit lipid peroxidation by competing with the propagation reaction for lipid hydroperoxides produced during initiation. These results also indicate that cytochrome P-450 may function in a variety of roles during lipid peroxidation in addition to its participation in propagation.

The ability of ferric cytochrome P-450 to catalyze the propagation of lipid peroxidation from lipid hydroperoxides is clearly demonstrated in Tables VIII and IX. Addition of 0.3 nmol/ml of ferric cytochrome P-450 to a reaction mixture in which lipid hydroperoxides are being generated in situ by soybean lipoxigenase, resulted in an 11-fold increase in MDA formation and a 3-fold increase in lipid hydroperoxide formation (Table VIII). The results in Table IX demonstrate the role that ferric cytochrome P-450 plays in the propagation of NADPH-dependent lipid peroxidation. In the reaction mixture, lipid hydroperoxides are generated by the ADP-perferryl ion initiation complex (24). Addition of 0.3 nmol/ml of ferric cytochrome P-450, which cannot be reduced by the protease solubilized NADPH-cytochrome P-450 reductase (52) results in an 8-fold increase in MDA formation and 16-fold increase in lipid hydroperoxide formation. Thus, ferric cytochrome P-450 is apparently an excellent propagator of lipid peroxidation from lipid hydroperoxides produced during initiation of NADPH-dependent lipid peroxidation.

As summarized in Fig. 2, the results presented in this paper clearly demonstrate that NADPH-dependent lipid peroxidation occurs in two sequential steps. The first step, initiation, is catalyzed by the ADP-perferryl ion. The second step, propagation, is dependent upon the lipid hydroperoxides formed during initiation, and results in the rapid formation of reactive products of lipid peroxidation.
intermediates and products of lipid peroxidation. Propagation is the EDTA-Fe²⁺, DTPA-Fe²⁺, or ferric cytochrome-P-450-catalyzed breakdown of lipid hydroperoxide to form reactive intermediates of lipid peroxidation. Propagation accounts for more than 90% of the products formed during lipid peroxidation and is predominantly radical in nature. Propagation is perhaps a Fenton-type reaction resulting primarily in the formation of lipid alkoxy radicals. Low levels of hydroperoxy radicals are also possibly formed as indicated by the presence of O²⁻ during propagation.

REFERENCES


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The mechanism of NADPH-dependent lipid peroxidation. The propagation of lipid peroxidation.

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