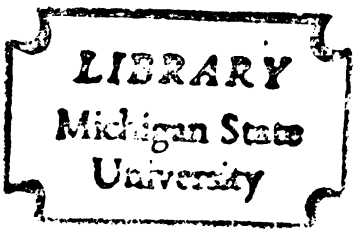


This is to certify that the

dissertation entitled

The Role of Chelated Iron in the Mechanism of Enzymatic Promotion of Lipid Peroxidation

presented by

Bruce A. Svingen

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Biochemistry

Major professor

Date August 18, 1981

The Role of Chelated Iron in the Mechanism of Enzymatic Promotion of Lipid Peroxidation

by

Bruce A. Svingen

A DISSERTATION

Submitted to

Michigan State University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

1981

ABSTRACT

The Role of Chelated Iron in the Mechanism of Enzymatic Promotion of Lipid Peroxidation

by
Bruce A. Svingen

Iron-dependent peroxidation of microsomal phospholipids promoted by either NADPH-cytochrome P450 reductase or xanthine oxidase occurs by similar, if not identical, mechanisms. Lipid peroxidation occurs by two distinct sequential radical chain reaction initiating reactions, lipid hydroperoxide-independent and -dependent initiation. Lipid hydroperoxide-independent initiation is characterized by the formation of low levels of lipid hydroperoxides in previously peroxide free lipids. Lipid hydroperoxide-independent initiation of lipid peroxidation may be promoted by the ADP-perferryl ion. In NADPH-dependent lipid peroxidation the ADP-perferryl ion is formed by the direct reduction of the ADP-ferric ion complex catalyzed by NADPH-cytochrome P450 reductase and the subsequent reaction of the ADP-ferrous ion complex with O_2. In superoxide-dependent lipid peroxidation the ADP-perferryl ion is generated by the direct reaction of superoxide with the ADP-ferric ion complex. Lipid hydroperoxide-independent initiation of lipid peroxide is superoxide dismutase sensitive. Lipid hydroperoxide-independent initiation apparently does not involve either singlet oxygen or the free hydroxyl radical. Lipid hydroperoxide-independent initiation accounts for approximately 10-15% of total peroxidative products formed during enzymatically promoted iron-dependent lipid peroxidation.

Lipid hydroperoxide-dependent initiation of lipid peroxidation is dependent upon the presence of previously formed lipid hydroperoxides. Lipid hydroperoxide-dependent initiation is the metal catalyzed heterolysis of lipid hydroperoxides to form radical products. Lipid hydroperoxide-dependent

initiation can be efficiently promoted by either EDTA-ferrous ion or DTPA-ferrous ion complexes. In NADPH-dependent lipid peroxidation the reduced chelates are formed by direct enzymatic reduction of the ferric chelate. The NADPH-dependent reaction is thus not superoxide dismutase sensitive. In superoxide-dependent lipid peroxidation the formation of the ferrous ion-chelate complex is dependent upon the reaction of superoxide with the ferric ion-chelate complex and the reaction is, therefore, superoxide dismutase sensitive. The hydroxyl radical is not formed during the iron-promoted breakdown of lipid hydroperoxides. However small amounts of singlet oxygen are formed secondarily to lipid peroxy radical formation. Lipid hydroperoxide-dependent initiation of lipid peroxidation accounts for 80-90% of the total peroxidative products formed during enzymatically promoted iron-dependent lipid peroxidation. Lipid hydroperoxide-dependent initiation can also be promoted by ferric cytochrome P450.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF TABLES (Continued) Page

LIST OF FIGURES

ABBREVIATIONS

ADP	Adenosine 5'-diphosphate
ADP-Fe^{+3} (Fe^{+2})	Ferric (ferrous) ion chelated by ADP
BHT	Butylated hydroxytoluene
DMPO	5,5-dimethyl-1-pyrroline-N-oxide
DPBF	Diphenylisobenzofuran
DPF	2,5-diphenylfuran
DTPA	Diethylenetriaminepentaacetic acid
DTPA-Fe^{+3} (Fe^{+2})	Ferric (ferrous) ion chelated by DTPA
EDTA	Ethylenediamintetraacetic acid
EDTA-Fe^{+3} (Fe^{+2})	Ferric (ferrous) ion chelated by EDTA
ESR	Electron Spin Resonance
$Fe^{+2}O$	Ferryl ion
$Fe^{+2}O_2$, $Fe^{+3}O_2^-$.	Perferryl ion
HO•	Hydroxyl radical
LH	Lipid molecule
L•	Lipid alkyl radical
LO•	Lipid alkoxy radical
LOOH	Lipid hydroperoxide
LOO•	Lipid peroxy radical
MDA	Malondialdehyde
$^1O_2$	Singlet oxygen
$O_2^{\cdot-}$	Superoxide
PUFA	Polyunsaturated fatty acid
SOD	Superoxide dismutase
TBA	Thiobarbituric acid

LITERATURE REVIEW

Mostly through the excellent work of Gee, Bolland, Bateman, and co-workers, of the British Rubber Producers Research Association, lipid peroxidation has been shown to occur by a free radical mechanism [1-12]. Studies on both autoxidative and enzymatically-promoted lipid peroxidation by these investigators have established the basic reactions of lipid peroxidation. The reactions of free radical lipid peroxidation are outlined below (LH, polyunsaturated fatty acids (PUFA); LOOH, lipid hydroperoxide; L•, lipid alkyl radical; LOO•, lipid peroxy radical) [13,14]:

Initiation:

$$LH + O_2 \longrightarrow \text{free radicals} \qquad (1)$$

$$LOOH \longrightarrow \text{free radicals} \qquad (2)$$

Propagation:

$$L^\bullet + O_2 \longrightarrow LOO^\bullet \qquad (3)$$

$$LOO^\bullet + LH \longrightarrow LOOH + L^\bullet \qquad (4)$$

Termination:

$$L^\bullet + L^\bullet \longrightarrow \text{Stable (nonradical) products} \qquad (5)$$

$$LOO^\bullet + LOO^\bullet \longrightarrow \text{Stable (nonradical) products} \qquad (6)$$

$$L^\bullet + LOO^\bullet \longrightarrow \text{Stable (nonradical) products} \qquad (7)$$

Reactions 1 and 2 represent two possible means for the initiation of lipid peroxidation. Reaction 1 is initiation independent of lipid hydroperoxides and reaction 2 is lipid hydroperoxide-dependent initiation. The relative importance of reactions 1 and 2 in the initiation of enzymatically promoted lipid peroxidation is matter of some controversy.

Polyunsaturated fatty acids are essentially inert to reaction 1 as written. The kinetic inertness of PUFA to reaction 1 arises from the basic concepts of spin conservation during reaction. Spin conservation imposes restrictions on reaction mechanisms such that under normal circumstances reactions occur only between reactants of the same spin multiplicity. In reaction 1, as written, the ground states of the reactants are of dissimilar multiplicity. The oxygen ground state is of triplet multiplicity and the PUFA ground state is of singlet multiplicity. Thus the reaction is spin forbidden and unlikely to occur as written. On the basis of quantum theory it has been predicted that reaction 1 is very endothermic, has an extremely high activation energy and is unlikely to occur to any extent under normal biological conditions [15,16]. The kinetic inertness of lipid hydroperoxides to reaction 2, thermal homolysis, has been experimentally demonstrated [17,18]. Thermal homolysis of hydroperoxides is an extremely slow reaction under any but quite drastic conditions. Thus it is unlikely that, under biological conditions, lipid peroxidation would be initiated by the unpromoted form of either reaction 1 or 2.

However, when either reaction 1 or 2 is promoted by a metal ion it becomes kinetically feasible as a mechanism for the initiation of lipid peroxidation. The participation of a metal in reaction 1 imparts radical characteristics to the reaction and thus removes the spin restrictions imposed on the unpromoted reaction [19]. The metal promotion of reaction 2, reductive activation, has been extensively studied and has been demonstrated to be quite a rapid reaction [17]. Oxidized heme promotion of reaction 2 has also been shown to be quite facile [17,20-24].

The actual mechanism of initiation in enzyme-dependent lipid peroxidation will be dealt with in greater detail later. The relative contributions of the two reactions to the overall rate of initiation can vary dramatically. In hydroperoxide-free lipids initiation must occur by some form of reaction 1. However, in lipids containing lipid hydroperoxides the contributions of reaction 1 to initiation of lipid peroxidation are quickly overshadowed by those from reaction 2 [21,25]. In iron promoted peroxidation of hydroperoxide free lipid, kinetic analysis indicates that while the initial rate of peroxidation reflects the kinetics of reaction 1, reaction 2 quickly supplants it [26,27].

After initiation has occurred the propagation cycle of the overall mechanism begins. By various methods it has been estimated that each free radical formed goes through 8 to 14 propagation cycles [28]. Because propagation can also produce a nonradical product necessary for initiation, lipid hydroperoxides, it is easy to envision how lipid peroxidation can become a geometrically progressive reaction resulting in extensive membrane damage.

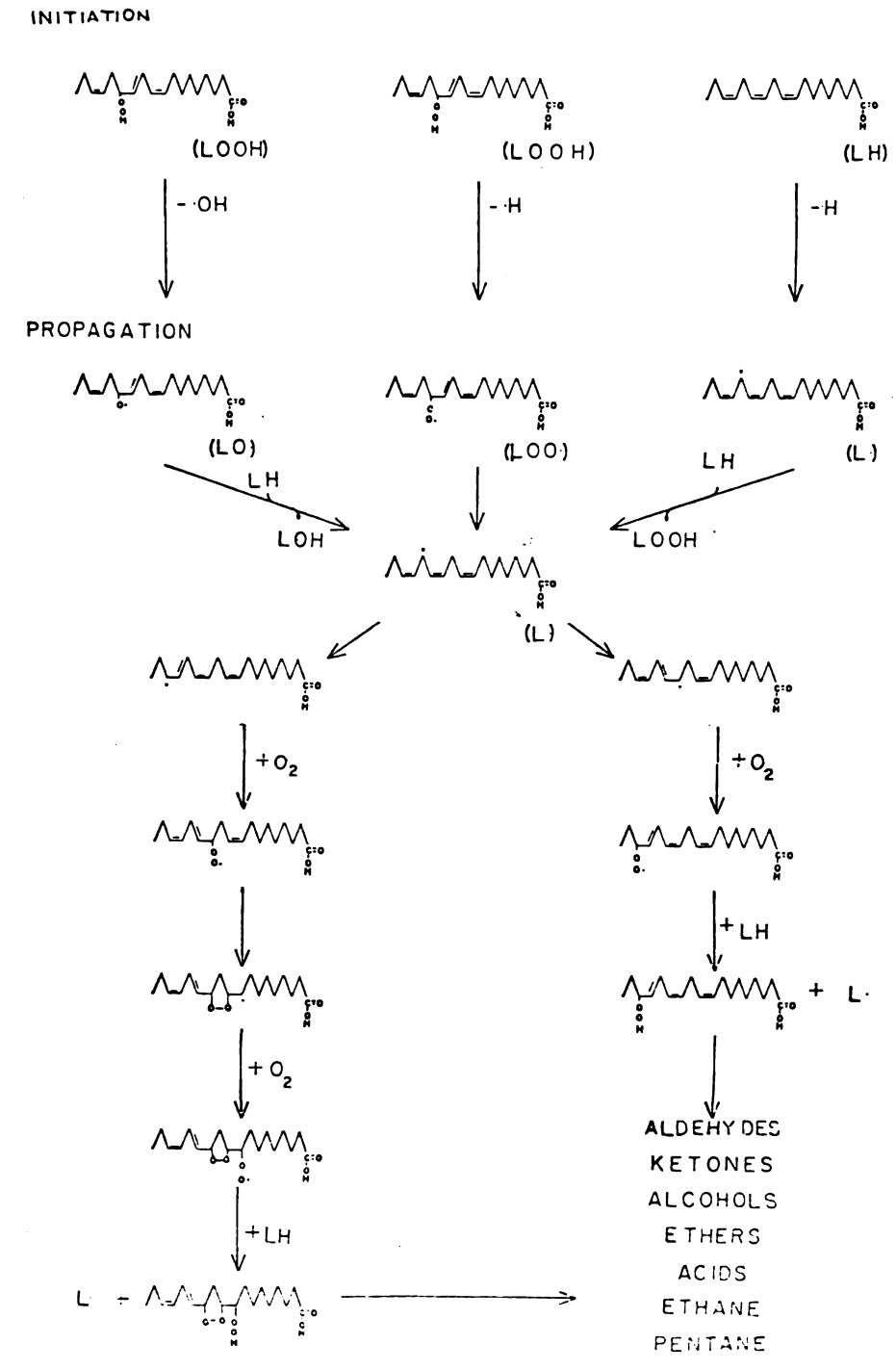
As shown, termination of the propagatory chain can occur by several reactions. Although the products of the termination reactions are by definition not free radicals, this should not be construed to indicate that termination products are non-reactive. For example, reaction 6 has been shown to generate singlet oxygen [29]. If formed, singlet oxygen can react with PUFA to produce lipid dioxetenes and hydroperoxides [30].

The application of the basic tenets of free radical reaction mechanisms to the specific problem of PUFA peroxidation has resulted in the proposal of a general mechanism of lipid peroxidation as

schematically presented in Fig. 1 [31]. There are two possible initiation mechanisms. In peroxide free PUFA, initiation is proposed to occur by abstraction of a methylene hydrogen from a PUFA molecule generating a PUFA alkyl radical. Alternatively, in lipids which already contain peroxide material, such as PUFA hydroperoxides, initiation may predominantly occur by hydroperoxide breakdown to form either PUFA alkoxy or peroxy radicals.

If initiation occurs by hydrogen abstraction to generate the PUFA alkyl radical the reaction then enters into the propagation stage of the mechanisms by the addition of dioxygen. The addition of oxygen is a diffusion limited process ($k=10^9 - 10^{10}$ M^{-1} sec^{-1}) when the oxygen partial pressure of the reaction mixture is 100 mm of Hg or greater [18]. The partial pressure of oxygen has great significance for the termination reactions expressed. If the partial pressure is 100 mm or greater, only termination reactions involving the peroxy radicals need be considered. If the oxygen partial pressure is below 100 mm the termination reactions involving both peroxy and alkyl radicals must be considered. If initiation occurs via generation of the PUFA peroxy radical, once again the classical reactions of propagation are directly entered into. If, however, initiation occurs by formation of the PUFA alkoxy radical, the next step is likely to be the abstraction of a methylene hydrogen from a neighboring PUFA generating a PUFA alcohol and a PUFA alkyl radical. The PUFA alkyl radical then enters the propagation stage of the mechanism as discussed above. The propagation of lipid peroxidation involves the abstraction of a PUFA methylene hydrogen by a PUFA peroxy radical. This reaction forms a PUFA hydroperoxide and a PUFA alkyl radical. The

Figure 1. Basic Scheme of Free Radical Lipid Peroxidation.


INITIATION
(LOOH)
(LOOH)
(LH)
-·OH
-·H
-H
PROPAGATION
(LO·)
(LOO·)
(L·)
LH
LOH
LH
LOOH
(L·)
+O2
+O2
+O2
+LH
+LH
+ L·
ALDEHYDES
KETONES
ALCOHOLS
ETHERS
ACIDS
ETHANE
PENTANE
TERMINATION

PUFA alkyl radical then adds oxygen to reform a PUFA peroxy radical. A significant modification of the propagation reaction occurs when a PUFA peroxy radical internally cyclizes to form a PUFA endoperoxide radical. The PUFA endoperoxide radical can then subsequently add dioxygen to generate a PUFA endoperoxide peroxy radical. The PUFA endoperoxide peroxy radical can participate in the propagation scheme the same as any other PUFA peroxy radical. However, the significance of this internal endoperoxide formation with the addition of a β-oxygen function (the peroxy function) is that this endoperoxide appears to be the material detected in the thiobarbituric acid assay for the rate and extent of lipid peroxidation [32-36]. As previously mentioned the termination reactions are greatly altered by the oxygen partial pressure. Additionally, as the reaction mixture becomes more complex the termination reaction becomes more complex and can involve constitutents other than PUFA radicals. In biological membranes termination reactions will include reaction between PUFA radicals and membrane constituents such as α-tocopherol, cholesterol and sulfhydryl groups of glutathione and proteins.

THE REQUIREMENT FOR IRON IN ENZYMATICALLY-PROMOTED LIPID PEROXIDATION

Several metals which undergo univalent redox reactions can participate in the promotion of autoxidative and enzymatic peroxidation of PUFA. Of these metals, the subgroup of cobalt, copper, iron and manganese are of biological significance. Of this group, iron has been found to be the most active promoter of lipid peroxidation both *in vitro* and *in vivo*. The unique role of iron stems from two basic points. First, the iron concentration in animal tissues is higher than any other

member of this group. Second, certain biological chelators of iron enhance the ability of iron to function as a promoter of lipid peroxidation. This second point is supported by several lines of evidence. Tappel et al. [20-23] showed that heme compounds, many of which are intimately associated with the phospholipids of biological membranes, are the best promoters of lipid peroxidation both in vitro and in vivo. Also, iron is required for oxygen activation to initiate lipid hydroperoxide formation upon which heme promotion of lipid peroxidation is dependent [37-40]. The role of metals in the promotion of autoxidation has been the subject of previous reviews [25,26,41,42] and will not be dealt with here. This review will deal with the role of iron in the initiation of enzymatic lipid peroxidation.

Many researchers investigating numerous and varied systems of enzymatically promoted in vitro lipid peroxidation have found that addition of iron or an iron-chelate complex is required for peroxidation. This requirement was first demonstrated in NADPH-dependent microsomal lipid peroxidation. Ernster and co-workers [37,43-45] found that the addition of iron chelated by ADP was required for NADPH-dependent microsomal lipid peroxidation. Subsequently, several other investigators confirmed that the addition of complexed iron was required for promotion of NADPH-dependent microsomal lipid peroxidation [38,40,46-54]. It was found that ADP could be replaced by other nucleotides and pyrrophosphate [38,40]. Extensive experimentation showed that although other chelates could be used, activity was maximal with ADP. Pederson et al. [55] found that the addition of EDTA-chelated iron and ADP-chelated iron to microsomes promoting NADPH-dependent lipid peroxidation resulted in an

increase in the rate of peroxidation over that observed in the presence of only ADP-chelated iron. Pederson et al. also observed that EDTA-chelated iron alone could not function in the promotion of NADPH-dependent microsomal lipid peroxidation. However, Lai and Piette [56,57] found that it could. Further endeavors to elucidate the mechanism of microsomal lipid peroxidation led to the identification of NADPH-cytochrome P450 reductase as the enzyme responsible for the promotion of NADPH-dependent microsomal lipid peroxidation [55,58]. Pederson et al. [55,58] demonstrated that protease solubilized NADPH-cytochrome P450 reductase in the presence of NADPH and ADP-chelated iron could promote the peroxidation of liposomes prepared from extracted microsomal lipid. These investigators also found that the promotion of lipid peroxidation in the above reaction mixture could be dramatically enhanced by the addition of EDTA-chelated iron. The enhancement observed in liposomes was much greater than that previously observed in microsomes. The findings of Pederson et al. were confirmed by Sugioko and Nakano [59] and Noguchi and Nakano [60] utilizing a similar liposomal reaction mixture to study the reconstitution of NADPH-dependent lipid peroxidation. In contrast, Pospelova et al. [61] found that NADPH-dependent peroxidation of liposomes could be promoted by either ADP-chelated iron or EDTA-chelated iron. The choice of iron chelate apparently made little difference since the rate and extent of peroxidation were similar in both cases.

Superoxide-dependent lipid peroxidation has also been shown to be promoted by iron complexes. Pederson and Aust [62] demonstrated that superoxide-dependent peroxidation of liposomes prepared from extracted

microsomal lipid was promoted by ADP-chelated iron. Again, as in NADPH-dependent liposomal peroxidation, the addition of EDTA-chelated iron together with ADP-chelated iron greatly enhanced the rates of peroxidation over those observed in the presence of ADP-chelated iron alone. These investigators found that EDTA-chelated iron alone could not promote superoxide-dependent lipid peroxidation. The findings of Pederson and Aust [62] were confirmed by Svingen et al [63]. Superoxide-dependent peroxidation of mitochondrial membranes is also promoted by ADP-chelated iron [64].

THE ROLE OF IRON IN HYDROPEROXIDE-INDEPENDENT INITIATION OF LIPID PEROXIDATION

Initiation of lipid peroxidation in hydroperoxide free PUFA is thought to occur by the abstraction of a methylene hydrogen from a PUFA molecule giving rise to a PUFA alkyl radical (see Fig. 1). Hydrogen abstraction occurs at the methylene carbon because the allylic position reduced the bond dissociation energy. The bond dissociation energy for methylene hydrogen is 41.4 Kcal per mole compared to 68.2 Kcal per mole for vinyl hydrogen and 1130.0 Kcal per mole for secondary hydrogen. Even though the methylene hydrogen can be considered partially activated it is obvious that a strong oxidizing agent must be involved in the abstraction. Of the oxidative reagents that have been proposed to be formed in biological systems, two are theoretically of the proper oxidative power to be involved in initiation of lipid peroxidation. The two oxidants most often proposed to be involved in initiation are the hydroxyl radical (HO•) and the perferryl ion ($Fe^{+2}O_2$). In this section of the review the experimental and theoretical support for the involvement of either HO• or $Fe^{+2}O_2$ in the initiation of lipid peroxidation

from the standpoint of iron-promotion of initiation will be examined. This section will be concluded with a theoretical examination of the identity of HO• and $Fe^{+2}O_2$. Are these two reactants perhaps members of the same group of iron activated oxygen intermediates and therefore perhaps indistinguishable?

Initiation of lipid peroxidation by HO• abstraction of PUFA methylene hydrogen has been proposed by several research groups. In all instances HO• participation in initiation of lipid peroxidation has been proposed for superoxide-dependent lipid peroxidation. The basic mechanism proposed for formation of HO• in superoxide-dependent lipid peroxidation is based on a combination of the reactions that constitute Fenton's Reagent and the Haber-Weiss reaction [65]. The mechanism proposed is perhaps most adequately described as a superoxide driven, iron promoted Haber-Weiss reaction. The general scheme for HO• production is schematically shown below.

$$O_2^{\cdot-} + Fe^{+3} \longrightarrow Fe^{+2} + O_2 \qquad (8)$$

$$2O_2^{\cdot-} + 2H^+ \longrightarrow H_2O_2 + O_2 \qquad (9)$$

$$Fe^{+2} + H_2O_2 \longrightarrow Fe^{+3} + OH^- + HO\cdot \qquad (10)$$

Experimental evidence supporting the above reaction scheme is most often derived from the ability of HO• traps, catalase and superoxide dismutase to inhibit not only lipid peroxidation but also other reactions that are characteristic of HO•, H_2O_2 and superoxide. The three reactions were sequentially ordered on the basis of inhibition of lipid peroxidation and secondary reactions characteristic of superoxide, H_2O_2 and HO•. The participation of iron in the reaction sequence is based on its required presence for the occurrence of reactions characteristic of HO•. This

indicates that the unpromoted Haber-Weiss reaction does not occur to any significant extent. The requirement for iron reduction is shown by the inability of H_2O_2 and ferric ion to catalyze lipid peroxidation at neutral pH.

Fong et al. [39] proposed that NADPH-dependent lipid peroxidation promoted by NADPH-cytochrome P450 reductase in either microsomes or liposomes occurred via an iron promoted Haber-Weiss reaction. These authors found that lipid peroxidation, as measured by the release of acid phosphatase from lysosomes, required ADP-Fe^{+3} and was inhibited by superoxide dismutase, catalase and hydroxyl radical traps. From their data these authors proposed the following mechanism of HO• generation:

$$2O_2 + NADPH \longrightarrow NADP^+ + H^+ + 2\ O_2^{\bullet-} \quad (11)$$

$$2O_2^{\bullet-} + 2H^+ \longrightarrow H_2O_2 + O_2 \quad (9)$$

$$H_2O_2 + O_2^{\bullet-} \longrightarrow O_2 + OH^- + HO\bullet \quad (12)$$

$$O_2^{\bullet-} + Fe^{+3} \longrightarrow Fe^{+2} + O_2 \quad (8)$$

$$Fe^{+2} + H_2O_2 \longrightarrow Fe^{+3} + OH^- + HO\bullet \quad (10)$$

Reaction 11 would be catalyzed by the microsomal enzyme NADPH-cytochrome P450 reductase. The requirement for ADP was proposed to be for the solubility of iron in a neutral aqueous solution. The authors proposed that HO• was formed via reactions 11, 9, 8 and 10. Little if any HO• formation was proposed to occur via reactions 11, 9 and 12, the uncatalyzed Haber-Weiss reaction. If HO• is generated it is most likely via the mechanism the authors propose since it is now widely accepted that the uncatalyzed Haber-Weiss reaction does not occur [66-68] and its participation in reactions of biological interest would be negligible

[69-71]. Further investigations into this reaction system by King et al. [72] supported the basic mechanism originally proposed by Fong et al [39].

There are some critical questions that must be addressed when the proposal of Fong et al. [39] is examined. First, if microsomes are heavily contaminated with catalase [73,74] how can the addition of exogenous catalase inhibit a H_2O_2-dependent reaction? It has been shown that the endogenous catalase content of microsomes is so great that unless azide is added to the microsomal suspension H_2O_2 production cannot be demonstrated [73,74]. The inhibition observed by Fong et al. upon addition of catalase is perhaps due to the presence of a stabilizing antioxidant, such as thymol, in the commercial catalase preparation used. Most commercial catalase preparations contain a stabilizing antioxidant which must be removed prior to use. The stabilizing agents can often be quickly and conveniently removed by column chromatography of the commercial enzyme preparation over a desalting column. Additionally, other investigators have shown that stabilizer-free catalase does not inhibit but actually stimulates superoxide-dependent lipid peroxidation [64,75]. Second, Noguchi and Nakano [60] have demonstrated that the reduction of ADP-chelated iron by NADPH-cytochrome P450 reductase is not superoxide-dependent and is not inhibited by superoxide dismutase. If this is true then the addition of superoxide dismutase to the reaction mixture should not inhibit but perhaps actually stimulate lipid peroxidation if it occurs by the proposed mechanism. Stimulation would arise from increased H_2O_2 production in the presence of superoxide dismutase while the rate of iron reduction would not be affected since this could be directly catalyzed by the reductase. Stimulation would occur only if

the rate limiting step is not the reduction of iron. Finally, the use of HO• traps to indicate the participation of HO• in a reaction is at best a tennable position. The inhibition of a reaction by HO• traps does not necessarily indicate that the observed reaction occurs via HO• but simply that an oxidant capable of oxidizing the trap participates in the reaction being studied. This subject will be more fully discussed later.

Lai et al. [56,57,76] also proposed that NADPH-dependent microsomal lipid peroxidation was initated via HO•. These authors proposed that HO• was formed in essence by a superoxide driven, EDTA-chelated iron promoted Haber-Weiss reation. The mechanism of HO• generation proposed by Lai et al. is the same as that proposed by Fong et al. [39], save for the iron chelator used and that the iron chelate must be added in the ferrous form. The mechanism of NADPH-dependent microsomal generation of HO• proposed by Lai et al. is given below:

$$2O_2 + NADPH \longrightarrow NADP^+ + H^+ + 2O_2^{\bullet-} \qquad (11)$$

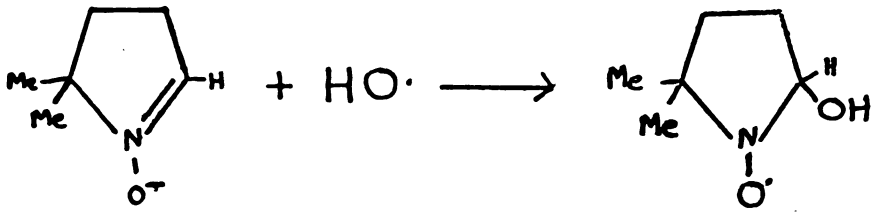
$$2O_2^{\bullet-} + 2H^+ \longrightarrow H_2O_2 + O_2 \qquad (9)$$

$$EDTA\text{-}Fe^{+2} + H_2O_2 \longrightarrow EDTA\text{-}Fe^{+3} + OH^- + HO\bullet \qquad (13)$$

Reaction 11 is again catalyzed by NADPH-cytochrome P450 reductase. The mechanism suggested by Lai et al. is based on experimental evidence that: 1) lipid peroxidation, as measured by malondialdehyde formation, can be inhibited by the addition of the spin trap 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) [77] to a microsomal reaction mixture, 2) the electron spin resonance (ESR) signal generated by the reaction of DMPO with a free radical present in the microsomal reaction mixture is identical to that formed when HO• and DMPO react; 3) spin adduct signal

intensity parallels the progress of malonaldehyde formation; 4) formation of the spin trap adduct signal is enhanced by the addition of superoxide dismutase to the reaction mixture; 5) addition of the HO· trap thiourea inhibits spin adduct formation; and 6) spin adduct signal formation requires EDTA-Fe^{+2}.

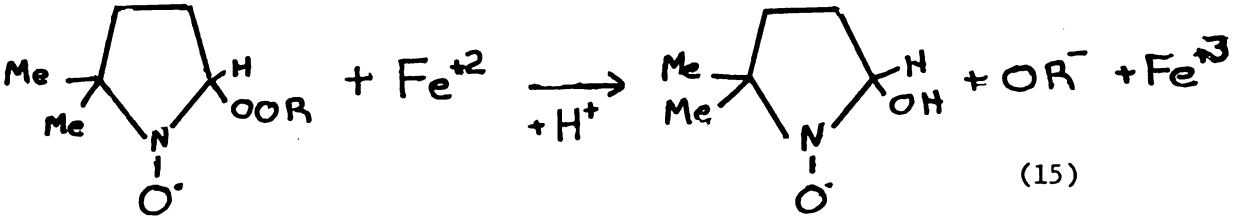
The inhibition of lipid peroxidation and ESR signal generation are proposed to be due to the following reaction:



(14)

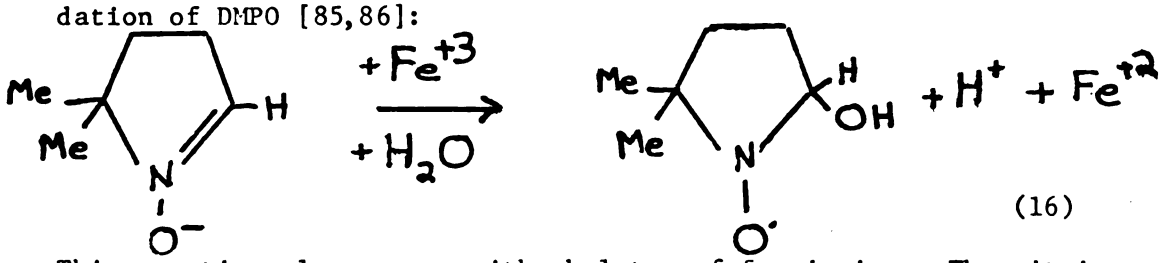
In examining the data that led Lai et al. to their final conclusions, a few critical pieces of information must be kept in mind. First, several researchers have previously demonstrated that NADPH-cytochrome P450 reductase in the presence of NADPH can reduce EDTA-chelated ferric ion [60,78-80]. Several researchers have also shown that superoxide, which may be produced by NADPH-cytochrome P450 reductase during its catalytic cycle, can reduce EDTA-chelated ferric ion [81-83]. The observed requirement for EDTA-chelated ferrous ion for lipid peroxidation and spin adduct formation is thus curious. Second, other investigators [37-39,43-55] have been unable to demonstrate the promotion of NADPH-dependent microsomal lipid peroxidation in the presence of EDTA-iron alone. Third, the rates observed by Lai et al. are very low compared to the rates reported by others [37-39,43-55], almost at the level of background or autoxidative rates observed by others. Finally, spin traps such as that used by Lai et al., can enter into

several side reactions in complex reaction mixtures. The use of proper controls must be stringently observed if reliable experimental data is to be obtained [84]. For example, in the presence of EDTA-chelated ferrous ion the DMPO-OH signal observed may have actually arisen from the breakdown of the DMPO-superoxide or DMPO-hydroperoxide spin adduct. The same would be true for DMPO adducts of lipid hydroperoxy radicals. These reactions are given below:



(15)

Or the hydroxyl radical spin adduct can be formed by the ferric ion oxidation of DMPO [85,86]:



(16)

This reaction also occurs with chelates of ferric ion. Thus it is obvious that the application of spin trapping techniques in a biological system can quickly become complex and that the use of properly designed control reactions is essential to the obtainment of valid experimental data.

Perferryl ion ($Fe^{+2}O_2 \rightleftharpoons Fe^{+3}O_2^{\cdot-}$)-promoted, hydroperoxide-independent initiation of lipid peroxidation has been proposed by investigators for both NADPH-dependent and superoxide-dependent lipid peroxidation. Perferryl ion-promoted initiation of NADPH-dependent microsomal lipid peroxidation was first proposed by Ernster and co-workers [37,43-45]. The proposal that the perferryl ion could promote

the initiation of lipid peroxidation was based on the similarities between lipid peroxidation and the numerous biological oxidation systems studied by Mason in which the perferryl ion had been proposed to mediate the reaction [87,88]. Perferryl ion-promoted initiation of superoxide-dependent lipid peroxidation in both microsomes and liposomes was first proposed by Svingen et al. [63]. The mechanism of superoxide-dependent lipid peroxidation proposed by Svingen et al. was based on similarities to the NADPH-dependent reaction system. In both systems chelation of iron by ADP or a similar nucleotide or pyrrophosphate was required.

Initiation of lipid peroxidation by an activated dioxygen-transition metal complex was first proposed by Heaton and Uri for a cobaltous-stearate reaction mixture that could promote PUFA peroxidation [89]. The close parallels between cobalt and iron chemistry led these authors to propose that a similar reaction occurred when the ferrous ion was substituted for the cobaltous ion. Experimentally this relationship has been borne out and much of the chemistry of the perferryl ion has been first investigated in a cobalt model system. As previously discussed, reversible dioxygen binding by transition metals occurs with the reduced form of a transition metal that can undergo a one electron redox reaction. Dioxygen activation occurs because complexation with the transition metal imparts free radical characteristics to the oxygen molecule. This free radical characteristic of dioxygen circumvents the spin restrictions that are present for the reaction of ground state dioxygen with organic molecules, such as PUFA, and allows the reaction to occur. The reduction states of the dioxygen-ferrous ion complex can perhaps best be represented by the following set of equivalent structures:

$$Fe^{+2} + O_2 \longleftrightarrow Fe^{+2}O_2 \longleftrightarrow Fe^{+3}O_2^{\cdot-} \quad (17)$$

The greatest contribution may come from the resonance form shown as the oxidized metal-superoxide complex. The formal charge on the iron atom of the perferryl ion is +6. Thus the perferryl ion is quite electronegative and as expected it is predicted to be a strong oxidant. The reaction(s) by which activated dioxygen-ferrous ion complexes are proposed to initiate lipid peroxidation may be one of the following reactions [26]:

$$Fe^{+3}O_2^{\cdot-} + LH \longrightarrow Fe^{+3}O_2H^- + L\cdot \quad (18)$$

$$Fe^{+3}O_2^{\cdot-} + LH \longrightarrow Fe^{+2} + HO\cdot_2 + L\cdot \quad (19)$$

$$Fe^{+3}O_2^{\cdot-} + LH \longrightarrow Fe^{+3}OH + LO\cdot \quad (20)$$

$$Fe^{+3}O_2^{\cdot-} + LH \longrightarrow Fe^{+3} - L^- + HO_2\cdot \quad (21)$$

At the present time there is no experimental evidence favoring one initiation reaction over another, however, application of ESR spin trapping techniques may be useful in elucidating the relative importance of these reactions.

Experimental evidence indicating that the initiation of lipid peroxidation is promoted by the perferryl ion relies heavily on the observed requirement for ferrous ion and oxygen or ferric ion and superoxide for the initiation of lipid peroxidation. Unfortunately, proposals for perferryl ion-promotion of initiation have often had to rely on corroborative negative data indicating that other intermediates, such as $HO\cdot$, H_2O_2, superoxide alone or iron alone, cannot promote or do not participate in the promotion of initiation of lipid peroxidation. Thus, it often appears that the proposal for perferryl ion promotion of initiation of lipid peroxidation is used as a stop-gap measure filling a void

in our knowledge. However, the circumstantial evidence that the perferryl ion-promotes initiation of lipid peroxidation is perhaps admissable since to date the perferryl ion has not been isolated and circumstantial evidence is all that is available. The direct demonstration of perferryl ion-promotion of initiation of lipid peroxidation must wait for further experimental and theoretical development.

In their original paper, Hochstein and Ernster [37] did not observe a requirement for iron in the promotion of NADPH-dependent microsomal lipid peroxidation but rather an ADP requirement. However, subsequent investigations by these authors demonstrated that the commercial preparation of ADP used in their experiments was contaminated with iron and that both ADP and iron were required for the promotion of NADPH-dependent microsomal lipid peroxidation [43]. The perferryl ion was proposed to be formed in two steps. First, NADPH-dependent reduction of ADP-chelated ferric ion via a microsomal flavoprotein and second, the addition of dioxygen to the ADP-chelated ferrous ion complex. The requirement for ADP was proposed to be to chelate the ferric ion and keep it in solution at neutral pH. If it were not for chelation the ferric ion would precipitate as the hydroxide and the concentration of ferric ion would be drastically reduced.

Pederson and Aust [55,58,75] also proposed that the perferryl ion promoted the initiation of NADPH-dependent lipid peroxidation. These investigators studied not only NADPH-dependent microsomal lipid peroxidation but also the reconstitution of microsomal lipid peroxidation in liposomes utilizing NADPH-cytochrome P450 reductase. In the microsomal reaction mixture these investigators observed the same requirements for promotion of lipid peroxidation as did Ernster and co-workers

[37,43-45]. Pederson and Aust also found that peroxidation could be further stimulated by the addition of EDTA-chelated ferric ion to a reaction mixture containing ADP-chelated iron. However, EDTA-chelated iron alone could not promote initiation of lipid peroxidation. These authors found that the NADPH-cytochrome P450 reductase promoted peroxidation of liposomes required both ADP-chelated ferric iron and EDTA-chelated ferric iron for maximal rates of peroxidation. The rates of peroxidation observed in the presence of ADP-chelated iron alone were very low as compared to NADPH-dependent ADP-chelated iron-promoted microsomal lipid peroxidation. No peroxidation was observed in the presence of EDTA-chelated ferric iron alone. However, addition of EDTA-chelated ferric ion together with ADP-chelated iron greatly stimulated the rate of peroxidation. The proposal that the ADP-perferryl ion promoted initiation was based to some extent on negative data.

Pederson and Aust could not demonstrate the participation of H_2O_2 or HO• in NADPH-dependent lipid peroxidation. The absolute requirement for ADP-chelated feric iron and NADPH, coupled with the known ability of NADPH-cytochrome P450 reductase to reduce ADP-chelated iron [60,79], indicated that ADP-chelated ferrous ion was probably directly involved in initiation. Other researchers had previously demonstrated that ferrous ion alone could initiate lipid peroxidation [90,91]. Because of the requirement for reduced iron in an oxygenated solution Pederson and Aust proposed that one of a variety of reactive intermediates formed between ferrous ion and oxygen was involved in the initiation of lipid peroxidation. One such reactive intermediate is the perferryl ion. It was previously demonstrated that these reactive intermediates could promote reactions such as aromatic hydroxylations [92-94] and bio- and

chemiluminescence in aqueous solution [95,96]. Thus, these reactive intermediates may perhaps be capable of initiating lipid peroxidation. Pederson and Aust did not propose a function for EDTA-chelated iron other than it replaced some microsomal component that was absent in liposomes prepared from the Folch lipid extract [97] of microsomes.

There are some critical questions posed by the results of Pederson and Aust [55,59,62,75]. First, why are both ADP-chelated iron and EDTA-chelated iron required for lipid peroxidation? The answer is fairly obvious and is the one put forth by the authors themselves. The irons function to promote different reactions. The function each iron plays is dictated by the characteristics of its chelation. Chelation effects the redox potential of the iron. The redox potential of the iron not only affects the stability of the perferryl ion [98] but also the likelihood that the iron will participate in such reactions as reductive activation of hydroperoxides [99,100]. Second, in their investigation of superoxide-dependent lipid peroxidation, they also found that both forms of iron chelates were necessary for the promotion of liposomal peroxidation [62,75]. What is the role for these iron chelates in the mechanism of superoxide-dependent lipid peroxidation? Pederson and Aust propose no role for these iron chelates and instead propose that lipid peroxidation is initiated via singlet oxygen addition to PUFA. These authors proposed that singlet oxygen was produced by superoxide dismutation. However, the conclusion that singlet oxygen is responsible for initiation is based on data showing that the singlet oxygen trap 2,5-diphenylisobenzofuran inhibited lipid peroxidation. This data may be in error as it has been suggested that 2,5-diphenylisobenzofuran is a free radical trap in addition to being a singlet oxygen trap [72].

Svingen et al. [63,78] have proposed that the ADP-perferryl ion is involved in the initiation of both NADPH-dependent lipid peroxidation and superoxide-dependent lipid peroxidation. Using an iodometric method for the determination of lipid hydroperoxides [33] these authors demonstrated that lipid hydroperoxides could be generated in essentially lipid hydroperoxide free liposomes, prepared from extracted microsomal lipid, by ADP-chelated ferrous ion, by ADP-chelated ferric ion in the presence of NADPH and NADPH-cytochrome P450 reductase or by ADP-chelated ferric ion in the presence of a superoxide generating system, such as xanthine and xanthine oxidase. Lipid hydroperoxide formation could not be promoted by ferrous ion, ferric ion, ADP-ferric ion or by either EDTA-chelated ferric or ferrous ion. Promotion of lipid hydroperoxide formation in the above reaction mixtures was essentially totally inhibited by superoxide dismutase but was not inhibited by catalase or HO• traps. Since superoxide alone has been shown to be unable to initiate lipid peroxidation [63,78] these authors, in light of the requirement for ADP-chelated iron concluded that promotion of initiation of lipid peroxidation occurred via the ADP-perferryl ion. The perferryl ion could be formed by reduction of ADP-chelated ferric ion followed by dioxygen addition or by the reaction of ADP-chelated ferric ion with superoxide. While EDTA-chelated ferric or ferrous ion could not promote initial formation of lipid hydroperoxides these authors found that addition of EDTA-chelated ferrous ion, or EDTA-chelated ferric ion in the presence of reducing equivalents, could greatly stimulate ADP-perferryl ion promotion of lipid peroxidation. Svingen et al. proposed, as did Pederson and Aust [75] that the type of chelator used had a dramatic

effect on the participation of iron in lipid peroxidation. As previously stated, chelation was proposed not only to facilitate iron participation in promotion of lipid peroxidation but to also moderate the role played by iron by changing the redox potential of iron. Chelation also has a dramatic effect on the stability of the perferryl ion not only from a redox standpoint but also from the standpoint of steric hinderance towards further autoxidation [98,101-104]. The effect of chelation is cyclical in nature. The perferryl ion is most stable when chelated by weak ligands such as phosphate anions or very strong chelators such as in the oxygen carrying hemoproteins. Between the two extremes of chelation there lies a whole spectrum of chelation effects with their corresponding effects on perferryl ion stability. Chelates that donate electron density to the ferrous ion strengthen the iron-oxygen bond in the perferryl ion and thus stabilize the perferryl ion. In the opposite sense, chelates that withdraw electron density from the ferrous ion reduce the stability of the perferryl ion. Applying this theory to the effects of chelation expressed in lipid peroxidation it can be seen that ADP, a relatively weak chelator which may increase electron density on the iron center and thus increases perferryl ion stability, gives an iron complex which is an active promoter of hydroperoxide-independent initiation of lipid peroxidation. On the other hand, chelation of ferrous ion by a stronger chelator, EDTA, would be expected to reduce the stability of the perferryl ion, if not forego its formation altogether. As predicted chelation of ferrous ion by EDTA renders the ferrous ion unable to promote hydroperoxide-independent initiation of lipid peroxidation. This theory is also supported by the demonstrated ability of free ferrous, where the ligand would be water,

to promote hydroperoxide independent initiation of lipid peroxidation [105]. The foundations of this theory will continue to be tested as more and more chelators of iron are examined for their effect on the ability of ferrous ions to promote lipid peroxidation.

Like Pederson and Aust [55,58,62,75], Svingen et al. [63,78] found that both ADP-chelated and EDTA-chelated iron were required for maximal rates of lipid peroxidation in liposomes. This was true for either NADPH-dependent or superoxide-dependent lipid peroxidation. If only the ADP-perferryl ion participates in the LOOH-independent initiation of lipid peroxidation the authors are faced with the question, what is the role played by EDTA-chelated iron? Svingen et al. proposed that EDTA-chelated iron promoted the breakdown of initially formed lipid hydroperoxides. The EDTA-chelated iron-promoted breakdown of lipid hydroperoxides is essentially the same as reductive activation of organic hydroperoxides by ferrous ions [107-110]. Reductive activation of lipid hydroperoxides generates lipid alkoxy radicals and can be considered hydroperoxide-independent initiation of lipid peroxidation. (Lipid hydroperoxide-dependent initiation of lipid peroxidation will be the subject of the final section of this review.)

Finally, it has been proposed that the perferryl ion promotes lipid hydroperoxide formation in the lipoxygenase catalysis of lipid hydroperoxide generation in PUFA [111]. Nakano and Sugioka [111] have proposed that the perferryl ion promotes the abstraction of hydrogen from the methylene carbon of PUFA giving rise to a PUFA alkyl radical. This is essentially the same reaction mechanism proposed for the initiation of lipid peroxidation by the ADP-perferryl ion in lipid hydroperoxide free lipids, Fig. 1. The generation of a PUFA alkyl radical during the

catalytic cycle of lipoxygenase has been demonstrated by ESR spin trapping techniques [112,113]. Continued investigation into the mechanisms of lipoxygenase catalysis may shed some light on the mechanism of ADP-perferryl ion promoted initiation of lipid peroxidation. It has already been shown that the lipoxygenase reaction is free radical in nature and is inhibited by superoxide dismutase but not by HO· or $^1O_2$ traps. The mechanism for lipoxygenase thus appears to be similar to a controlled form of NADPH-dependent or superoxide-dependent ADP-perferryl ion-promoted initiation of lipid peroxidation.

As can be seen from the above discussions there are two basic mechanisms that have been proposed for the initiation of iron promoted lipid peroxidation in hydroperoxide free lipids. Initiation via the perferryl ion and initiation via the hydroxyl radical. The two mechanisms of initiation have been proposed separately for the same reaction system, NADPH-dependent microsomal lipid peroxidation. Investigators have found strong experimental evidence to support both proposals. Such widely disparate conclusions drawn from the investigations of the same reaction mixture raise the question of whether grossly different experimental techniques are being employed? However, the answer to this question is an emphatic no. The possibility then arises that the researchers are perhaps looking at different aspects of the same reaction system. Perhaps the researchers should be examining the energetics of the initiation reaction and not concentrating so hard on giving a physical description to the intermediates involved. This approach is supported by experimental findings that the hydroxyl radical formed by Fenton's Reagent and the perferryl ion formed by ferrous ion autoxidation may not be as discrete entities as once believed. It is now

apparent that rather than discrete entities these two reactive intermediates are more likely to be part of a broad continuum of iron-oxygen reactive intermediates. Attempts at identification of the iron-oxygen intermediates may be more reflective of the experimental techniques employed than the actual intermediate.

The experimentation of Walling et al. [114] and of Groves et al. [115-117] indicates that free HO• may not be formed by the Fenton's Reagent under any but strongly acidic conditions. Rather some type of iron-hydroxyl radical intermediate is probably formed. It is only under conditions of low pH that Fenton's Reagent promoted reactions give product distributions truly characteristic of a free radical reaction. As the polarity and/or pH of the reaction mixture is altered the reaction products are less characteristic of a free radical mechanism and show the growing influence of a stereospecific reaction. The stereospecific nature of several Fenton's Reagent-promoted hydroxylations led Groves et al. [115-117] to propose that except under strong acid conditions the oxidative intermediate that promotes hydroxylation is the ferryl ion ($Fe^{+2}O$) and not HO•. The ferryl ion is known to be a strong oxidant and it has been proposed to be the promoter of several ferrous ion dependent oxidation reactions [118-121]. Ferrous ion dependent hydroxylations are essentially identical to Fenton's Reagent promoted hydroxylations run at neutral pH. The formation of ferryl ion from ferrous ion has been proposed to occur by the following mechanism. (Note that the first step of the mechanism involves formation of the perferryl ion) [122,123].

$$Fe^{+2} + O_2 \longrightarrow Fe^{+2}O_2 \qquad (22)$$

$$Fe^{+2}O_2 + Fe^{+2} \longrightarrow Fe^{+2}O_2Fe^{+2} \qquad (23)$$

$$Fe^{+2}O_2Fe^{+2} \longrightarrow 2\ Fe^{+2}O \qquad (24)$$

The ferryl ion could be formed in both the NADPH-dependent and superoxide-dependent lipid peroxidation reaction mixtures studied by Pederson et al. [55,58,62,75], Svingen et al. [27,63,78] or McCay and co-workers [48,49,52,72].

LIPID HYDROPEROXIDE-DEPENDENT INITIATION OF LIPID PEROXIDATION

Once low levels of lipid hydroperoxides are present in a lipid matrix the predominant mechanism of initiation involves their breakdown to form free radicals.

$$LOOH \longrightarrow \text{free radicals} \qquad (2)$$

Thus when lipid hydroperoxide formation promoted by the ADP-perferryl ion reaches a significant level its contribution to total peroxidation is quickly overshadowed by the contributions from lipid hydroperoxide-dependent mechanisms of initiation. The mechanisms of free radical generation from lipid hydroperoxides, especially by heavy metal promoted reactions, have been extensively studied and have been the subject of several reviews [124-126]. The predominance of metal promoted hydroperoxide-dependent initiation over oxygen activation mechanisms of initiation has led to special difficulties in the investigation of the mechanism of initiation of lipid peroxidation. The problem that arises for the biologist or biochemist is that during isolation of samples, and their subsequent handling, hydroperoxides are often formed in the membranes by autoxidation. Unless special precautions are taken to minimize or eliminate lipid hydroperoxide formation during sample manipulations one may draw the conclusion, and perhaps rightly so, that only lipid hydroperoxide-dependent initiation occurs in enzymatic lipid

peroxidation. Contamination of samples by lipid hydroperoxides can completely mask the activated oxygen mechanism of initiation.

The mechanisms of hydroperoxide-dependent initiation of free radical reactions have been extensively discussed in detail [124-126]. Free radical generation from lipid hydroperoxides occurs by three general mechanisms:

1. Thermal or Unimolecular Homolysis:

$$LOOH \longrightarrow LO\cdot + HO\cdot \qquad (25)$$

Hydroperoxides which are generated by autoxidation or other means undergo unimolecular homolysis at 37°C at an extremely slow rate. In fact it has been estimated that if H_2O_2 loss was by unimolecular homolysis only, its half life at body temperatures would be 10^{11} years [18]. Thus from all indications it is very doubtful that this mechanism of free radical generation contributes appreciably to lipid hydroperoxide-dependent initiation of lipid peroxidation.

2. Molecule Induced Homolysis (MIH) or Molecule Assisted Homolysis (MAH) - Bimolecular Homolysis:

$$LOOH + LH \longrightarrow LO\cdot + L\cdot + H_2O \qquad (26)$$

In this mechanism there is not only bond breakage but also bond formation, thus the energy of activation and the endothermicity of the reaction are both greatly reduced as compared to reaction 25. Reactions that occur via MIH are much faster than unimolecular reactions. The rate of MIH reactions are increased by polar solvents especially those which can hydrogen bond. However, the importance of free radical generation from lipid hydroperoxides via MIH mechanisms is likely to be insignificant compared to the rate of lipid peroxidation in biological

systems where the presence of heavy metals, most notably iron, can promote reductive activation of lipid hydroperoxides.

3. Reductive Activation

$$Fe^{+2} + LOOH \longrightarrow Fe^{+3} + LO\cdot + OH\cdot \qquad (27)$$

$$Fe^{+3} + LOOH \longrightarrow Fe^{+2} + LOO\cdot + H^{+} \qquad (28)$$

The metal promoted decomposition of organic hydroperoxides and peroxidic material has been extensively studied. The reductive activation reaction with ferric iron (28) is a relatively slow reaction because it requires prior ionization of the hydroperoxides. Hydroperoxides as a family have a pKa of 10.8 [125]. Free ferric ion is insoluble above pH 4. Since the two reactants have opposite pH maxima for activity it is fairly obvious why this is a slow reaction [127,128]. The rate of the ferric ion reaction (28) can be greatly affected by chelation. This effect may be due to increased solubility at higher pHs, but the system is more complex. Chelation has been found to enhance, suppress, or not affect the rate of reaction 28 depending on the nature of the chelate, the metal, and the chelate-metal complex formed [129]. For instance, it is well known that many ferric hemeproteins can promote the breakdown of lipid hydroperoxides in an extremely efficient manner. In general, the ferrous ion dependent reaction is much more rapid than the ferric ion reaction. However, as with the ferric ion reaction, the ferrous ion reaction is greatly affected by chelation [100]. In biological systems, either *in vivo* or *in vitro*, it is likely that reductive activation is the predominant mechanism of free radical generation in lipid hydroperoxide-dependent initiation of lipid peroxidation.

The ability of metal ions and heme compounds to catalyze the oxidation of unsaturated fatty acids has been extensively studied. Tappel

et al. [129-131] found that lipid peroxidation catalyzed by hematin compounds is a basic pathological reaction *in vivo* and a deteriorative reaction *in vitro*. These investigators found that heme compounds are the most powerful catalysts of lipid peroxidation found in animal tissues. Additionally, hemeproteins are often found intimately associated with lipid membranes.

Svingen et al. [63,78] demonstrated that in both NADPH-dependent and superoxide-dependent peroxidation of liposomes EDTA-chelated iron promoted reductive activation of lipid hydroperoxides. These investigators found that EDTA-chelated iron promotion of lipid peroxidation was dependent upon the reduction of the ferric ion complex. The EDTA-ferric ion complex can be reduced either by superoxide or directly by NADPH-cytochrome P450 reductase in the presence of NADPH. EDTA-chelated iron promotion of lipid peroxidation requires lipid hydroperoxides and reduction of the iron complex. The EDTA-ferrous ion promoted lipid hydroperoxide-dependent initiation reaction was found to be free radical in nature, not to occur via a perferryl ion (activated dioxygen) intermediate, as it was not inhibited by superoxide dismutase, and to not involve HO•.

Svingen et al. [63,78] demonstrated the integral relationship of ADP-perferryl ion hydroperoxide-independent initiation of lipid peroxidation and the EDTA-ferrous ion hydroperoxide-dependent initiation of lipid peroxidation. These authors found, as one might guess, that the latter was dependent on the former. Svingen et al. also demonstrated that hydroperoxide-dependent initiation accounts for greater than 90% of the total peroxidic products formed. Thus it is apparent that once a significant concentration of lipid hydroperoxides is initially formed

the predominant mechanism of initiation then becomes reductive activation of lipid hydroperoxides.

Svingen et al. [63,78] also directly demonstrated that in microsomal lipid peroxidation, where only ADP-chelated iron is required, the microsomal cytochromes, most notably cytochrome P450, promote the heterolytic activation of ADP-perferryl ion generated lipid hydroperoxides. These investigators showed that inhibition of microsomal lipid peroxidation by non-antioxidant inhibitors of cytochrome P450 could be completely reversed by the addition of EDTA-chelated iron. Since EDTA-chelated iron must be reduced before it will promote reductive activation of lipid hydroperoxides it is apparent that the drug substrates inhibit lipid peroxidation by interacting with cytochrome P450 and not by competition for reducing equivalents [63]. The inhibition of cytochrome P450 promoted heterolytic activation of lipid hydroperoxides by oxidizable substrates of cytochrome P450 and the lipid hydroperoxide-dependent co-oxidation of those drug substrates demonstrates that inhibition is due to a peroxidase mechanism in which the substrate is oxidized and the lipid hydroperoxide is likely reduced to an alcohol. Reduced cytochrome P450 can also function as a peroxidase, reducing lipid hydroperoxides to lipid alcohols.

CHAPTER 1

INITIATION OF LIPID PEROXIDATION

SUMMARY

An investigation into the mechanism of lipid peroxidation promoted by xanthine oxidase showed a dependence upon superoxide, singlet oxygen and adenosine 5'-diphosphate chelated iron (ADP-Fe^{+3}). In the absence of ADP-Fe^{+3} or in the presence of superoxide dismutase there is complete inhibition of enzymatically promoted peroxidation. Initiation of peroxidation likely occurs through a complex formed by ADP, Fe^{+3} and superoxide. Use of the singlet oxygen trapping agent 2,5-diphenylfuran showed that singlet oxygen does not participate in the LOOH-independent initiation of peroxidation but rather in its LOOH-dependent initiation. The mechanism of NADPH-cytochrome P450 reductase-promoted and ADP-Fe^{+2}-promoted lipid peroxidation parallel that of xanthine oxidase in that LOOH-independent initiation occurs through a superoxide dismutase-sensitive reaction and that singlet oxygen is present only during LOOH-independent initiation of lipid peroxidation. Superoxide dismutase sensitivity may result from the scavenging of $O_2^{\cdot-}$ which disassociates from the reduced iron-oxygen complex leaving leaving oxidized metal:

$$\text{ADP-Fe}^{+3} + e^- \quad \text{ADP-Fe}^{+2} + O_2 \quad \text{ADP-Fe}^{+2}\text{-}O_2 \quad \text{ADP-Fe}^{+3}\text{-}O_2^{\cdot-} \quad \text{ADP-Fe}^{+3} + O_2^{\cdot-} \qquad (29)$$

INTRODUCTION

The role that superoxide ($O_2^{\cdot-}$) and singlet oxygen, $^1O_2(^1{}_g)$ play in the peroxidation of unsaturated lipids is a subject of current intensive research. The number of biological systems known to produce $O_2^{\cdot-}$ and catalyze lipid peroxidation is expanding. Included are the oxidation of

xanthine by xanthine oxidase (62), the cyclic reduction and oxidation of paraquat (methyl viologen) by microsomal NADPH-cytochrome P450 reductase and oxygen [132,133] and perhaps the same oxidation-reduction cycle of the anthraquinone structure of the anticancer drug adriamycin [134].

A mechanism of superoxide-dependent lipid peroxidation promoted by xanthine-xanthine oxidase has been proposed by Pederson and Aust [62] based on the dismutation of superoxide to give singlet oxygen according to the scheme [135]:

$$2O_2^{\cdot-} + 2H^+ \longrightarrow {}^1O_2 + H_2O_2 \qquad (30)$$

Singlet oxygen was proposed to be the direct initiator of lipid peroxidation by a concerted addition-abstraction reaction with the diene bonds of unsaturated lipid giving rise to lipid hydroperoxides [30]. Enzymatic peroxidation required ADP-Fe^{+3} and was enhanced by the addition of Fe^{+3} chelated by EDTA.

Initiation of xanthine-xanthine oxidase-promoted lipid peroxidation mediated through $^1O_2$ was also proposed by Kellogg and Fridovich [136]. They reported that peroxidation was dependent upon $O_2^{\cdot-}$ and H_2O_2 and proposed the following scheme for the generation of $^1O_2$:

$$O_2^{\cdot-} + H_2O_2 \longrightarrow OH^- + OH\cdot + {}^1O_2 \qquad (31)$$

Contrary to these two schemes, King et al. [72] found no evidence for the production of $^1O_2$ by the xanthine-xanthine oxidase system in the absence of active lipid peroxidation. Indeed, whether the self dismutation of $O_2^{\cdot-}$ gives rise to $^1O_2$ was questioned by Nilsson and Kearns [137]. King et al. [72] proposed that $^1O_2$ was produced in both xanthine-xanthine oxidase and NADPH-cytochrome P450 reductase-promoted lipid peroxidation only after lipid peroxidation was initiated and that $^1O_2$ was perhaps formed from the breakdown of lipid hydroperoxides (LOOH).

In this chapter a unified mechanism for lipid hydroperoxide-independent initiation of lipid peroxidation promoted by NADPH-cytochrome P450 reductase, and xanthine oxidase in the presence of ADP-Fe^{+3} and by ADP-Fe^{+2} alone is proposed. Lipid hydroperoxide-independent initiation of lipid peroxidation is dependent on the production of an ADP-perferryl ion complex (ADP-Fe^{+2}-O_2 ⇌ ADP-Fe^{+3}-$O_2^{\cdot-}$). This initiation complex shows superoxide dismutase sensitivity. Singlet oxygen participation in lipid peroxidation arises from the breakdown of lipid hydroperoxides promoted by reduced iron chelates (ADP-Fe^{+2}, EDTA-Fe^{+2}). Once produced, $^1O_2$ reacts with unsaturated lipid to produce lipid hydroperoxides. There are several LOOH-dependent reactions, only some of which produce $^1O_2$. These reactions give rise to the observed sensitivity to $^1O_2$ trapping agents, and account for the observed trapping agents' partial inhibition of lipid peroxidation.

MATERIALS AND METHODS

Chemicals: Milk xanthine oxidase, Sigma Type I (E.C. No. 1.2.3.2.), superoxide dismutase, bovine erythrocyte (E.C. No. 1.15.1.1.), soybean lipoxygenase (E.C. No. 1.13.1.13), adenosine 5'-diphosphate (ADP), cytochrome c, Sigma Type IV, nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) and xanthine were obtained from Sigma Chemical Company. 2,5-Diphenylfuran (DPF) was obtained from Eastman Organic Company. Trans-1,2-dibenzoyl ethylene was obtained from Aldrich Chemical Company. All other reagents used were of analytical grade.

Microsomes and Microsomal Lipid: Microsomal phospholipid and NADPH-cytochrome P450 reductase were prepared by the methods of Pederson et al. [55]. NADPH-cytochrome P450 reductase specific activity, determined by the method of Pederson et al. [55], was 45-52 units/mg.

Superoxide dismutase activity was measured by the method of McCord and Fridovich [138].

Lipid hydroperoxides were prepared by the action of soybean lipoxygenase on liposomes of extracted microsomal lipid. The liposomes were prepared by sonication and diluted to a final concentration of 1.0 µmole lipid phosphate per ml in 0.05 M Tris-Cl pH 9.0 at 37°C. Sodium deoxycholate, 0.04% w/v, was included in the incubation system to enhance the reaction. The incubation was initiated by addition of lipoxygenase, at 100 µg per ml. The buffer was saturated with oxygen and the incubation was carried out under an oxygen atmosphere. At the end of a 45 minute incubation, the phospholipid hydroperoxides were extracted and stored by the method of Pederson and Aust [55]. Lipid hydroperoxide content was measured iodometrically by the method of Buege and Aust [33].

Iron Chelates: Chelated iron solutions were prepared in 0.05 M Tris-Cl pH 7.5 at 37°C. When preparing the ferrous chelates buffers were saturated with argon. The molar ratio of the ADP-iron solution was 17:1 while that of the EDTA-iron was 1.1:1.

Liposomes: Control liposomal incubation systems contained 1.0 µmole lipid phosphate per ml in 0.05 M Tris-Cl pH 7.5 at 37°C. When lipid peroxidation was promoted by xanthine oxidase, buffers were saturated with O_2. Incubations were carried out at 37°C in a metabolic shaker bath under an air atmosphere. Thiobarbituric acid (TBA)-reactive material was determined by the method of Bernheim et al. [139]. To eliminate nonenzymatic chromophore formation during the assay, 0.03 volume of 2% butylated hydroxytoluene (in ethanol) was added to the TBA reagent. Lipid hydroperoxides were assayed as previously indicated

[33]. Where indicated superoxide dismutase was added in 0.05 M Tris-Cl pH 7.5 at 37°C and DPF as a 0.02 ml of a solution prepared in acetone. Addition of 0.02 ml of acetone to any of the incubations had no effect on activity.

Other Methods: The oxidation of DPF by $^1O_2$ was confirmed by following the decrease in fluorescence of DPF at 368 nm, using an excitation wavelength of 333 nm. Confirmation of product formation was obtained by thin layer chromatography (TLC) of the chloroform extract of the incubation systems. TLC was performed on Silica Gel G plates developed in the dark in a hexane-dioxane (3:1) solvent system. DPF was visualized by its fluorescence. The $^1O_2$ oxidation product, cis-1, 2-dibenzoylethylene, was visualized by spraying the plate with 0.5% 2,4-dinitrophenyl hydrazine in 2 M HCl and identified by comparison to a cis-1,2-dibenzoylethylene standard prepared from the trans-1,2-dibenzoylethylene isomer by the method of Lutz and Wilder [140].

Total lipid phosphate was measured by the method of Bartlett [141]. Protein was determined by the method of Lowry et al. [142] standardized with bovine serum albumin (Pentex) using $E^{1\%}_{1\ cm}$ at 280 nm equal to 6.6.

RESULTS

Xanthine Oxidase-Promoted Lipid Peroxidation

Lipid peroxidation, assayed as TBA-reactive material, during xanthine oxidation by xanthine oxidase in the presence of ADP-Fe^{+3} is inhibited 95% by superoxide dismutase (Table 1). This is in agreement with the results of Pederson and Aust [62] and King et al [72]. Lipid hydroperoxide production was inhibited 76% by superoxide dismutase (Table 1).

TABLE 1

INHIBITION OF XANTHINE OXIDASE-PROMOTED LIPID PEROXIDATION BY SUPEROXIDE DISMUTASE (SOD) AND 2,5-DIPHENYLFURAN (DPF)

Control reaction mixtures were as described under "Methods" with the following additions: 1.7 mM ADP, 0.1 mM $FeCl_3$, 0.33 mM xanthine and where indicated 1.0 units SOD/ml or 0.2 mM DPF. Incubations and assays were performed as described under "Methods". Results are reported as initial rates.

		nmoles MDA min ml^{-1}	nmoles LOOH min ml^{-1}
Control		0.02	1.8
	+DPF	0.04	0.0
	+SOD	0.01	0.9
+xanthine oxidase		0.68	6.6
+xanthine oxidase	+DPF	0.60	4.5
+xanthine oxidase	+SOD	0.03	1.6

This suggests a dependence upon a superoxide dismutase-sensitive initiation reaction in this system.

Inhibition of xanthine oxidase-promoted lipid peroxidation by the singlet oxygen trapping agent DPF is considerably less than that observed with superoxide dismutase (Table 1). Inhibition of the formation of TBA-reactive material was 13% while the inhibition of LOOH production was 32%. That DPF can only partially inhibit xanthine-xanthine oxidase-promoted lipid peroxidation is substantiated by the observations of Pederson and Aust [62] and King et al. [72] that even at saturating concentrations of singlet oxygen trapping agents diphenylisobenzofuran (DPBF) or DPF respectively, lipid peroxidation is inhibited by only approximately 50%.

Addition of EDTA-Fe^{+3} is necessary for maximal peroxidation in xanthine oxidase-promoted lipid peroxidation [62]. This addition apparently has no effect on the initiation mechanism of lipid peroxidation as evidenced by the consistent effects of DPF and superoxide dismutase on a system containing EDTA-Fe^{+3} (Tables 2 and 3). 2,5-Diphenylfuran inhibits by only 6% while superoxide dismutase essentially completely eliminates formation of TBA-reactive material. Lipid hydroperoxide production in the presence of superoxide dismutase is reduced by 57%. In the presence of DPF, LOOH formation is reduced by 14%.

NADPH-Cytochrome P450 Reductase-Promoted Lipid Peroxidation

Promotion of lipid peroxidation by NADPH-cytochrome P450 reductase in the presence of NADPH and ADP-Fe^{+3} is shown in Table 4. Addition of superoxide dismutase reduces the NADPH-dependent formation of TBA-reactive material by more than 86%. This is in good agreement with the inhibition of NADPH-dependent lipid peroxidation in microsomes by

TABLE 2

INHIBITION OF XANTHINE OXIDASE PROMOTED

LIPID PEROXIDATION BY SOD IN THE PRESENCE OF EDTA-Fe^{+3}

Control reaction mixtures were as described under "Methods". The following additions were made where indicated: 1.7 mM ADP, 0.11 mM EDTA, 0.2 mM $FeCl_3$, 0.33 mM xanthine and 1.0 units SOD/ml. Incubations and assays were performed as in Table 1.

		nmoles MDA $min\ ml^{-1}$	nmoles LOOH $min\ ml^{-1}$
Control		0.05	2.4
	+SOD	0.04	0.5
+xanthine oxidase		2.10	22.5
+xanthine oxidase	+SOD	0.05	10.1

TABLE 3

INHIBITION OF XANTHINE OXIDASE-PROMOTED LIPID PEROXIDATION BY DPF IN THE PRESENCE OF EDTA-Fe^{+3}

Control reaction mixtures were as in Table 2. 0.2 mM DPF was added where indicated. Incubations and product assays were performed as in "Methods".

		nmoles MDA $min\ ml^{-1}$	nmoles LOOH $min\ ml^{-1}$
Control		0.05	1.0
	+DPF	0.02	0.1
+xanthine oxidase		1.41	19.5
+xanthine oxidase	+DPF	1.32	16.8

TABLE 4

INHIBITION OF NADPH-CYTOCHROME P450 REDUCTASE PROMOTED LIPID PEROXIDATION BY SOD

Control reaction mixtures were as described under "Methods". The following additions were made where indicated: 0.1 units NADPH-cytochrome P450 reductase/ml, 1.7 mM ADP, 0.1 mM $FeCl_3$ and where indicated 1.0 units/ml SOD. The reactions were initiated by the addition of 0.1 mM NADPH. Incubations and assays were carried out as in "Methods".

		nmoles MDA $min\ ml^{-1}$	nmoles LOOH $min\ ml^{-1}$
Control		0.01	0.5
	+SOD	0.01	0.1
+NADPH		0.15	1.8
+NADPH	+SOD	0.02	0.1

superoxide dismutase reported by King et al. [72]. Lipid hydroperoxide production is inhibited by superoxide dismutase by 94%.

Addition of DPF inhibited the formation of TBA-reactive material by 13% and LOOH by 21%, as shown in Table 5. As observed in xanthine oxidase-promoted initiation, DPF was a much less effective inhibitor than superoxide dismutase.

As with the xanthine oxidase system, addition of EDTA-Fe^{+3} enhanced NADPH-cytochrome P450 reductase-promoted lipid peroxidation. The effects of adding DPF and superoxide dismutase to this system (Table 6) paralleled those found in the system lacking EDTA-Fe^{+3} (Table 4). Inhibition of the formation of TBAreactive material by superoxide dismutase was essentially complete. Lipid hydroperoxide formation was inhibited 92% by superoxide dismutase. 2,5-diphenylfuran inhibited the formation of TBA-reactive material by 20% while inhibiting LOOH formation by 37%.

The results with NADPH-cytochrome P450 reductase-promoted lipid peroxidation closely parallel those found in the xanthine oxidase system. This is true for the action of both DPF and superoxide dismutase. This suggests that the mechanism of these two systems is closer than previously proposed by Pederson et al. [55], Pederson and Aust [62], King et al. [72] and Kellogg and Fridovich [136].

ADP-Fe^{+2} Promoted Lipid Peroxidation

The two systems previously discussed both show an absolute requirement for the ADP-Fe^{+3} and an enhancement of peroxidation upon the addition of EDTA-Fe^{+3} [55,62]. These iron forms may exist in either the oxidized or reduced state. Addition of the two iron forms in either of these oxidation states showed that ADP-Fe^{+2} alone was capable of promoting

TABLE 5

INHIBITION OF NADPH-CYTOCHROME P450 REDUCTASE PROMOTED LIPID PEROXIDATION BY DPF

Reaction mixtures were as described for Table 3. 0.2 mM DPF was added where indicated. Incubations and assays were performed as in "Methods".

		nmoles MDA $min\ ml^{-1}$	nmoles LOOH $min\ ml^{-1}$
Control		0.03	0.6
	+DPF	0.01	0.5
+NADPH		0.15	1.9
+NADPH	+DPF	0.13	1.5

TABLE 6

INVOLVEMENT OF SUPEROXIDE AND SINGLET OXYGEN IN NADPH-CYTOCHROME P450 REDUCTASE-DEPENDENT LIPID PEROXIDATION

Control mixtures were as described under "Methods". The following additions were made where indicated: 1.7 mM ADP, 0.11 mM EDTA, 0.2 mM $FeCl_3$, 0.1 unit NADPH-cytochrome P450 reductase/ml and 0.2 mM DPF or 1.0 unit SOD/ml as indicated. Incubations and assays were performed as in "Methods".

		nmoles MDA min ml^{-1}	nmoles LOOH min ml^{-1}
Control		0.01	0.1
	+DPF	0.01	0.1
	+SOD	0.01	0.1
+NADPH		2.50	15.0
+NADPH	+DPF	2.00	9.4
+NADPH	+SOD	0.02	1.1

peroxidation in unperoxidized liposomes (Table 7). The results of addition of inhibitors are similar to those seen in the xanthine oxidase and NADPH-cytochrome P450 reductase promoted lipid peroxidation systems (Tables 1-6). Superoxide dismutase inhibits ADP-Fe^{+2}-promoted formation of TBA-reactive material by 70% while DPF inhibits by only 17%. Superoxide dismutase inhibits LOOH formation by 78% and DPF inhibits LOOH formation by 33%.

Lipid Hydroperoxide-Dependent Initiation of Lipid Peroxidation

Lipid hydroperoxides can react with reduced metals, in this case iron, to form products such as lipid free radicals, alkoxy free radicals and lipid hydroperoxy free radicals capable of initiating lipid peroxidation. Oxidized metals react at a much slower rate and their reaction with LOOH is minimal. The results reported in Table 8 show that both ADP-Fe^{+2} and EDTA-Fe^{+2} can promote the LOOH-dependent initiation of peroxidation as detected by malondialdehyde (MDA) formation. However, EDTA-Fe^{+2} appears to be a better promoter since it not only promotes an increase in MDA but also in LOOH, which is both a reactant and product of the reaction. ADP-Fe^{+2}, on the other hand, is a poor promoter of reductive activation as shown by the rapid loss of LOOH. This is in agreement with the findings of Pederson and Aust [55,62] and those reported here that in the peroxidation of extracted microsomal lipid ADP-iron alone is not sufficient for maximal rates of peroxidation. To maximize rates it was found that EDTA-Fe^{+3} must be added. The EDTA-iron evidently functions in the ferrous form to promote the breakdown of LOOH formed by the action of ADP-Fe^{+2}, Table 7. Our findings emphasize the central position of LOOH-dependent initiation reactions in the overall scheme of lipid peroxidation.

TABLE 7

INVOLVEMENT OF SUPEROXIDE AND SINGLET OXYGEN IN THE PROMOTION OF LIPID PEROXIDATION BY CHELATED IRON

Control reaction mixtures were as described under "Methods". The following additions were made where indicated: 1.0 unit SOD/ml, 0.2 mM DPF, 1.7 mM ADP with 0.1 mM $FeCl_3$, 1.7 mM ADP with 0.1 mM $FeCl_2$. Reactions were initiated by addition of the appropriate iron forms. Incubations and assays were performed as described in "Methods".

		nmoles MDA min ml^{-1}	nmoles LOOH min ml^{-1}
Control		0.00	0.0
+DPF		0.00	0.0
+SOD		0.00	0.0
+EDTA-Fe^{+3}		0.00	0.0
+EDTA-Fe^{+2}		0.00	0.0
+ADP-Fe^{+3}		0.00	0.0
+ADP-Fe^{+2}		0.30	1.3
+ADP-Fe^{+2}	+DPF	0.25	0.9
+ADP-Fe^{+2}	+SOD	0.09	0.3

TABLE 8

LIPID HYDROPEROXIDE DEPENDENT INITIATION OF LIPID PEROXIDATION FROM LOOH CATALYZED BY ADP AND EDTA CHELATED IRON

Lipid hydroperoxides were generated as described under "Methods". Incubation mixtures contained 0.1 mole lipid hydroperoxide/ml in 0.05 M Tris-Cl pH 7.5 at 37°C (1.02 mole lipid hydroperoxides/ mole lipid phosphate). 0.1 mM Fe^{+2} was added as indicated either as ADP-Fe^{+2} (17:1 mole ratio) or EDTA-Fe^{+2} (1.1:1 mole ratio). Reactions were initated by addition of the appropriate iron forms. Lipid peroxidation was determined as in "Methods".

	nmoles MDA $min\ ml^{-1}$	*nmoles LOOH $min\ ml^{-1}$
Partially peroxidized liposomes		
No additions	0.02	+ 6.6
+ADP-Fe^{+2}	1.56	-18.8
+EDTA-Fe^{+2}	1.16	+ 6.4

*Initial rates of increase (+) or decrease (-) of LOOH during incubation

It has been suggested that $^1O_2$ may also be a product of hydroperoxide breakdown [143]. Singlet oxygen so produced could function in the reactions of lipid peroxidation by addition to lipid diene bonds producing LOOH. The results reported in Table 9 support the concept that $^1O_2$ is a product of LOOH decomposition and is an intermediate in the reactions of lipid peroxidation. Using EDTA-Fe^{+2} which has been shown to be an efficient promoter of LOOH-dependent initiation of lipid peroxidation (Table 8) but incapable of LOOH-independent initiation of lipid peroxidation (Table 7) the participation of $^1O_2$ in the ferrous complex promoted breakdown of LOOH was investigated using the $^1O_2$ trapping agent DPF. Lipid hydroperoxide-dependent initiation, as determined by formation of TBA-reactive material, was inhibited 20% by DPF. In the presence of DPF, LOOH formation was inhibited and a loss of LOOH was observed. It is apparent that $^1O_2$ is produced and reacts with PUFA. Addition of superoxide dismutase to the same EDTA-Fe^{+2}-LOOH mixture had no effect on the formation TBA-reactive material but did inhibit LOOH formation to the point that a loss in LOOH content was observed. Thus $O_2^{\cdot}$ may participate in these reactions but to a lesser extent than does $^1O_2$.

The nature of the ADP-Fe^{+2} complex was examined by investigating the inhibition of its restricted abilities to promote LOOH-dependent initiation by DPF and SOD, Table 9. This is a more difficult situation to analyze since ADP-Fe^{+2} can promote LOOH-independent as well as LOOH-dependent initiation. DPF does not inhibit LOOH-independent initiation but does inhibit LOOH-dependent initiation to some extent as evidenced by a decrease in LOOH content. Addition of superoxide dismutase to the incubation mixture which will inhibit LOOH-independent initiation by

TABLE 9

INVOLVEMENT OF SUPEROXIDE AND SINGLET OXYGEN IN THE PROMOTION OF LIPID PEROXIDATION IN PARTIALLY PEROXIDIZED LIPOSOMES

Incubation mixtures were as described in the legend to Table 8 with the following additions where indicated: 0.2 mM DPF or 1.0 unit superoxide dismutase/ml. Reactions were initiated by addition of the appropriate iron form. Lipid peroxidation was determined as in "Methods".

		nmoles MDA $min\ ml^{-1}$	*nmoles LOOH $min\ ml^{-1}$
Partially peroxidized liposomes:			
no additions		0.02	+ 6.6
+DPF		0.04	- 7.0
+SOD		0.02	+ 2.4
+EDTA-Fe^{+2}		1.16	+ 6.4
+EDTA-Fe^{+2}	+DPF	0.96	- 4.8
+EDTA-Fe^{+2}	+SOD	1.20	- 5.0
+ADP-Fe^{+2}		1.56	-18.8
+ADP-Fe^{+2}	+DPF	1.60	-28.0
+ADP-Fe^{+2}	+SOD	1.24	-13.2

*Initial rates of increase (+) or decrease (-) of LOOH during incubation.

ADP-Fe^{+2} gives a true indication of the LOOH-dependent initiating abilities of ADP-Fe^{+2}. The addition of superoxide dismutase clearly shows that ADP-Fe^{+2} is a less efficient promoter of LOOH-dependent initiation than is EDTA-Fe^{+2}. The results also show that the limited LOOH-dependent initiation of peroxidation promoted by ADP-Fe^{+2} is not inhibited by superoxide dismutase. Thus the active agent in LOOH-dependent initiation promted initiation by ADP-Fe^{+2} must be significantly different from that involved in LOOH-independent initiation.

DISCUSSION

Lipid peroxidation as determined by TBA-reactive material and LOOH may be divided into two sequential parts. Such a division was first proposed by Tam and McCay [52] as the result of time course studies in microsomal lipid peroxidation. They observed a precursor-product relationship between LOOH and the TBA-reactive material produced during peroxidation. We have been able to define and separate LOOH-independent and LOOH-dependent initiation of lipid peroxidation. Lipid hydroperoxide independent initiation is the generation of LOOH accompanied by low levels of TBA-reactive material. The rate of formation of TBA-reactive material is lower than usually associated with enzymatic peroxidation, while LOOH formation may be quite rapid. Lipid hydroperoxide-dependent initiation is the breakdown of the initially formed LOOH into reactive intermediates capable of continuing the free-radical reactions of lipid peroxidation. Lipid hydroperoxide-independent initiation of peroxidation, as detected by LOOH formation, can be promoted by ADP-Fe^{+2}, by either NADPH-cytochrome P450 reductase or xanthine oxidase activity in the presence of ADP-Fe^{+3} or by soybean lipoxygenase as demonstrated in this paper. Lipid hydroperoxides so

formed may be used in the examination of LOOH-dependent initiation of lipid peroxidation. Lipid hydroperoxide-dependent initiation of lipid peroxidation promoted by EDTA-Fe^{+2}, which is incapable of LOOH-independent initiation (Table 7), was demonstrated in partially peroxidized lipids. EDTA-Fe^{+2} caused the rapid formation of TBA-reactive material from hydroperoxides while at the same time promoting the formation of LOOH, Table 8. The separation of LOOH-independent and LOOH-dependent initiation of lipid peroxidation allows a more definitive investigation into the mechanism of lipid peroxidation.

Superoxide dismutase inhibits the formation of TBA-reactive material by xanthine oxidase promoted LOOH-independent initiation by greater than 90% while inhibiting the formation of LOOH by more than 75% (Table 1). 2,5-Diphenylfuran, on the other hand, inhibits by only 13% and 32% respectively. Lipid hydroperoxide-independent initiation of lipid peroxidation promoted by xanthine oxidase must therefore be dependent upon a superoxide dismutase-sensitive intermediate. The xanthine oxidase system demonstrates an absolute requirement for ADP-Fe^{+2} [62]. Addition of EDTA-Fe^{+3} does not change the system's dependence upon $O_2^{\cdot}$, ADP-Fe^{+3} or its sensitivity to superoxide dismutase (Table 2). Singlet oxygen does not appear to be involved in LOOH-independent initiation. However, it is formed during active lipid peroxidation [72] and may result in some further peroxidation as evidenced by DPF inhibition.

Lipid peroxidation promoted by NADPH-cytochrome P450 reductase also exhibits an absolute requirement for ADP-Fe^{+3} [58]. The inhibitory effects of superoxide dismutase and DPF on the NADPH-cytochrome P450 reductase-promotion of LOOH-independent initiation of lipid peroxidation (Tables 4 and 5) were similar to those observed for xanthine oxidase-

promoted LOOH-independent initiation lipid peroxidation. Again, addition of EDTA-Fe^{+3} enhances peroxidation without changing the characteristics of the system (Table 6). This demonstrates that LOOH-independent initiation occurs through a superoxide dismutase-sensitive reaction and that $^1O_2$ is not involved in LOOH-independent initiation. The requirement for ADP-Fe^{+3} and the effects of superoxide dismutase and DPF form a common denominator between the two systems. ADP-Fe^{+3} appears to function in like manner in both systems, even though the enzymes are quite different. Xanthine oxidase produces $O_2^{\cdot}$ and NADPH-cytochrome P450 reductase participates in electron transport. Both systems are capable of the reduction of ADP-Fe^{+3} and it was shown that ADP-Fe^{+2} can promote LOOH-independent initiation lipid peroxidation, Table 7. Investigation of peroxidation promoted by ADP-Fe^{+2} showed that it closely parallels that promoted by both xanthine oxidase and NADPH-cytochrome P450 reductase. Inhibition by superoxide dismutase was nearly complete while DPF exhibited only partial inhibition.

The LOOH-independent initiation mechanism appears to be common for these three systems and is dependent upon a superoxide dismutase-sensitive intermediate. The ability of ADP-Fe^{+2} alone to promote LOOH-independent initiation of lipid peroxidation indicates its direct participation in the initiation reaction. It has been proposed that LOOH-independent initiation of lipid peroxidation in the NADPH-cytochrome P450 reductase system occurs through an ADP-perferryl ion, ADP-Fe^{+2}-O_2 [58]. The perferryl ion complex is generated by the reduction of ADP-Fe^{+3} to ADP-Fe^{+2} by NADPH-cytochrome P450 reductase and subsequent interaction with molecular oxygen.

The same initiation complex may be involved in xanthine oxidase-promoted LOOH-independent initiation. It was shown that $^1O_2$ is not involved in the LOOH-independent initiation of peroxidation, Table 1. The requirement for ADP-iron in xanthine oxidase-dependent lipid peroxidation [62] and the fact that O_2^- cannot directly react with polyunsaturated fatty acids leads to the proposal that the active initiation complex is formed by the reaction of $O_2^{\cdot-}$ with ADP-Fe^{+3}. The interaction of $O_2^{\cdot-}$ with ADP-Fe^{+3} perhaps gives rise to the ADP-perferryl ion, the proposed promoter of LOOH-independent initation in NADPH-dependent peroxidation. The initiation complex could be produced via $O_2^{\cdot-}$ during xanthine oxidation by xanthine oxidase by the scheme:

$$O_2^{\cdot-} + \text{ADP-Fe}^{+3} \qquad (\text{ADP-Fe}^{+3}\text{-}O_2^{\cdot-}) \qquad (\text{ADP-Fe}^{+2}\text{-}O_2) \qquad (32)$$

This initiation complex would account for the requirement for ADP-Fe^{+3} in the xanthine oxidase and the NADPH-cytochrome P450 reductase systems and for the sensitivity of both to superoxide-dismutase. Dismutase activity with iron bound $O_2^{\cdot-}$ has been proposed by Richter et al. [144].

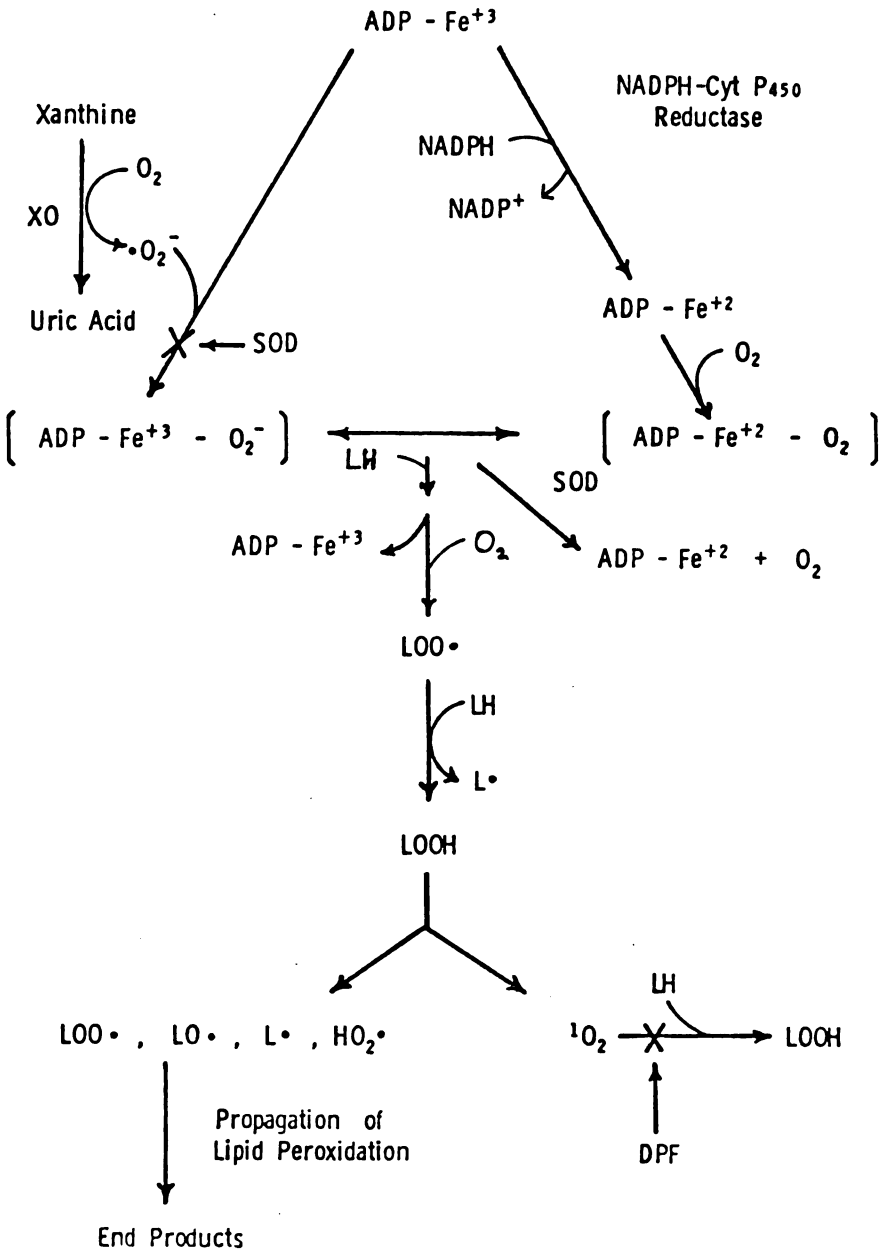
Singlet oxygen is not involved in the LOOH-independent initiation of lipid peroxidation but is likely involved in the LOOH-dependent initiation of lipid peroxidation based on the inhibition of peroxidation by DPF. During LOOH-dependent initiation $^1O_2$ may be produced by the breakdown of the initially formed lipid hydroperoxides. Howco et al. [143] have reported that the breakdown of linoleic acid hydroperoxides by hemeproteins involves the production of $^1O_2$ in substantial quantities. Singlet oxygen is produced in the breakdown of LOOH as evidenced by the decreased formation of TBA-reactive material from partially peroxidized lipids in the presence of DPF (Table 9). For EDTA-Fe^{+2}, which is only

capable of promoting LOOH-dependent initiation, production of TBA-reactive material from partially peroxidized lipids was inhibited by 20% in the presence of DPF. Lipid hydroperoxide-dependent initiation of lipid peroxidation promoted by EDTA-Fe^{+2} was also slightly inhibited by superoxide dismutase as evidenced by a decrease in LOOH when superoxide dismutase is added. This indicates that $O_2^{\cdot}$ may play some role in LOOH-dependent initiation. That ADP-Fe^{+2} can promote LOOH-independent initiation as well as LOOH-dependent initiation is evidenced by the difference in the formation of TBA-reactive material from lipid hydroperoxides in the presence of superoxide dismutase or DPF (Table 9). The presence of DPF, which does not inhibit LOOH-independent initiation, does not affect the production of TBA-reactive material. Superoxide dismutase does inhibit LOOH-independent initiation and allows the determination of the LOOH-dependent initiation promoting abilities of ADP-Fe^{+2} (Table 9). This suggests that inhibition of LOOH-independent initiation by superoxide dismutase occurs by the breakdown of the initiation complex (the ADP-perferryl ion), leaving ADP-Fe^{+2} which can promote LOOH-dependent initiation.

These findings are summarized in Figure 2. Lipid hydroperoxide independent initiation takes place through a common initiation complex, the ADP-perferryl ion. The initiation complex may be producing by complexing $O_2^{\cdot}$ with ADP-Fe^{+3} or by the reduction of ADP-Fe^{+3} to ADP-Fe^{+2} and subsequent interaction with oxygen. The initiation complex produces low levels of lipid hydroperoxides and some TBA-reactive material upon reaction with unsaturated lipid. The initiation complex is sensitive to superoxide dismutase but not DPF. Lipid hydroperoxide-dependent

initiation involves the breakdown of the initial hydroperoxides into reactive intermediates of peroxidation. Since $^1O_2$ is only one of several intermediates less than 50% inhibition by singlet oxygen trapping agents is to be expected.

Figure 2. NADPH-Dependent and $O_2^{\cdot-}$-Dependent Promotion of Peroxidation of Extracted Microsomal Lipid Vesicles.


ADP - Fe+3
NADPH-Cyt P450 Reductase
Xanthine
NADPH
XO
O2
NADP+
•O2-
Uric Acid
ADP - Fe+2
SOD
O2
[ADP - Fe+3 - O2-]
[ADP - Fe+2 - O2]
LH
SOD
ADP - Fe+3
O2
ADP - Fe+2 + O2
LOO•
LH
L•
LOOH
LOO• , LO• , L• , HO2•
1O2
LH
LOOH
Propagation of Lipid Peroxidation
DPF
End Products

CHAPTER 2

HYDROXYL RADICAL-DEPENDENT INITIATION OF LIPID PEROXIDATION

SUMMARY

Fenton's reagent, aqueous ferrous ion and H_2O_2, can promote the peroxidation of liposomes prepared from extracted microsomal lipid. Conditions for optimal rates of peroxidation were found to be an iron to H_2O_2 ratio of 2 to 1 and a reaction mixture pH of 7. Lipid peroxidation promoted by the Fenton's reagent was inhibited by mannitol, benzoate and thiourea. However, the observed inhibition may not directly demonstrate the participation of the hydroxyl radical in the promotion of lipid peroxidation since the addition of several buffers and metal chelators inhibited Fenton's reagent promoted lipid peroxidation to a similar extent. It appears that HO•-dependent initiation occurs only under well defined reaction conditions where there are no metal chelators or other complexeing substances.

INTRODUCTION

The hydroxyl radical has been proposed to initiate lipid peroxidation by the abstraction of methylene hydrogen from polyunsaturated fatty acids. The involvement of HO• in the initiation of enzyme-promoted lipid peroxidation has been suggested by several researchers. McCay et al. [39,48,49,52,72] and Piette et al. [56,57,76] have proposed that HO• initiates NADPH-dependent superoxide ($O_2^{\bullet-}$) mediated peroxidation of microsomal lipid. Fridovich et al. [136,145] have also proposed that HO• is involved in the initiation of $O_2^{\bullet-}$-dependent lipid peroxidation promoted by xanthine oxidase. Proposals that HO• is involved in lipid peroxidation is usually supported by data indicating that peroxidation

involves the sequential generation of $O_2^{\cdot}$, H_2O_2 and HO•. The sequence of intermediate generation has been demonstrated by the ability of HO• traps, catalase and superoxide dismutase to sequentially inhibit lipid peroxidation.

There are two reaction mechanisms that are generally proposed as the means of HO• generation in biological systems. First, Fridovich et al. [136,145] have proposed that HO• is generated by an uncatalyzed Haber-Weiss reaction.

$$2OH_2^{\cdot} + 2H^+ \longrightarrow H_2O_2 \qquad (9)$$

$$O_2^{\cdot} + H_2O_2 \longrightarrow OH^- + O_2 + HO\cdot \qquad (12)$$

However, current research indicates that the uncatalyzed Haber-Weiss reaction does not occur to any significant extent under biological conditions [66-71]. The second and perhaps the more feasible mechanism for the formation of HO• in a biological system is a reaction mechanism that may best be described as an iron-promoted Haber-Weiss reaction proposed by McCay et al. [39,48,49,52,72] and Piette et al. [56,57,76] for the HO• initiation of lipid peroxidation.

$$O_2^{\cdot} + Fe^{+3} \longrightarrow O_2 + Fe^{+2} \qquad (8)$$

$$2O_2^{\cdot} + 2H^+ \longrightarrow H_2O_2 + O_2 \qquad (9)$$

$$Fe^{+2} + H_2O_2 \longrightarrow Fe^{+3} + OH^- + HO\cdot \qquad (10)$$

Both free iron and chelated iron have been proposed to promote the Haber-Weiss reaction.

Recent experimental work by Walling et al. [114] and Groves et al. [115-117] has yielded some valuable information about the course of the reaction as written above. Groves et al. and Walling et al. question the existence of the free HO• when generated by iron-promoted H_2O_2 breakdown. These authors have shown that the free HO• only exists

under acid conditions and in "pure" solution. When the pH of the solution is raised to physiological pH or the solution deviates from pure water the free HO• is no longer present. Instead the oxidative moiety formed in ferrous ion promoted H_2O_2 breakdown appears to be a ferric ion hydroxyl radical complex. This ferric ion hydroxyl radical complex has been termed the ferryl ion based on the formal oxidation state of the iron (Fe^{+4}). The ferryl ion is a powerful oxidant and the reactivity attributed to the HO• can also be attributed to the ferryl ion. The presence of the ferryl ion is indicated by data showing directive effects observed during ring hydroxylation. These directive effects are the antithesis of free HO• reactions. Thus in a biochemical system these authors would favor a proposal that initiation occurs via the ferryl ion. Indeed the initiation of oxidative free radical reactions by the ferryl ion has been proposed for many well known reactions [87,88]. The ferryl ion can also be formed from the perferryl ion as outlined below in the scheme for the autoxidation of ferrous ion [23,24, 122,123].

$$Fe^{+2} + O_2 \longrightarrow Fe^{+2}O_2 \qquad (22)$$

$$Fe^{+2}O_2 + Fe^{+2} \longrightarrow Fe^{+2}O_2Fe^{+2} \qquad (33)$$

$$Fe^{+2}O_2Fe^{+2} \longrightarrow 2Fe^{+2}O \qquad (34)$$

Considering that Aust et al. [75] have proposed that both NADPH-dependent and O_2^--dependent lipid peroxidation are initiated by the perferryl ion and not HO•, equations 22,33,34 raise an interesting paradox. Are the perferryl ion, the ferryl ion and the HO• part of the same continuem of reactive intermediates and as such interchangeable. If this is so, then is experimental data supportive of one not supportive of all. Also, if the data is supportive of only one of the intermediates may not such

support be reflective of the experimental viewpoint rather than actual experimental fact.

In this chapter the ability of Fenton's Reagent to promote the initiation of lipid peroxidation and the effect of varying experimental parameters such as concentration of reactants and chelation of iron on lipid peroxidation will be examined.

MATERIALS AND METHODS

Chemicals: Catalase, Type I, was purchased as a lyopholized powder from Sigma Chemical Company. As a lyopholized powder the catalase contained no antioxidant preservations and required no further purification. Chelex 100, a cationic exchange resin, was purchased from Pharmacia Chemical Company. All other chemicals used were of analytical grade and were used without further purification.

Microsomal lipid and liposomes: Total extraction of microsomal lipids was carried out by the method of Folch et al. [97]. Procedural precautions taken to minimize autoxidation of the lipid during handling and storage have been previously described [27]. Liposomes were prepared as described previously [55]. Aqueous solutions were prepared using distilled-deionized water that had been passed over a Chelex 100 column (50 x 2.5 cm). Distilled-deionized water was prepared from distilled water by passing it through a mixed-bed ion exchange resin (Boeringher Company). All buffer solutions were passed over a Chelex column a second time after pH adjustment. The pH of aqueous and buffered solutions was adjusted using 1M HCl and NaOH solutions that were passed over a Chelex 100 column after preparation. All aqueous solutions were shown to be free of contaminating iron [146].

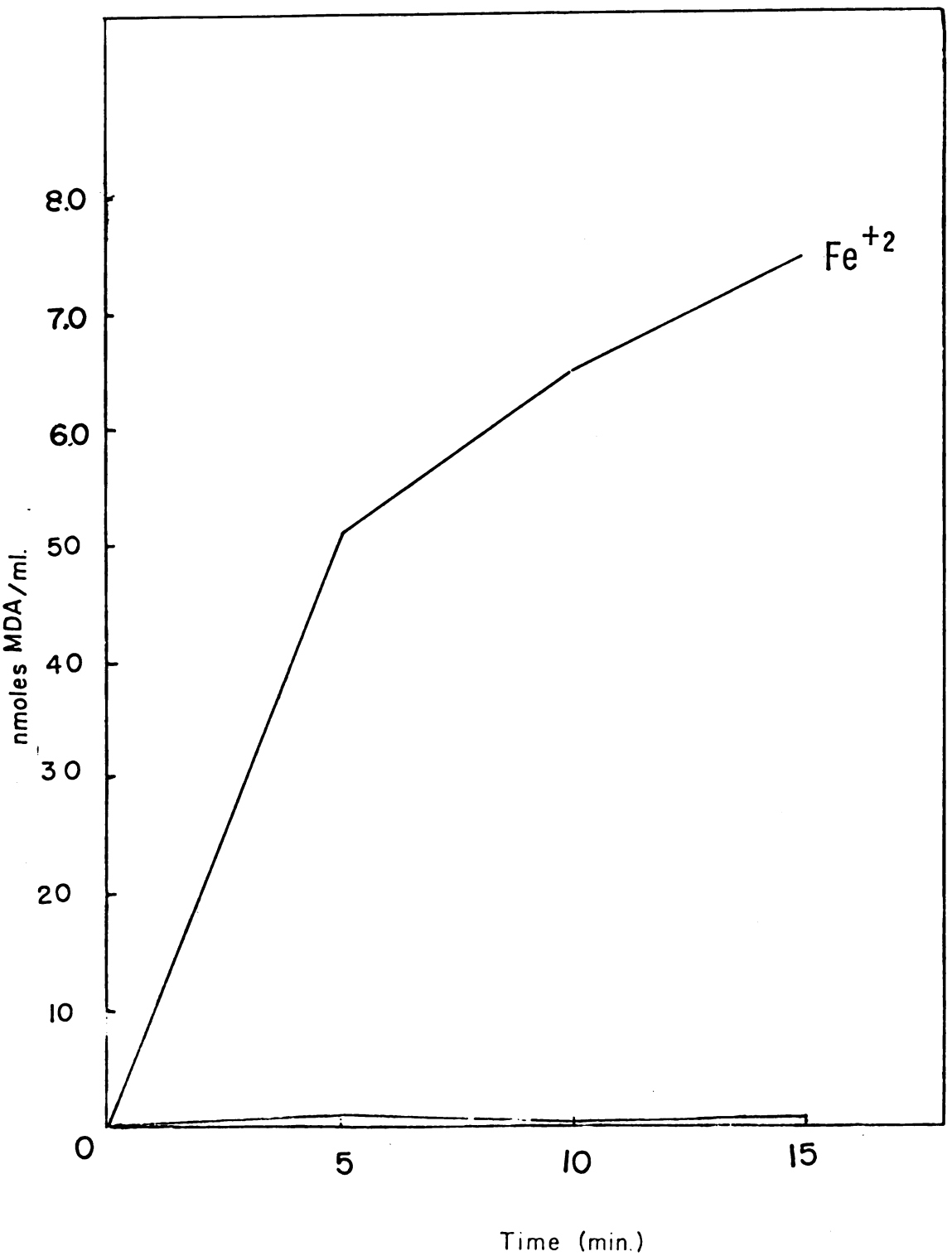
Reaction mixtures in which lipid peroxidation was promoted by Fenton's reagent contained 1.0 μmole lipid phosphate per ml (in the form of liposomes) and H_2O_2 and $FeCl_2$ in the concentrations indicated in the appropriate figures and tables. Reaction mixtures were prepared using chelexed-distilled deionized 0.3 M NaCl at the pH indicated in the appropriate figures and tables. Reactions were initiated by the addition of $FeCl_2$. Other additions to the reaction mixtures were as indicated in the figures and tables. Incubations were carried out at 37°C under an air atmosphere in a Dubnoff metabolic shaking bath. Lipid peroxidation was measured by the formation of TBA-reactive material, malondialdehyde (MDA), and lipid hydroperoxides by the method of Buege and Aust [33]. Rates of MDA and lipid hydroperoxide formation reported are initial rates.

Other methods: ADP and EDTA-chelated ferric ion solutions were prepared in chelexed-distilled deionized water. The molar ratios of the chelator to ferric ion are indicated in the appropriate tables. The pH of the iron complex solutions was adjusted to pH 7.5 by methods described for aqueous solutions above. Ferrous ion solutions, whether free or chelexed, were prepared as above except the chelexed-distilled deionized water was degassed and purged with argon prior to use. This method of preparation minimized ferrous ion autoxidation prior to addition to an air saturated reaction mixture. Total lipid phosphate was measured by the method of Bartlett et al. [141].

RESULTS

Fenton's reagent can promote the peroxidation of liposomes prepared from extracted microsomal lipid (Fig. 3). The rate of peroxidation as measured by MDA formation is greater than that observed for either

Figure 3. Time Course of Fenton's Reagent-Promoted Lipid Peroxidation - Reaction mixtures contained 1.0 μmole phosphate per ml, 0.1 mM H_2O_2 and 0.2 mM $FeCl_2$ in 0.3 M NaCl, pH 7.0. Incubations and assays were performed as described under "Methods."


8.0
7.0
6.0
50
40
30
20
10
0
nmoles MDA/ml.
Fe^{+2}
5
10
15
Time (min.)

NADPH-dependent or O_2^--dependent peroxidation of liposomes [27,55,63]. The rate of MDA formation is 4.45 nmoles/min ml^{-1} as compared to 3.5 nmoles/min ml^{-1} for the two enzymatically-promoted reaction mixtures. The rate of MDA formation is linear up to 5 minutes after initation by the addition of $FeCl_2$.

The effect of varying H_2O_2 to $FeCl_2$ concentration ratios on the promotion of lipid peroxidation by Fenton's reagent is shown in Fig. 4. The rate of peroxidation demonstrates a sharp maximum at a H_2O_2 to $FeCl_2$ ratio of 1:2.

The effect of H_2O_2 concentration on the Fenton's Reagent promotion of lipid peroxidation is shown in Fig. 5. The maximum rate of peroxidation occurs with a H_2O_2 concentration of 0.1 mM. Taken together with the data from Fig. 4 this would fix the concentration of $FeCl_2$ at which maximum peroxidation occurs at 0.2 mM.

The pH profile for Fenton's reagent promotion of lipid peroxidation is presented in Fig. 6 (Figure values have been corrected for rates of MDA formation in the absence of H_2O_2). As can be seen from the data the reaction demonstrates a relatively sharp pH maxima at 7.0.

The data presented in Figs. 3-6 define the basic reaction mixture used for the rest of the experimentation. Reaction mixtures containing 1.0 µmole lipid phosphate per ml, 0.1 mM H_2O_2 and 0.2 mM $FeCl_2$ in 0.3 M NaCl, pH 7.0 gave the maximal rate of Fenton's reagent promotion of lipid peroxidation.

The ability of accepted HO• trapping agents to inhibit Fenton's Reagent promotion of lipid peroxidation is shown in Table 10. None of the HO• trapping agents inhibits peroxidation in a linear concentration

Figure 4. The Dependence of Fenton's Reagent-Promoted Lipid Peroxidation on H_2O_2 Concentration - Reaction mixtures contained 1.0 μmole lipid phosphate per ml and 0.2 mM $FeCl_2$ in 0.3 NaCl, pH 7.0. The concentration of H_2O_2 in the reaction mixtures was as indicated in the figure. Incubations and assays were performed as described under "Methods."

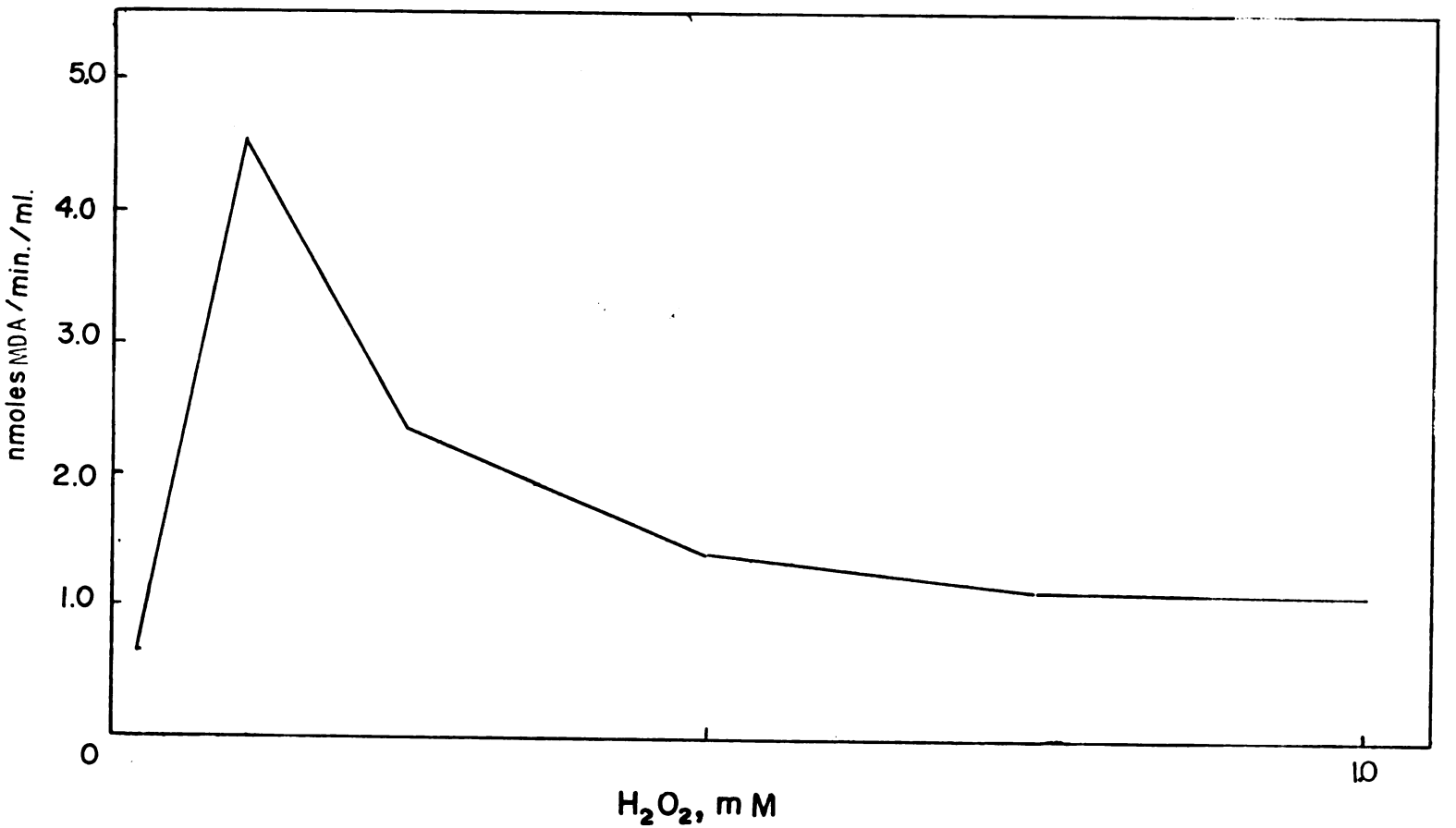

5.0
4.0
3.0
2.0
1.0
0
nmoles MDA / min. / ml.
1.0
H_2O_2, m M

Figure 5. The Effect of H_2O_2 to Ferrous Ion Concentration on Fenton's Reagent-Promoted Lipid Peroxidation - Reaction mixtures contained 1.0 μmole lipid phosphate per ml, 0.2 mM $FeCl_2$ and the appropriate H_2O_2 concentration as indicated in the figure in 0.3 M NaCl, pH 7.0. Incubations and assays were performed as described under "Methods."

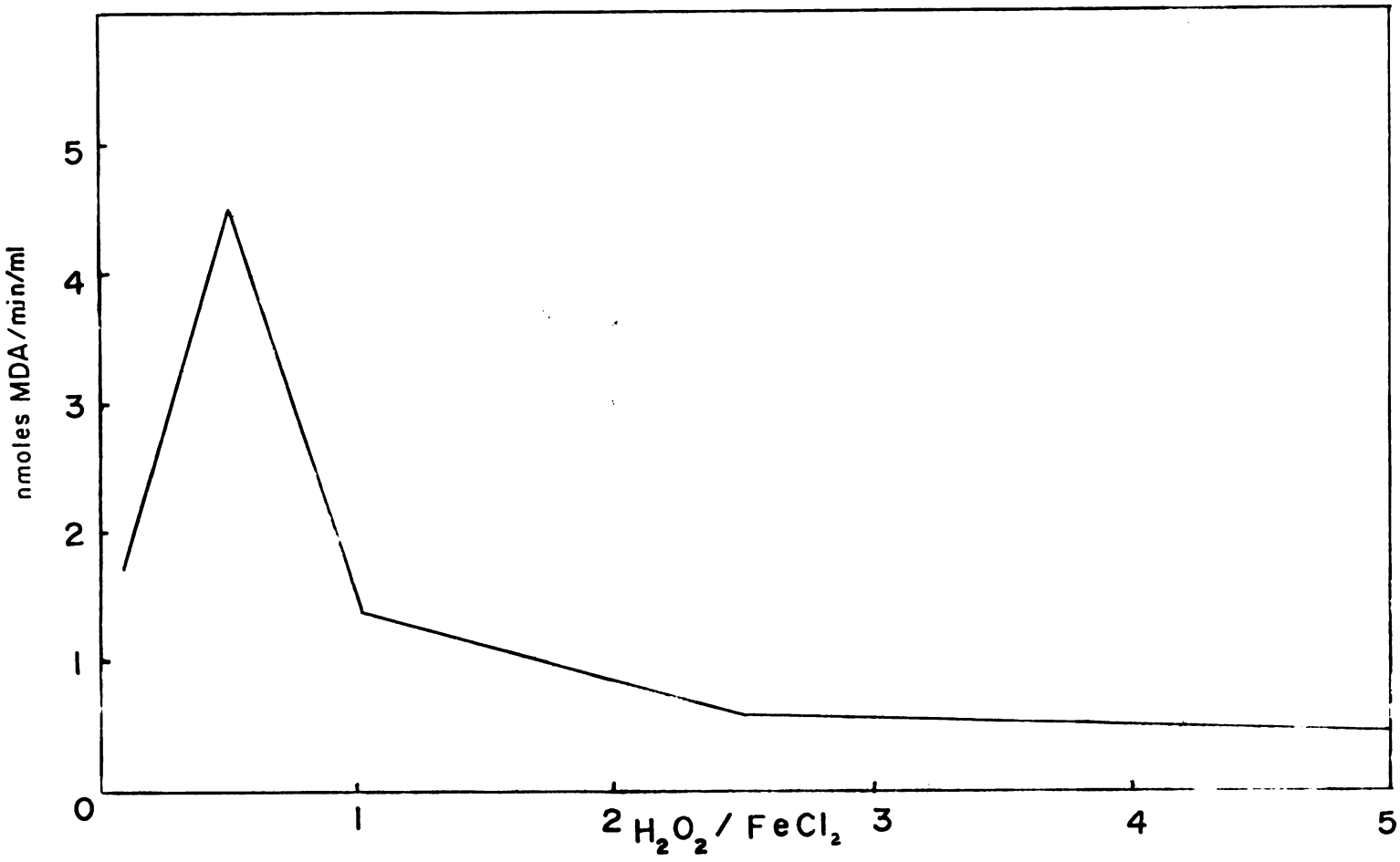

nmoles MDA/min/ml
0
1
2
3
4
5
2 H_2O_2 / $FeCl_2$ 3
4
5

Figure 6. The pH Profile of Fenton's Reagent-Promoted Lipid Peroxidation - Reaction mixtures contained 1.0 μmole lipid phosphate per ml, 0.1 mM H_2O_2 and 0.2 mM $FeCl_2$ in 0.3 M NaCl. The pH of the NaCl was as indicated in the figure. Incubations and assays were performed as described under "Methods."

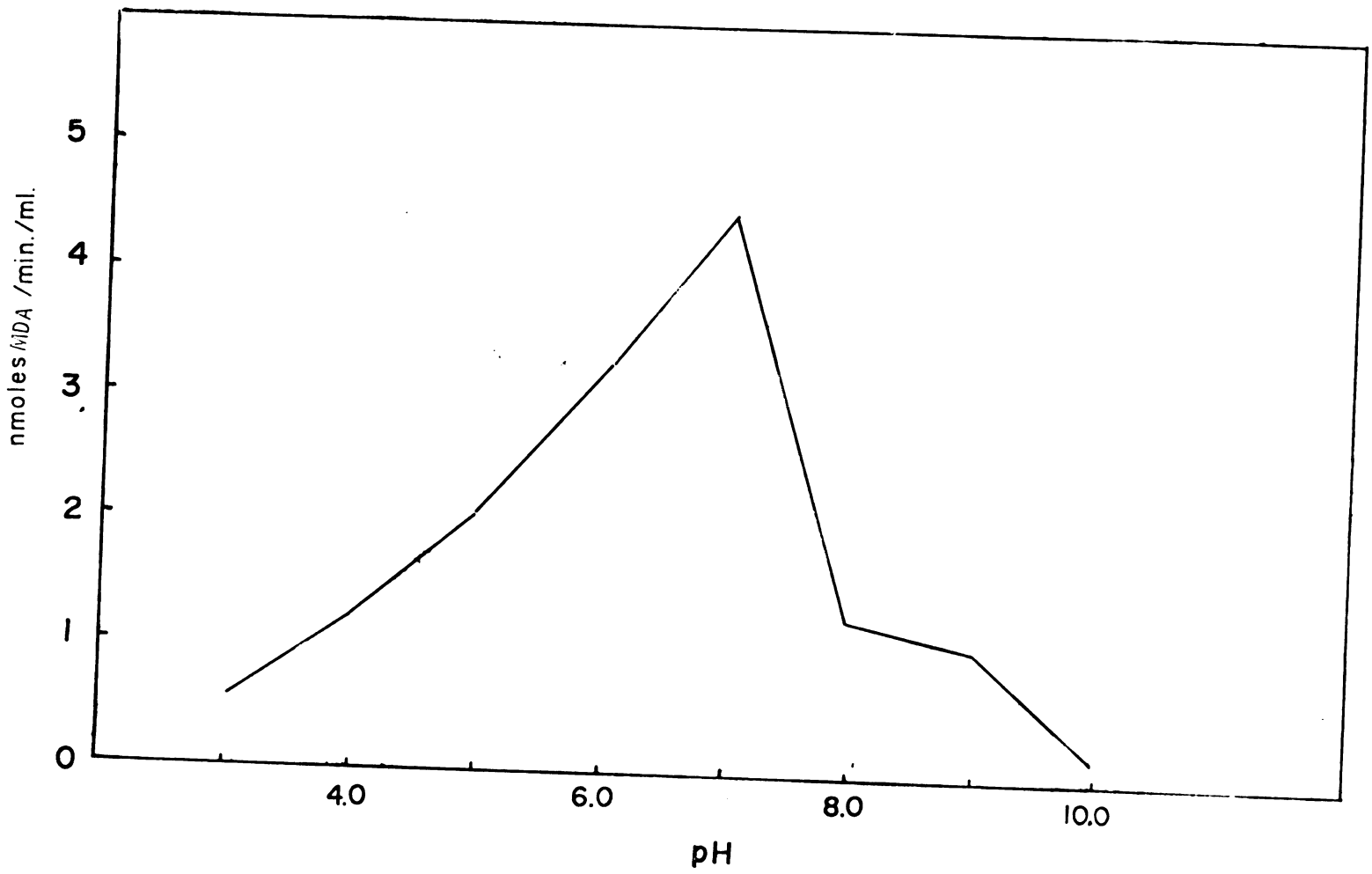

nmoles NIDA /min./ml.
5
4
3
2
1
0
4.0
6.0
8.0
10.0
pH

TABLE 10

THE EFFECT OF HO• TRAPPING AGENTS AND CATALASE ON FENTON'S REAGENT-PROMOTED LIPID PEROXIDATION

Reaction mixtures contained 1.0 μmole lipid phosphate per ml, 0.1 mM H_2O_2 and 0.2 mM $FeCl_2$ in 0.3 M NaCl, pH 7.0. Additions to and deletions from the reaction mixtures were made as indicated in the table. Incubations and assays were performed as described under "Methods."

Reaction Mixtures		nmol MDA $min\ ml^{-1}$
Control		3.11
-$FeCl_2$		0.01
+Benzoate	0.1 mM	3.11
	1.0 mM	3.11
	10.0 mM	0.43
+Mannitol	0.1 mM	3.29
	1.0 mM	3.14
	10.0 mM	0.14
+Thiourea	0.1 mM	3.09
	1.0 mM	1.07
	10.0 mM	0.12
+Catalase	1.0 unit/ml	0.14

dependent manner. Two of the HO• trapping agents, benzoate and mannitol appear to exhibit a threshold of inhibition. At 0.1 mM or 1.0 mM neither compound inhibits peroxidation. However upon increasing the HO• trapping agent concentration to 10.0 mM benzoate and mannitol inhibit Fenton's reagent-promoted lipid peroxidation by 87% and 94% respectively. Thiourea was a more efficient inhibitor of Fenton's reagent-promoted lipid peroxidation. Thiourea inhibited peroxidation by 0.6%, 66% and 96% respecively at concentrations Of 0.1 mM, 1.0 mM and 10.0 mM. Since thiourea also inhibited ascorbate ferric iron promoted lipid peroxidation to similar extents, the specificity of thiourea for inhibiting HO• dependent reactions is perhaps less than formerly believed.

The addition of 1.0 unit catalase per ml or the replacement of $FeCl_2$ by $FeCl_3$ resulted in a 96% decrease on the rate of peroxidation (Table 10). Thus the requirement for both H_2O_2 and Fe^{+2} is apparent.

The effect of chelation of ferrous ion by phosphate buffer or by specific metal chelators on the rate of Fenton's reagent promotion of lipid peroxidation is shown in Table 11. The variable effects observed upon addition of 10.0 mM mannitol to the various reaction mixtures indicates that the mechanism of promotion may be changing as the metal is chelated. The addition of 10 mM mannitol to a reaction mixture containing uncomplexed ferrous ion results in complete inhibition of the promotion of lipid peroxidation. The addition of sodium phosphate buffer at pH 7.0, conditions under which the iron will be chelated by the buffer, resulted inhibition of the rate of lipid peroxidation. Phosphate buffer at pH 7.0 inhibited peroxidation by 42%, 72% and 99% at 1.0 mM, 10.0 mM and 50.0 mM phosphate concentration respectively.

TABLE 11

EFFECT OF CHELATION ON FENTON'S REAGENT-PROMOTED LIPID PEROXIDATION

Reaction mixtures contained 1.0 μmole lipid phosphate per ml and 0.1 mM H_2O_2 in 0.3 M NaCl, pH 7.0. Additions to the reaction mixtures were as indicated in the table. Incubations and assays were performed as described under "Methods."

Reaction Mixtures	nmol MDA min ml^{-1}
Control	0.01
+0.2 mM $FeCl_2$	3.38
+0.2 mM $FeCl_2$ + 10 mM mannitol	0.01
+0.2 mM $FeCl_2$ + 1 mM sodium phosphate, pH 7.0	1.95
+0.2 mM $FeCl_2$ + 10 mM sodium phosphate, pH 7.0	0.95
+0.2 mM $FeCl_2$ + 50 mM sodium phosphate, pH 7.0	0.02
+0.2 mM $FeCl_2$ + 50 mM sodium phosphate, pH 3.5	1.66
+0.2 mM $FeCl_2$ + 1 mM sodium phosphate, pH 2.14	3.25
+0.2 mM $FeCl_2$ + 1 mM sodium phosphate + 10 mM mannitol, pH 2.14	1.81
+0.22 mM EDTA-0.2 mM $FeCl_2$	0.00
+0.44 mM EDTA-0.2 mM $FeCl_2$	0.00
+0.4 mM ADP-0.2 mM $FeCl_2$	2.43
+0.4 mM ADP-0.2 mM $FeCl_2$ + 10 mM mannitol	0.01
+0.8 mM ADP-0.2 mM $FeCl_2$	1.91
+0.8 mM ADP-0.2 mM $FeCl_2$ + 10 mM mannitol	1.91
+1.2 mM ADP-0.2 mM $FeCl_2$	1.47
+1.6 mM ADP-0.2 mM $FeCl_2$ + 10 mM mannitol	1.27
+3.4 mM ADP-0.2 mM $FeCl_2$	1.23
+3.4 mM ADP-0.2 mM $FeCl_2$ + 10 mM mannitol	1.04

However, inhibition decreased with decreasing pH. At a decreased pH the chelation of ferrous ion should be lessened. At pH 3.5 50 mM phosphate buffer inhibited peroxidation by only 51% as compared to 99% at pH 7.0. At pH 2.14, 1.0 mM phosphate buffer inhibited peroxidation by only 4% as compared to 42% at pH 7.0. Thus it appears that pH can effect the nature of phosphate chelation and thus the mechanism of Fenton's reagent promotion of lipid peroxidation. The change in mechanism may also be reflected in the decreasing ability of mannitol to inhibit peroxidation at lower pHs. At pH 2.14, 10 mM mannitol inhibits Fenton's reagent promotion of lipid peroxidation by only 46%. Chelation of ferrous ion by the strong metal chelator EDTA completely inhibited Fenton's reagent promotion of lipid peroxidation at pH 7.0.

Chelation of ferrous ion by ADP, a weaker ferrous ion chelator than EDTA, gave a graded inhibition response. Fenton's reagent-promoted lipid peroxidation was inhibited by 28%, 47%, 56% and 64% when ferrous ion was chelated by ADP in a 2-fold, 4-fold, 8-fold and 17-fold excess of ADP to iron respectively. Thus as chelate concentration, and perhaps extent of chelation, increases Fenton's reagent promotion of lipid peroxidation decreases. Chelation of ferrous ion by ADP at pH 7.0 also appears to drastically change the mechanism of promotion of lipid peroxidation. The addition of 10 mM mannitol to the reaction mixture containing ADP to iron in a 2-fold excess of ADP results in the complete inhibition of lipid peroxidation. However, mannitol is completely uneffective at inhibiting peroxidation promoted by ADP-ferrous complex at other ADP to iron ratios. Thus it would appear that the promotion of peroxidation is no longer the same as that observed in the presence of uncompleted ferrous ion.

DISCUSSION

Fenton's reagent, aqueous H_2O_2 and ferrous ion, was found capable of promoting rapid peroxidation of liposomes prepared from lipid extracted from rat hepatic microsomes. The reaction proceded under conditions that nominally reflected biological conditions. Conditions for the maximal rate of Fenton's reagent promotion of lipid peroxidation were found to be 1.0 μmole lipid phosphate per ml, 0.1 mM H_2O_2 and 0.2 mM $FeCl_2$ in 0.3 M NaCl, pH 7.0. It appears that peroxidation is promoted by HO• formed via the reductive activation of H_2O_2 by Fe^{+2} since the HO• trapping agents benzoate, mannitol and thiourea inhibit peroxidation. However, it is of interest that benzoate and mannitol do not inhibit peroxidation until they reach a concentration of 10 mM. There is no apparent relationship between inhibition and HO• trap concentration at lower concentrations. From this data a question of specificity of the HO• radical traps is raised. If the HO• traps are specific it would have been expected that inhibition would be linearly related to inhibitor concentration. This was not observed. Also thiourea which has been reported to be a specific HO• trap [147] not only inhibited Fenton's reagent-promoted lipid peroxidation but also ascorbate-ion promoted lipid peroxidation. Thus appears that thiourea may be a general antioxidant and inhibition of lipid peroxidation by thiourea cannot be taken to indicate participation of HO•. The same may be said for benzoate and mannitol. It appears that they may be inhibiting lipid peroxidation by acting as general antioxidants.

The question raised is, "Does a specific HO• trap exist?" From the data presented here there does not appear to be a specific HO• trap. The compounds used as HO• traps inhibit reactions by acting as

co-oxidants. These co-oxidants can be viewed as competitive inhibitors. The addition of a co-oxidant of the proper redox potential to any reaction will result in the inhibition of the reaction. Thus just because the HO• traps inhibit the reaction, it should not be construed as evidence for the participation of HO• in the reaction. Inhibition by these compounds simply means that an oxidant strong enough to competitively oxidize the HO• trap and the normal substrate of the reaction is formed during the reaction. Inhibition by co-oxidants indicates the energetics of the reaction but says nothing about the physical identity of intermediates involved. Thus the HO• traps may also inhibit ferryl ion-dependent reactions since the ferryl ion is energically equivalent to to the HO•.

Chelation of the ferrous ion dramatically effects the mechanism of promotion of lipid peroxidation. Chelation of the ferrous ion by EDTA can completely inhibit Fenton's reagent promotion of lipid peroxidation. This could be due to the lowering of the redox potential of iron upon chelation and thus increased autoxidation to the EDTA-ferric complex. The EDTA-ferric complex reductive activation of H_2O_2 would be very slow and thus the promotion of initiation of lipid peroxidation would be minimal [127,128]. Alternatively, or perhaps in addition, Walling has shown that metal chelators such as EDTA inhibit HO•-dependent reactions by intercepting the HO• at the site of formation [148]. Thus EDTA may inhibit Fenton's Reagent-promoted lipid peroxidation by acting as a co-oxidant. The chelation effects of phosphate buffer are harder to understand. It is doubtful that phosphate is acting as a co-oxidant. However, chelation of ferrous ion by phosphate changes its redox potential and also the stability of the peferryl and

ferryl ion [98,101-104]. The effects of phosphate buffer are variable with concentration and pH indicating that the effect is due to chelation. The effects observed may indicate that Fenton's reagent promotion of lipid peroxidation is via the ferryl ion and not free HO•. Chelation of the ferrous ion by ADP appears to completely change the mechanism of lipid peroxidation. As had been observed for phosphate chelation, the effect of ADP chelation on Fenton's reagent-promoted lipid peroxidation is not linear with concentration. At a low ratio of ADP-Fe^{+2} (2:1) Fenton's reagent-promoted peroxidation is still totally inhibitable by 10 mM mannitol. However, with ADP:Fe^{+2} ratios of 4:1 to 17:1 peroxidation rates are less and 10 mM mannitol has little, if any, effect on the rate of peroxidation. It would appear that the mechanism of initiation of lipid peroxidation changes upon chelation by ADP.

The changes in the observed mechanism of initiation of lipid peroxidation could indicate that a metal-oxygen intermediate is responsible for initiation and not the free HO•. The dramatic changes upon ADP chelation and the lack of mannitol inhibition indicates that initiation promoted by this system occurs via an intermediate of different energetics from the ferryl ion or HO•. It is possible that in the presence of ADP initiation occurs by an ADP-perferryl ion intermediate. In vivo all metals are likely to be complexed by molecules such as ADP. It would thus be unlikely that the free HO• is responsible for initiation of lipid peroxidation in vivo. Initiation of lipid peroxidation would more likely occur via an activated oxygen-metal complex such as the perferryl or feryl ions.

CHAPTER 3

LIPID HYDROPEROXIDE-DEPENDENT INITIATION OF NADPH-DEPENDENT PEROXIDATION OF LIVER MICROSOMAL PHOSPHOLIPIDS

SUMMARY

NADPH-dependent lipid peroxidation occurs in two distinct sequential radical steps. The first step, lipid hydroperoxide-independent initiation, is the ADP-perferryl ion-promoted formation of low levels of lipid hydroperoxides. The second step, lipid hydroperoxide-dependent initiation, is the iron-promoted breakdown of lipid hydroperoxides, formed during hydroperoxide-independent initiation, generating reactive intermediates and products characteristic of lipid peroxidation. Lipid hydroperoxide-dependent initiation results in the rapid formation of thiobarbituric acid reactive material and lipid hydroperoxides. Lipid hydroperoxide-dependent initiation can be promoted by ethylenediaminetetraacetate chelated ferrous ion, diethylenetriamine pentaacetic acid chelated ferrous ion or by ferric cytochrome P450. However, cytochrome P450 is destroyed during the promotion of lipid hydroperoxide breakdown.

INTRODUCTION

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 [37,38,40,43,44,46-49].

Only recently, however, has it been shown that NADPH-dependent lipid peroxidation is promoted by NADPH-cytochrome P450 reductase. The role of NADPH-cytochrome P450 reductase in lipid peroxidation was

established in part by the ability of antibody to the reductase to inhibit NADPH-dependent microsomal lipid peroxidation [55]. The use of purified NADPH-cytochrome P450 reductase allowed the reconstitution of ADP-iron requiring NADPH-dependent lipid peroxidation in liposomes [55] and a more detailed investigation into the mechanism of peroxidation.

Several mechanisms by which NADPH-dependent lipid peroxidation is promoted have been proposed. Pederson et al. [55] and others [37,43-45] have proposed that initiation occurs via an ADP-perferryl ion promoted 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 P450 reductase and the subsequent reaction of ADP-Fe^{+2} with molecular oxygen.

Other investigators have proposed the participation of several different forms of reduced oxygen in the initiation of NADPH-dependent lipid peroxidation. Fong et al. [39], McCay et al. [48,49], and King et al. [72] proposed that NADPH-dependent microsomal lipid peroxidation occurred via an ADP-ferrous ion facilitated Haber-Weiss reaction [65]. They found that NADPH-dependent lipid peroxidation was dependent on ADP-Fe^{+3}, $O_2^{\cdot-}$, H_2O_2 and HO•. The actual initiating species was proposed to be HO•. The authors therefore suggested the following scheme for NADPH-dependent lipid peroxidation involving $O_2^{\cdot-}$ produced by NADPH-cytochrome P450 reductase and HO•:

$$2O_2^{\cdot-} + 2H^+ \longrightarrow H_2O_2 \qquad (9)$$

$$H_2O_2 + O_2^{\cdot-} \longrightarrow O_2 + OH^- + HO\cdot \qquad (12)$$

$$O_2^{\cdot-} + \text{ADP-}Fe^{+3} \longrightarrow O_2 + \text{ADP-}Fe^{+2} \qquad (35)$$

$$\text{ADP-}Fe^{+2} + H_2O_2 \longrightarrow \text{ADP-}Fe^{+3} + OH^- + HO\cdot \qquad (36)$$

The flux of HO• produced via reactions 9, 35 and 36 was proposed to be much greater than by reactions 9 and 12, 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 $O_2^{\bar{\cdot}}$-dependent lipid peroxidation. First, the participation of HO• in initiation of $O_2^{\bar{\cdot}}$-dependent lipid peroxidation has not been confirmed [58,62-64,75,111]. Second, since the proposed mechanism is dependent upon H_2O_2, catalase would be expected to inhibit $O_2^{\bar{\cdot}}$-dependent lipid peroxidation. However, others have found that catalase enhances $O_2^{\bar{\cdot}}$-dependent lipid peroxidation [62,64]. Third, it is unlikely that HO• would diffuse from its site of formation before reacting as would be required by the proposed mechanism [114]. Fourth, the rate of nonenzymatic dismutation of $O_2^{\bar{\cdot}}$ at physiological pH may be minor in comparison to other reactions of $O_2^{\bar{\cdot}}$ [68,82,149]. Finally, the concentration of H_2O_2 obtained during microsomal NADPH-oxidation in the absence of azide may be too low to account for the rate of enzymatic lipid peroxidation observed [73,74]. It must also be realized that microsomes are contaminated with catalase.

Pederson et al. [55] showed that the reconstitution of NADPH-dependent lipid peroxidation in liposomes in the presence of purified protease solubilized NADPH-cytochrome P450 reductase required ADP-iron [55,58]. 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.

Using similar techniques, it was demonstrated that lipid peroxidation occurs in two sequential steps, LOOH-independent initiation and LOOH-dependent initiation (Chapter 1). The role of ADP-Fe^{+3} in the LOOH-independent initiation of NADPH-dependent lipid peroxidation was more clearly defined. The ADP-perferryl ion was proposed to catalyze the rapid initial formation of LOOH. Perferryl ion-promoted LOOH-independent initiation was found to be superoxide dismutase sensitive. Lipid hydroperoxide-independent initiation did not occur via $^1O_2$ since $^1O_2$ trapping agents showed negligible inhibition. The enhancement of NADPH-dependent liposomal peroxidation by the addition of EDTA-Fe^{+3} was confirmed.

The results presented in this chapter will clearly define the second step of lipid peroxidation, LOOH-dependent initiation. We will show that specific iron chelates, other than ADP-Fe^{+2} promote LOOH-dependent initiation of lipid peroxidation at the expense of initially formed LOOH. The EDTA-Fe^{+2} complex promotes the breakdown of LOOH to form radical and reactive intermediates of lipid peroxidation. The reactive intermediates generated appear to include $O_2^{\cdot}$, however HO$\cdot$ does not appear to be involved in NADPH-dependent lipid peroxidation. Significant amounts of $^1O_2$ are also produced during LOOH-dependent initiation. Diethylenetriamine pentaacetic acid (DTPA)-Fe^{+2} can replace EDTA-Fe^{+2} in the promotion of LOOH-dependent initiation. In microsomes, where EDTA-Fe^{+3} is not required, ferricytochrome P450 is shown to be the endogenous promoter of LOOH-dependent initiation. Cytochrome P450 is destroyed during the promotion of LOOH-dependent initiation.

MATERIALS AND METHODS

Chemicals: The materials used in these studies were obtained from the following sources: 2,5-diphenylfuran, Eastman Organic Company;

bovine serum albumin, Pentex Chemical Company; ADP, butylated hydroxytoluene, cytochrome c (Type IV), NADPH, and diethylenetriamine pentaacetic acid, Sigma Chemical Company; SKF 525-A, Smith, Kline and French Laboratories; aminopyrine, K and K Laboratories; cumene hydroperoxide, Aldrich Chemical Company. All other chemicals used were of analytical grade. All chemicals were used without further purification except aminopyrine, which was twice recrystallized from hexane before use.

Microsomes and Microsomal Lipid: Rat hepatic microsomes were isolated from 175-225 g male Sprague Dawley rats (Spartan Research Animals, Haslett, MI), by the method of Pederson et al. [55]. Microsomes were stored at -20°C under argon in 0.05 M Tris-HCl-20% glycerol (pH 7.5 at 37°C). The Tris-glycerol solutions were saturated with argon prior to use. Total microsomal lipid was extracted from freshly prepared microsomes by the method of Folch et al. [97]. All solvents were purged with argon and all operations were performed under argon at 4°C to minimize autoxidation of unsaturated lipids. The extracted lipid was stored at -20°C under argon in argon saturated $CHCl_3$:MeOH (2:1). Extracted lipids could be stored for several weeks without autoxidation as assayed by lipid hydroperoxide content [33].

Enzyme Sources: Bovine erythrocyte superoxide dismutase (E.C.1.15.1.1.) and soybean lipoxygenase (Type 1) (E.C.1.13.1.13.) were obtained from Sigma Chemical Company. Superoxide dismutase activity was measured by the method of McCord and Fridovich [138]. NADPH-cytochrome P450 reductase (E.C.2.3.6.4.) was isolated from freshly prepared rat hepatic microsomes by the method of Pederson et al. [55]. NADPH-cytochrome P450 reductase specific activity was 52 units per mg protein

as determined by the method of Pederson et al. [55]. Phenobarbital induced cytochrome P450 was purified to a specific content of 13.2 nmoles per mg protein by the method of Guengerich [150].

Reaction Mixtures: Lipid hydroperoxides were generated in extracted microsomal lipid by the action of soybean lipoxygenase on detergent treated liposomes. Liposomes were prepared by sonication in distilled-deionized water at 4°C by the method of Pederson et al. [55] and diluted to a final concentration of 1.0 μmole lipid phosphate per ml in oxygenated 0.05 M Tris-HCl, pH 9.0, at 25°C. Sodium deoxycholate, 0.04% w/v, was added to the reaction mixture to accelerate the rate of LOOH formation [151]. Incubations were performed at 25°C in oxygen saturated buffers under an oxygen atmosphere in a metabolic shaking water bath. Reactions were initiated by the addition of soybean lipoxygenase (100 μg per ml). At the end of a 45 minute incubation period, lipids were extracted from the aqueous medium by the method of Folch et al. [97]. Lipid hydroperoxide content was measured as described by Buege and Aust [33]. Lipid hydroperoxide containing lipids were stored at -20°C under argon in argon saturated $CHCl_3$:MeOH(2:1). Under these conditions the LOOH containing lipid could be stored for up to two months without further degradation as assayed by the formation of thiobarbituric acid (TBA)-reactive material [33].

NADPH-dependent microsomal lipid peroxidation reaction mixtures contained 0.5 mg microsomal protein per ml, 1.7 mM ADP, 0.1 mM $FeCl_3$ and 0.1 mM NADPH in 0.05 M Tris-HCl (pH 7.5 at 37°C). Reactions were initiated by the addition of NADPH. Incubations were carried out at 37°C under an air atmosphere in a metabolic shaking water bath. Lipid peroxidation was measured by the formation of the TBA-reactive material,

malondialdehyde (MDA), and LOOH [33]. Just prior to use 0.03 volumes of a 2% butylated hydroxytoluene (BHT) ethanolic solution was added to the TBA-TCA-HCl reagent to prevent nonspecific chromophore formation during the assay procedure. Additions were made to the reaction mixtures as indicated in the tables. Rates of MDA and LOOH formation reported are initial rates.

NADPH-cytochrome P450 reductase-promoted liposomal peroxidation reaction mixtures contained 1.0 μmole lipid phosphate per ml, 0.1 unit NADPH-cytochrome P450 reductase per ml, 1.7 mM ADP, and 0.1 mM $FeCl_3$ and 0.1 mM NADPH in 0.05 M Tris-HCl (pH 7.5 at 37°C). Reactions were initiated by the addition of NADPH. Incubations and assays were performed as for NADPH-dependent microsomal lipid peroxidation.

Other Methods: ADP, EDTA and DTPA chelated iron solutions were prepared in 0.05 M Tris-HCl (pH 7.5 at 37°C). The iron chelate solutions were prepared in the following molar ratios: ADP:$FeCl_3$, 17.1; EDTA:$FeCl_3$, 1.1:1 and DTPA:$FeCl_3$, 1.1:1. Ferrous chelates were prepared using $FeCl_2$ in the same molar ratios as for ferric chelates. The buffers used in preparation of the ferrous chelates were saturated with argon before use to prevent autoxidation of the ferrous ion. Protein was determined by the method of Lowry et al. [142]. Total lipid phosphate was assayed by the method of Bartlett [141].

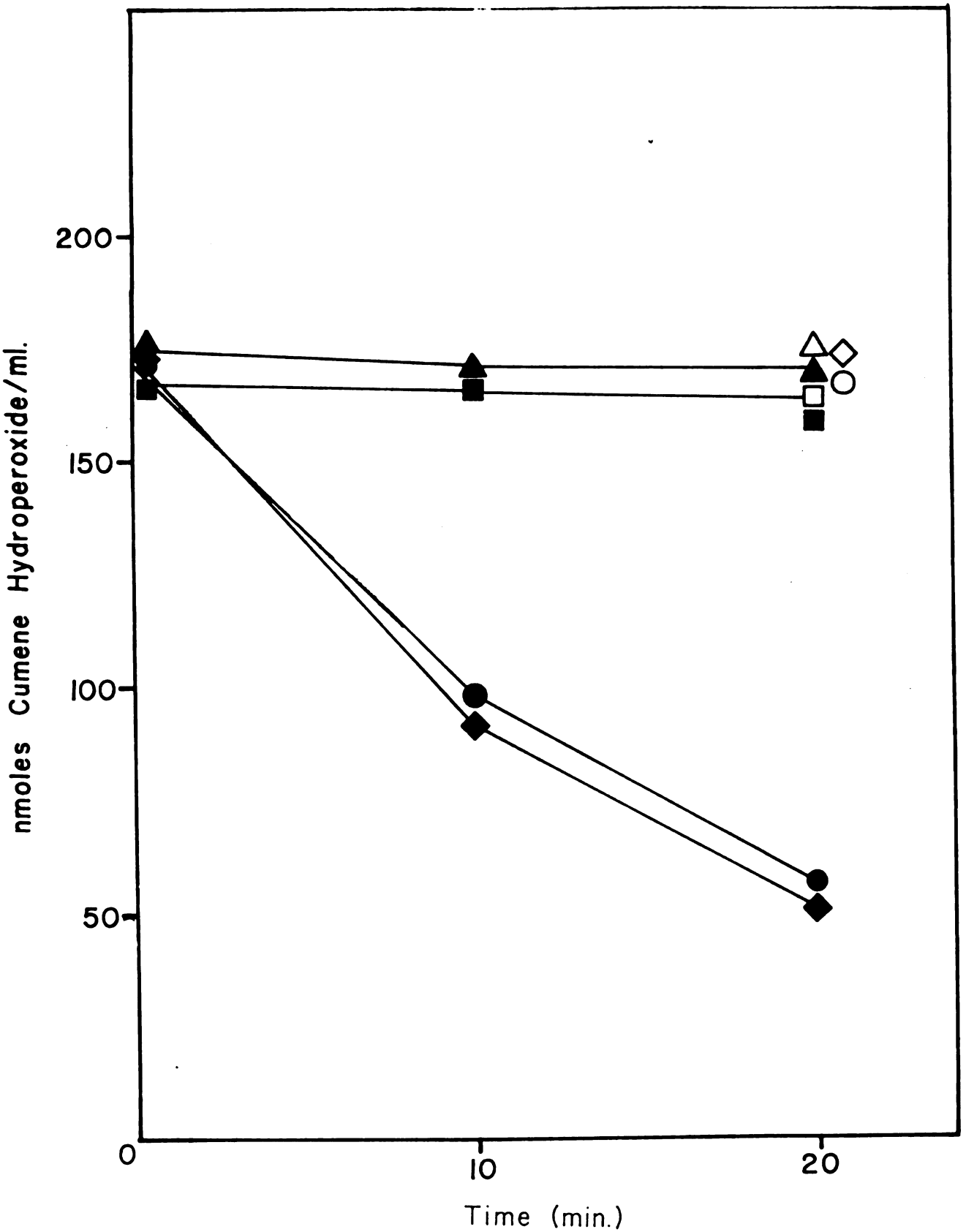
RESULTS

It has been previously proposed that lipid peroxidation can be divided into two distinct sequential reactions, LOOH-independent initiation and LOOH-dependent initation (Chapter 1). Lipid hydroperoxide-independent initiation was defined as the formation of LOOH accompanied by the minimal formation of MDA. Lipid hydroperoxide-independent

perferryl ion-promoted initiation was proposed to occur in both NADPH-dependent and $O_2^{\cdot}$-dependent lipid peroxidation. Lipid hydroperoxide-dependent initiation was suggested to involve the iron promoted breakdown of the initially formed LOOH generating reactive intermediates and products of lipid peroxidation. Lipid hydroperoxide-dependent initiation was proposed to result in the rapid formation of MDA and additional LOOH. Initial results (Chapter 1) indicated that in the peroxidation of extracted microsomal lipid the participation of EDTA-iron in LOOH-dependent initiation was of major significance while participation by ADP-iron was minimal.

It would be difficult to assess LOOH breakdown in a matrix where additional LOOH could be formed as in liposomes. Thus, cumene hydroperoxide (CHP) was used to assess the ability of different iron chelates to promote hydroperoxide breakdown. The choice of CHP was based on its ability to support NADPH-independent microsomal lipid peroxidation [152]. Cumene hydroperoxide apparently replaces the initially formed LOOH in the sequence of reactions. In NADPH-dependent liposomal peroxidation, ADP-Fe^{+3} and EDTA-Fe^{+3} are required for maximal activity [55]. To assess the participation of the ferric and ferrous forms of these iron chelates in the promotion of LOOH-dependent initiation, these agents were incubated with CHP and their affect on the breakdown of CHP was determined (Fig. 7). The ferrous ion chelates were formed by enzymtic reduction with NADPH-cytochrome P450 reductase. As shown in Figure 7, neither ADP-Fe^{+3}, EDTA-Fe^{+3}, ADP-Fe^{+2} nor the action of NADPH-cytochrome P450 reductase alone can promote the breakdown of CHP. Only EDTA-Fe^{+2} can promote the rapid breakdown of CHP. From these results it appears that only EDTA-Fe^{+2} is likely to function as the promoter of LOOH-dependent initiation of lipid peroxidation.

Figure 7. The NADPH-Dependent Decomposition of Cumene Hydroperoxide in the Presence of Chelated Iron - Experimental reaction mixtures (solid symbols) contained approximately 175 nmoles cumene hydroperoxide/ml as shown, 0.1 units NADPH-cytochrome P450 reductase/ml and 0.2 mM NADPH in 0.05 M Tris HCl, pH 7.4 at 37 1/4C. Control mixtures (open symbols) lacked NADPH. Chelated iron reagents were added as follows: no additions (Δ), 1.7 mM ADP and 0.1 mM $FeCl_3$ (●), 0.11 mM EDTA and 0.1 mM $FeCl_3$ (○), 1.7 mM ADP, 0.11 mM EDTA and 0.2 mM $FeCl_3$ (◆). Hydroperoxide levels were assayed by the iodometric assay described under "Methods."


200
150
100
50
0
10
20
nmoles Cumene Hydroperoxide/ml.
Time (min.)

The ability of these iron chelates to promote lipid peroxidation from LOOH was assessed by their addition to liposomes containing LOOH and measuring both MDA and LOOH formation (Table 12). Only EDTA-Fe^{+2} can promote LOOH-dependent initiation of lipid peroxidation. The initial rate of MDA formation, 1.16 nmoles/min/ml, reflects significant peroxidation since only 10% or less of total peroxidation is reflected by MDA formation [31,153]. The rate of LOOH formation, 9.3 nmoles/min/ml, also reflects significant peroxidation. It must be realized that during LOOH-dependent initiation LOOH are both reactant and product. For example, if LOOH breakdown occurs at a rate similar to CHP breakdown in the presence of EDTA-Fe^{+2}, 7.0 nmoles/min/ml, an increase in LOOH content of 9.3 nmoles/min/ml actually reflects a rate of LOOH formation of 16.3 nmoles/min/ml.

The ability of ADP-Fe^{+2} to promote LOOH-dependent initiation of lipid peroxidation is considerably less than that apparent in Table 12 since ADP-Fe^{+2} can also promote perferryl ion-dependent lipid peroxidation (Chapter 1). ADP-Fe^{+2} can promote LOOH-independent initiation of lipid peroxidation to the extent of 0.6 nmoles MDA/min/ml and 1.8 nmoles LOOH/min/ml (Chapter 1). If these rates of perferryl ion-dependent initiation are subtracted from the values in Table 1 for ADP-Fe^{+2} promotion of LOOH-dependent initiation it is apparent that ADP-Fe^{+2} promotion of LOOH-dependent initiation is minimal compared to EDTA-Fe^{+2} promotion of LOOH-dependent initiation.

The nature of the products formed during LOOH-dependent initiation is indicated by the results presented in Table 13. It appears that $O_2^{\cdot}$ may be a minor reactive intermediate formed during LOOH-dependent initiation as is reflected by the superoxide dismutase inhibition of

TABLE 12

THE PROMOTION OF LOOH-DEPENDENT INITIATION OF LIPID PEROXIDATION BY IRON CHELATES

Reaction mixtures contained 1.0 μmole lipid phosphate/ml (containing 0.1 μmole LOOH/ml) in 0.05 M Tris-HCl, pH 7.5 at 37°C. The following additions were made to the reaction mixtures as indicated: 1.7 mM ADP and 0.1 mM $FeCl_3$; 1.7 mM ADP and 0.1 mM $FeCl_2$; 0.11 mM EDTA and 0.1 mM $FeCl_3$; 0.11 mM EDTA and 0.1 mM $FeCl_2$. Reactions were initiated by addition of the appropriate iron complex. Incubations and assays were performed as described under "Methods."

	nmoles MDA min ml^{-1}	nmoles LOOH min ml^{-1}
Control (No Additions)	0.01	0.7
+ADP-Fe^{+3}	0.02	1.1
+EDTA-Fe^{+3}	0.01	1.1
+ADP-Fe^{+2}	0.60	1.4
+EDTA-Fe^{+2}	1.16	9.3

TABLE 13

THE PROMOTION OF LOOH-DEPENDENT INITIATION OF LIPID PEROXIDATION BY EDTA-Fe^{+2}

Reaction mixtures contained 1.0 μmole lipid phosphate/ml (containing 0.1 μmole LOOH/μmole lipid phosphate) in 0.05 M Tris-HCl, pH 7.5 at 37°C. The following additions were made as indicated: 0.11 mM EDTA and 0.1 mM $FeCl_3$; 0.11 mM EDTA and 0.1 mM $FeCl_2$; 1.0 unit SOD/ml; 0.2 mM DPF; 1.0 mM BHT and 40 mM benzoate. Reactions were initiated by addition of the appropriate iron form. Incubations and reactions were performed as described under "Methods."

		nmoles MDA $min\ ml^{-1}$	nmoles LOOH $min\ ml^{-1}$
Control (No Additions)		0.01	0.7
+EDTA-Fe^{+3}		0.16	1.3
+EDTA-Fe^{+2}		1.31	10.2
+EDTA-Fe^{+2}	+SOD	1.28	7.1
+EDTA-Fe^{+2}	+DPF	1.16	7.2
+EDTA-Fe^{+2}	+BHT	0.16	1.2
+EDTA-Fe^{+2}	+Benzoate	1.26	10.3

LOOH-dependent initiation. Superoxide dismutase inhibits MDA formation by 2% while inhibiting LOOH formation by 30%. Singlet oxygen may also be formed during LOOH-dependent initiation, as evidenced by the DPF inhibition of MDA formation of 11% and LOOH formation by 29%. (While the ability of DPF to act as a free radical scavenger clouds these results to a certain extent we have been unable to show that DPF inhibits ascorbate dependent peroxidation, a totally free radical mechanism, by more than 1% at the concentrations used for the experiments reported here.) The free radical nature of the LOOH-dependent initiation reactions are demonstrated by the inhibition of both MDA and LOOH formation by BHT. Addition of BHT inhibits both MDA and LOOH formation by 88%. The hydroxyl radical apparently does not participate in the LOOH-dependent initiation of lipid peroxidation as evidenced by the lack of inhibition of the reaction upon the addition of 40 mM benzoate (Table 13). Experiments with mannitol, gave similar results. These results are in agreement with the work of Tyler (64).

The ability of EDTA-Fe^{+3} to promote LOOH-dependent initiation of lipid peroxidation when reduced by NADPH-cytochrome P450 reductase is demonstrated by the data presented in Table 14. Peroxidation, as detected by both MDA and LOOH formation, is significant only in the presence of NADPH and EDTA-Fe^{+3}. The addition of NADPH gives rise to the enzymatic formation of EDTA-Fe^{+2}. The ability of NADPH-cytochrome P450 reductase to reduce EDTA-Fe^{+3} was demonstrated in a reaction mixture containing the reductase, NADPH and EDTA-Fe^{+3}. NADPH oxidation occurred only in the presence of all three components. These results were substantiated by ferrous ion chromophore formation when an excess of bathophenanthroline was added to an anaerobic reaction mixture after a

TABLE 14

PROMOTION OF LOOH-DEPENDENT INITIATION OF NADPH-DEPENDENT LIPID PEROXIDATION

Reaction mixtures contained 1.0 μmole lipid phosphate/ml (containing 0.1 μmole LOOH/μmole lipid phosphate), 0.1 unit NADPH cytochrome P450 reductase/ml, 0.11 mM EDTA, 0.1 mM $FeCl_3$ and 0.1 mM NADPH in 0.05 Tris-HCl, pH 7.5 at 37°C. The following additions were made as indicated: 1.0 unit SOD/ml, 0.2 mM DPF, 40 mM benzoate and 1.0 mM BHT. Reactions were initiated by the addition of NADPH. Incubations and assays were performed as described under "Methods."

	nmoles MDA min ml^{-1}	nmoles LOOH min ml^{-1}
Control (No Additions)	0.01	0.01
+NADPH	1.11	5.5
+NADPH +SOD	1.03	4.7
+NADPH +DPF	0.95	3.9
+NADPH +BHT	0.00	0.0
+NADPH +Benzoate	1.12	6.3

five minute reaction time. It is apparent that the function of EDTA-Fe^{+3} in NADPH-dependent lipid peroxidation is to promote, in its reduced form, LOOH-dependent initiation of lipid peroxidation.

Investigation into the nature of the reactive intermediates formed during enzymatic promotion of LOOH-dependent initiation (Table 14) yielded results equivalent to those obtained in the nonenzymatic reaction system (Table 13). The addition of superoxide dismutase inhibited MDA formation by 8% and LOOH formation by 15%. The addition of DPF inhibited MDA and LOOH formation by 13% and 28%, respectively. Again, BHT showed essentially complete inhibition of LOOH-dependent initiation. Benzoate did not inhibit NADPH-promoted LOOH-dependent initiation of lipid peroxidation, but actually enhanced peroxidation as detected by LOOH formation. It appears that the enzyme promoted LOOH-dependent initiation reaction gives rise to radical intermediates of lipid peroxidation, however, HO$\cdot$ is not among them. In addition, $^1O_2$ is apparently formed during LOOH-dependent initiation of lipid peroxidation.

Lipid hydroperoxide-dependent initiation accounts for a significant portion of the MDA and LOOH formed during NADPH-dependent liposomal peroxidation (Table 15). In th presence of ADP-iron alone, the rate of MDA and LOOH formation in NADPH-dependent lipid peroxidation is 0.3 nmole/min/ml and 1.8 nmole/min/ml, respectively. When a promoter of LOOH-dependnt initiation, EDTA-Fe^{+3}, is included, MDA and LOOH formation are increased 11-fold.

NADPH-dependent lipid peroxidation in the reconstituted system, shown in Table 15, is characterized by the simulataneous occurrence of both LOOH-independent initiation and LOOH-dependent initiation reactions. Superoxide dismutase inhibits both MDA and LOOH formation by

TABLE 15

NADPH-DEPENDENT LIPOSOMAL PEROXIDATION

Reaction mixtures contained 1.0 μmole lipid phosphate/ml, 0.1 unit NADPH-cytochrome P450 reductase/ml, 1.7 mM ADP, 0.1 mM $FeCl_3$ and 0.1 mM NADPH in 0.05 M Tris-HCl, pH 7.5, at 37°C. The following additions were made where indicated: 0.11 mM EDTA and 0.1 mM $FeCl_3$, 1.0 unit SOD/ml, 0.2 mM DPF, 1.0 mM BHT and 40 mM benzoate where indicated. Reactions were initiated by the addition of NADPH. Incubations and assays were performed as under "Methods."

	nmoles MDA $min\ ml^{-1}$	nmoles LOOH $min\ ml^{-1}$
Control (No Additions)	0.03	0.1
+NADPH	0.30	1.8
+EDTA-Fe^{+3}	0.02	0.02
+NADPH +EDTA-Fe^{+3}	3.40	19.5
+NADPH +EDTA-Fe^{+3} +SOD	0.50	3.5
+NADPH +EDTA-Fe^{+3} +DPF	3.10	16.3
+NADPH +EDTA-Fe^{+3} +BHT	0.00	0.0
+NADPH +EDTA-Fe^{+3} +Benzoate	3.45	20.1

approximately 85% in a reconstituted reaction mixture promoting both forms of initiation. In contrast, in a similar system promoting only LOOH-dependent initiation (Table 14), superoxide dismutase inhibits MDA and LOOH formation by only 8% and 15%, respectively. Such a difference between the two reaction mixtures serves to emphasize the dependence of LOOH-dependent initation on perferryl ion-dependent LOOH-independent initiation. The addition of DPF to an NADPH-dependent reaction mixture promoting both forms of initiation (Table 15) inhibits MDA and LOOH formation by 9% and 16%, respectively. The inhibition observed is equivalent to that observed during the NADPH-dependent promotion of LOOH-dependent initiation alone (Table 14). These results show that $^1O_2$ participates in only LOOH-dependent initiation and not in perferryl ion-dependent LOOH-independent initiation. The addition of BHT completely inhibits both MDA and LOOH formation demonstrating the radical nature of perferryl ion-dependent initiation. The radical nature of the LOOH-dependent initiation reaction was demonstrated in Table 14. Similar to the results in Table 14, no effect on the rate of lipid peroxidation upon the addition of benzoate was observed (Table 15) indicating that HO$\cdot$ does not participate in either form of initiation in NADPH-dependent liposomal peroxidation.

The iron chelate diethylenetriamine pentaacetic acid (DPTA) has been used by some [154] to inhibit metal catalyzed lipid peroxidation. Considering that the structure of DTPA is similar to that of a dimer of EDTA, the ability of DPTA-Fe^{+3} to replace EDTA-Fe^{+3} in NADPH-dependent lipid peroxidation was examined (Table 16). The results demonstrated that DTPA-Fe^{+3} could effectively replace EDTA-Fe^{+3}. Addition of DTPA-Fe^{+3} in the absence of ADP-Fe^{+3} showed that DTPA-Fe^{+3} could not function

TABLE 16

PROMOTION OF LOOH-DEPENDENT INITIATION OF NADPH-DEPENDENT LIPID PEROXIDATION BY EDTA-Fe^{+3} AND DTPA-Fe^{+3}

Reaction mixtures contained 1.0 μmole lipid phosphate/ml, 0.1 unit NADPH cytochrome P450 reductase/ml and 0.1 mM NADPH in 0.05 M Tris-HCl, pH 7.5 at 37°C. The following additions were made where indicated: 0.11 mM EDTA and 0.1 mM $FeCl_3$; 0.11 mM DTPA and 0.1 mM $FeCl_3$; 1.7 mM ADP and 0.1 mM $FeCl_3$. Reactions were initiated by the addition of NADPH. Incubations and assays were performed as described under "Methods."

		nmoles MDA min ml^{-1}	nmoles LOOH min ml^{-1}
Control (No Additions)		0.01	0.2
	+EDTA-Fe^{+3}	0.01	0.3
	+DTPA-Fe^{+3}	0.01	0.2
+NADPH		0.02	0.4
+NADPH	+EDTA-Fe^{+3}	0.01	0.4
+NADPH	+DTPA-Fe^{+3}	0.01	0.2
+NADPH	+ADP-Fe^{+3}	0.35	1.9
+NADPH	+ADP-Fe^{+3} +EDTA-Fe^{+3}	3.46	19.6
+NADPH	+ADP-Fe^{+3} +DTPA-Fe^{+3}	3.19	18.9

in the LOOH-independent initiation of NADPH-dependent lipid peroxidation. The results indicate DTPA-Fe^{+3} functions as a promoter of LOOH-dependent initiation of lipid peroxidation. These findings may explain the kinetic findings of Thomas et al. [154] since they used DTPA to chelate contaminating iron in a system that is capable of reducing DTPA-Fe^{+3} to DTPA-Fe^{+2} via $O_2^{\cdot}$.

The involvement of cytochrome P450 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 17). SKF 525-A inhibits cytochrome P450 catalyzed reactions by preferentially binding to cytochrome P450 displacing other substrates [155]. The addition of EDTA-Fe^{+3}, which has been shown to enhance NADPH-dependent microsomal lipid peroxidation [55,63], completely reversed the SKF 525-A inhibition of NADPH-dependent microsomal lipid peroxidation, indicating that SKF 525-A is specifically inhibiting an endogenous microsomal agent capable of promoting LOOH-dependent initiation. The specificity of SKF 525-A indicates that one of the endogenous promoters of LOOH-dependent initiation in microsomes is cytochrome P450.

Aminopyrine was proposed to inhibit NADPH-dependent microsomal lipid peroxidation by competing for reducing equivalents [156,157]. The addition of 5 mM aminopyrine to a microsomal reaction mixture inhibited NADPH-dependent lipid peroxidation by 57% (Table 18). The addition of

TABLE 17

THE EFFECT OF EDTA-Fe^{+3} ON THE SKF 525-A INHIBITION OF NADPH-DEPENDENT MICROSOMAL LIPID PEROXIDATION

Reaction mixtures contained 0.5 mg microsomal protein/ml, 1.7 mM ADP, 0.1 mM $FeCl_3$ and 0.1 mM NADPH in 0.05 M Tris-HCl, pH 7.5 at 37°C. The following additions were made as indicated: 100 μM SKF 525-A, 0.11 mM EDTA and 0.1 mM $FeCl_3$. Reactions were initiated by the addition of NADPH. Incubations and assays were performed as described under "Methods."

		nmoles MDA $min\ ml^{-1}$
Control (-NADPH)		0.11
+NADPH		2.43
+NADPH	+SKF 525-A	0.81
+NADPH	+SKF 525-A +EDTA-Fe^{+3}	2.24

TABLE 18

THE EFFECT OF EDTA-Fe^{+3} ON THE AMINOPYRINE INHIBITION OF NADPH-DEPENDENT MICROSOMAL LIPID PEROXIDATION

Reaction mixtures contained 0.5 mg microsomal protein/ml, 1.7 mM ADP, 0.1 mM $FeCl_3$ and 0.1 mM NADPH in 0.05 M Tris-HCl, pH 7.5 at 37°C. The following additions were made as indicated: 5.0 mM aminopyrine, 0.11 mM EDTA and 0.1 mM $FeCl_3$. Reactions were initiated by the addition of NADPH. Incubations and assays were performed as described under "Methods."

		nmoles MDA $min\ ml^{-1}$
Control (-NADPH)		0.20
+NADPH		2.81
+NADPH	+Aminopyrine	1.20
+NADPH	+Aminopyrine +EDTA-Fe^{+3}	2.81

EDTA-Fe^{+3} completely reversed aminopyrine inhibition. Since EDTA-Fe^{+3} must be reduced to be active in the promotion of LOOH-dependent initiation of lipid peroxidation, 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 P450. Inhibition may be the result of cytochrome P450 peroxidase activity utilizing lipid hydroperoxides and oxidizable drugs as substrates in a manner analagous to the CHP dependent drug metabolism observed by others [158-160]. This data suggests that the endogenous promoter of LOOH-dependent initiation is cytochrome P450 as previously indicated by experiments with SKF 525-A.

The ability of ferric cytochrome P450 to promote LOOH-dependent initiation of lipid peroxidation was investigated by addition of cytochrome P450 to a reaction mixture in which LOOH were generated in situ by soybean lipoxygenase (Table 19). In detergent treated liposomes, lipoxygenase catalyzed initial rates of formation of 0.08 nmoles MDA/min/ml and 0.53 nmoles LOOH/min/ml. The addition of 0.3 nmol/ml ferric cytochrome P450 to the reaction mixture resulted in an 11-fold increase in the rate of MDA formation and a 3-fold increase in the rate of LOOH formation. From this data it appears that cytochrome P450 is an excellent promoter of LOOH-dependent initiation. The data indicates that on a per mole basis cytochrome P450 is a beter promoter of LOOH-dependent initiation than is EDTA-Fe^{+2}. However, as others have previously shown, cytochrome P450 is degraded during lipid peroxidation thereby limiting its promotional abilities [161,162].

TABLE 19

PROMOTION OF LOOH-DEPENDENT INITIATION OF LIPID PEROXIDATION BY FERRIC CYTOCHROME P450

Reaction mixtures contained 1.0 μmole lipid phosphate/ml, 100 μg lipoxygenase/ml and 0.04% sodium deoxycholate in 0.05 M Tris-HCl, pH 7.5 at 37°C. Ferric cytochrome P450, 0.3 nmole/ml, was added where indicated. Reactions were initiated by the addition of lipoxygenase. Incubations and assays were performed as decribed under "Methods."

	nmoles MDA $min\ ml^{-1}$	nmoles LOOH $min\ ml^{-1}$
Control (-Lipoxygenase)	0.02	0.1
+ferric cytochrome P450	0.05	0.3
+lipoxygenase	0.08	0.5
+lipoxygenase + ferric cytochrome P450	0.88	1.4

The complete reconstitution of NADPH-dependent microsomal lipid peroxidation in liposomes utilizing ferric cytochrome P450 as the promoter of LOOH-dependent initiation is shown in Table 20. In the absence of cytochrome P450, the ability of ADP-Fe^{+2} to initiate lipid peroxidation is evident (Chapter 1). The addition of 0.3 nmoles/ml cytochrome P450 as a promoter of LOOH-dependent initiation increases MDA and LOOH rates of formation by 8- and 16-fold, respectively. These results indicate the key role played by cytochrome P450 in NADPH-dependent lipid peroxidation.

DISCUSSION

The results presented here along with those previously reported (Chapter 1), suggest a mechanism of NADPH-dependent lipid peroxidation. Previously, it was shown that lipid peroxidation can be divided into two sequential series of radical reactions (Chapter 1). The first reaction is the ADP-perferryl ion promoted formation of LOOH. Perferryl ion-dependent initiation products were proposed to subsequentially undergo reductive activation reactions with EDTA-Fe^{+2} to generate radical intermediates of lipid peroxidation. Promotion of LOOH-dependent initiation resulted in the rapid formation of MDA and LOOH as is typical of iron-dependent enzymatic lipid peroxidation. In this chapter, it was demonstrated that LOOH-dependent initiation is promoted by EDTA-Fe^{+2}, DTPA-Fe^{+2} or ferric cytochrome P450. The promotion of LOOH-dependent initiation is responsible for up to 90% of the peroxidation observed.

NADPH-cytochrome P450 reductase promoted liposomal peroxidation requires both ADP-Fe^{+3} and EDTA-Fe^{+3} for maximum activity. In the enzymatic reaction both the ferric and the ferrous forms of the iron chelates exists. It was previously established that only ADP-Fe^{+2} can

TABLE 20

PROMOTION OF LOOH-DEPENDENT INITIATION OF NADPH-DEPENDENT LIPOSOMAL PEROXIDATION BY FERRIC CYTOCHROME P450

Reaction mixtures contained 1.0 μmole lipid phosphate/ml, 0.1 unit NADPH-cytochrome P450 reductase/ml, 0.1 mM NADPH, 1.7 mM ADP and 0.1 mM $FeCl_3$ in 0.05 M Tris-HCl, pH 7.5 at 37°C. Ferric cytochrome P450, 0.3 nmole/ml, was added where indicated. Reactions were initiated by the addition of NADPH. Incubations and assays were performed as described under "Methods."

	nmoles MDA min ml^{-1}	nmoles LOOH min ml^{-1}
Control (-NADPH)	0.01	0.1
+ferric cytochrome P450	0.04	0.1
+NADPH	0.21	0.4
+NADPH + ferric cytochrome P450	1.51	6.6

initiate the formation of LOOH (Chapter 1). In LOOH-dependent initiation the promoter must be able to efficiently promote the breakdown of LOOH to reactive intermediates of lipid peroxidation. Using CHP as a model organic hydroperoxide to assay promotion by the four possible iron complexes, it was apparent that only EDTA-Fe^{+2} could promote the efficient breakdown of the organic hydroperoxide (Figure 7). Thus, although EDTA-Fe^{+2} could not function in the perferryl ion promoted LOOH-independent initiation of lipid peroxidation it can promote LOOH-dependent initiation of lipid peroxidation.

The ability of EDTA-Fe^{+2} to promote LOOH-dependent initiation of lipid peroxidation in partially peroxidized liposomes was directly demonstrated in Tables 12 and 13. Of the four iron chelates investigated in Figure 7, only EDTA-Fe^{+2} could efficiently promote the formation of MDA and additional LOOH from LOOH in a lipid matrix. Although the formation of MDA and LOOH upon addition of ADP-Fe^{+2} appears to be significant, it must be recognized that ADP-Fe^{+2} can promote perferryl ion-dependent initiation of lipid peroxidation in unperoxidized fatty acid (Chapter 1). Considering the ability of ADP-Fe^{+2} to promote perferryl ion-dependent initiation of lipid peroxidation at rates similar to those observed in Table 12, promotion of LOOH-dependent initiation by ADP-Fe^{+2} is apparently minimal compared to that of EDTA-Fe^{+2} which cannot promote perferryl ion-dependent initiation of lipid peroxidation. The findings reported here indicate that only EDTA-Fe^{+2} is efficient in the promotion of LOOH-dependent initiation of lipid peroxidation.

The results presented in Table 13 showed that EDTA-Fe^{+2} promoted LOOH-dependent initiation was not mediated by a superoxide dismutase sensitive complex or $O_2^{\cdot}$, in contrast to ADP-Fe^{+2} promoted initiation.

Thus, the LOOH-dependent initiation reaction is apparently not promoted by an iron bound $O_2^{\cdot}$ since such a complex would likely be superoxide dismutase sensitive [144]. The LOOH-dependent initiation reaction is likely promoted by EDTA-Fe^{+2}. The minor inhibition by superoxide dismutase may reflect a small amount of $O_2^{\cdot}$ which is produced by air oxidation of the EDTA-Fe^{+2} complex according to the following reaction:

$$\text{EDTA-Fe}^{+2} + O_2 \rightleftharpoons \text{EDTA-Fe}^{+3} + O_2^{\cdot} \qquad (37)$$

Addition of superoxide dismutase would then shift the reaction equilibrium to the right, effectively decreasing the concentration of promoter. Tables 14 and 15 also suggest that minor amounts of $^1O_2$ may be produced during the LOOH-dependent initiation reaction, perhaps via the reaction of two lipid hydroperoxide radicals [29,163]. The minor inhibition exhibited by DPF indicates that $^1O_2$ is a product of perhaps only some of several possible reactions. The inhibition of peroxidation upon the addition of BHT to the reaction mixture indicates the radical nature of the reaction. The lack of inhibition upon the addition of benzoate to the reaction mixture indicates that HO$\cdot$ is not one of the radicals that mediate LOOH-dependent initiation of lipid peroxidation.

Using NADPH-cytochrome P450 reductase to promote the formation of EDTA-Fe^{+2} *in situ* (Table 14), it was demonstrated that the enzymatic reaction was identical to the nonenzymatic reaction (Table 13). Enzymatically promoted LOOH-dependent initiation was superoxide dismutase insensitive, showed some inhibition by DPF, showed no inhibition by benzoate, but was completely inhibited by BHT. Thus, the overall enzyme promoted reaction, mediated by EDTA-Fe^{+2}, was radical in nature, however, minor amounts of $^1O_2$ may be formed and participate in further peroxidation.

The effect of various inhibitors of lipid peroxidation on the total reconstitution of NADPH-dependent lipid peroxidation (Table 15) are consistent with the mechanism of initiation previously suggested (Chapter 1) and the mechanism of LOOH-dependent initiation being proposed here. Superoxide dismutase almost completely inhibits lipid peroxidation because it inhibits ADP-perferryl ion catalyzed initiation, as previously shown (Chapter 1). It was previously shown that superoxide dismutase has minimal effect on the LOOH-dependent initiaton reaction alone (Tables 13 and 14). The slight inhibition of peroxidation by the addition of DPF, indicates that DPF does not inhibit perferryl ion-dependent initiation, but does inhibit LOOH-dependent initiation to a minor extent (Tables 13 and 14) indicating that $^1O_2$ is a minor product of the reaction. Butylated hydroxytoluene completely inhibits NADPH-dependent lipid peroxidation indicating that perferryl ion-dependent initiation is radical in nature. The radical nature of LOOH-dependent initiation was shown in Tables 13 and 14. The lack of benzoate inhibition in NADPH-dependent lipid peroxidation indicates that HO$\cdot$ does not participate in either form of initiation. This data clearly shows that NADPH-dependent lipid peroxidation occurs in two successive radical steps each dependent upon metal catalysis.

The significant contribution of EDTA-Fe^{+2} promoted LOOH-dependent initiation to the total quantity of products formed during NADPH-dependent lipid peroxidation has been shown previously (Chapter 1) and is demonstrated by the data presented in Table 15. The addition of EDTA-Fe^{+3} to a NADPH-dependent liposomal peroxidation mixture increased the rate of MDA and LOOH formation by 11-fold over the rates in the presence of ADP-Fe^{+3} and NADPH alone. Thus, it appears that LOOH-dependent

initiation is a key reaction in lipid peroxidation accounting for more than 90% of total product formation.

Table 16 demonstrates that EDTA is not unique among the chelators of iron in its ability to facilitate iron promoted LOOH-dependent initiation of lipid peroxidation. DTPA-Fe^{+3} can replace EDTA-Fe^{+3} in NADPH-dependent lipid peroxidation in liposomes. ADP-Fe^{+3} is an efficient promoter of perferryl ion-dependent LOOH-independent initiation of lipid peroxidation but a very poor promoter of the breakdown of organic hydroperoxides (Figure 7) or the LOOH-dependent initiation of lipid peroxidation (Table 12). Chelation of iron by EDTA greatly stabilizes the ferric complex, lowering the reduction potential of the complex to 0.254 v [164,165] making the ferrous complex a relatively strong reducing agent. Chelation 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 should enhance the promotion of LOOH breakdown. The effect of iron chelation on the breakdown of H_2O_2 by Fenton type reagents has been previously demonstrated [166,167]. If LOOH-dependent initiation is a Fenton type reaction, efficient promotion by EDTA-Fe^{+2} 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 [168]. The data suggests that the key to the formation of an efficient promoter of LOOH-dependent initiation may be the lowering of the iron reduction potential upon chelation.

The ability of DTPA-Fe^{+3} to promote LOOH-dependent initiation may offer an alternative explanation to the experimental data reported

recently by Thomas et al. [154]. Thomas et al., proposed that $O_2^{\cdot}$-dependent lipid peroxidation occurred via the reaction of $O_2^{\cdot}$ with LOOH as follows:

$$O_2^{\cdot} + LOOH \longrightarrow LO\cdot + O_2 + OH^- \qquad (38)$$

The authors found that peroxidation was dependent on LOOH formed by autoxidation. The reaction constant for reaction 38 was found to be 7 x 10^3 M^{-1} sec^{-1}. However, the rate of self-dismutation of $O_2^{\cdot}$ at the pH of the investigation, 7.4, is at least 2 orders of magnitude greater than the rate constant for reaction 38. Thus, the importance of reaction 38 in $O_2^{\cdot}$-dependent lipid peroxidation may be minimal. The observation that peroxidation was dependent upon preformed LOOH, correlates well with the mechanism of iron promotion of LOOH-dependent initiation of lipid peroxidation proposed here. The kinetics of the observed reaction [155] also agree with the kinetics of EDTA-Fe^{+2} or DTPA-Fe^{+2} promoted LOOH-dependent initiation, as reported here. That is, the rate of LOOH formation during LOOH-dependent initiation, as defined here, is approximately equal to the rates of peroxidation observed by Thomas et al. These two observations together with the use by Thomas et al. 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 chelated promoted LOOH-dependent initiation of lipid peroxidation. The chelate involved likely would be DTPA-Fe^{+3}, which is reduced by $O_2^{\cdot}$ to DTPA-Fe^{+2} an active promoter of LOOH-dependent initiation as demonstrated in this paper.

Pederson et al. [55,58] proposed that EDTA-Fe^{+3} replaced an endogenous microsomal agent that participated in NADPH-dependent microsomal lipid peroxidation. Table 17 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 binding to cytochrome P450 and not by disruption of microsomal electron transport [155]. The reversal of SKF 525-A inhibition by addition of EDTA-Fe^{+3} indicates that cytochrome P450 may be one microsomal entity responsible for promotion of LOOH-dependent initiation in NADPH-dependent microsomal lipid peroxidation.

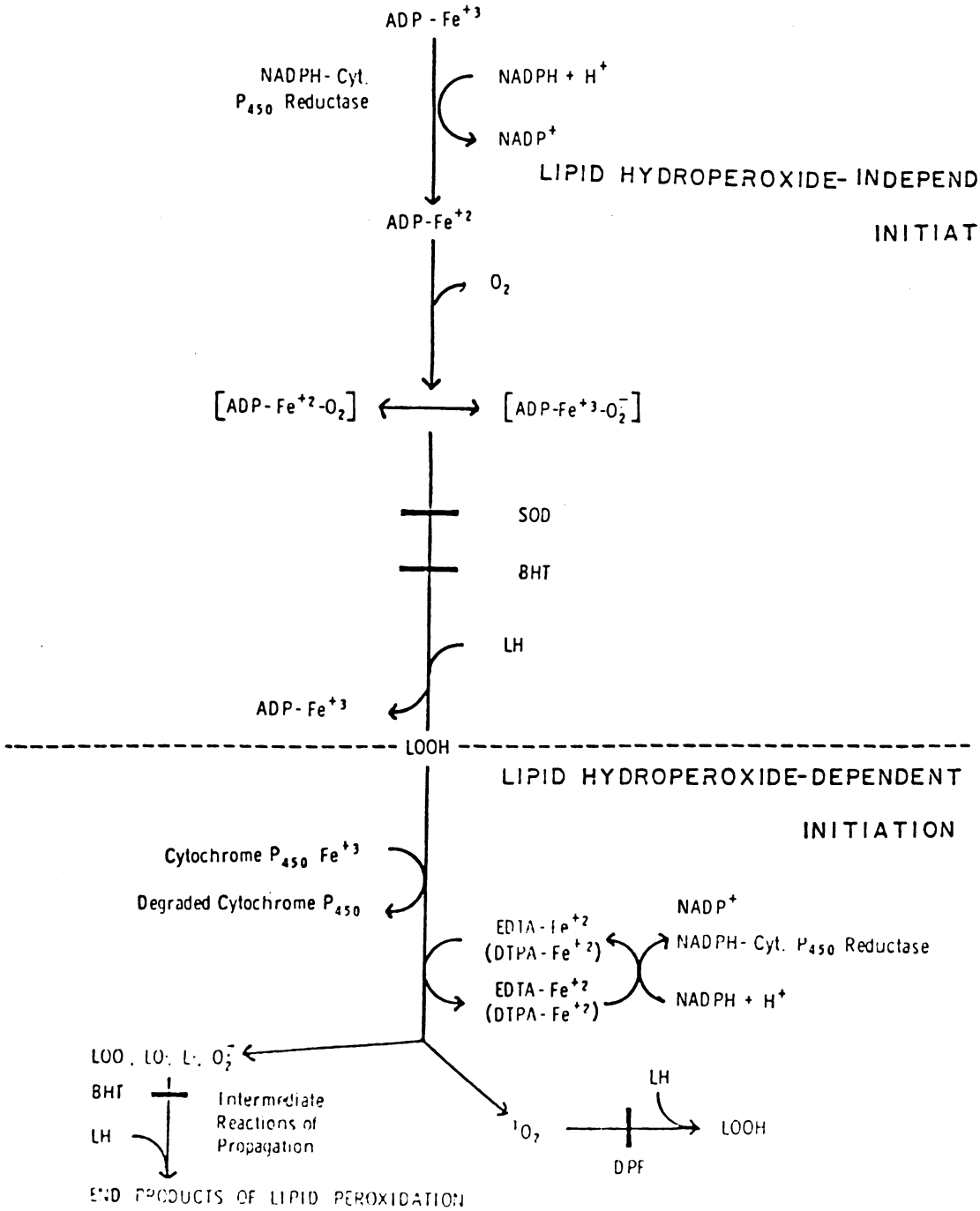
The proposed role of cytochrome P450 in microsomal lipid peroxidation is supported by the EDTA-Fe^{+3} reversal of aminopyrine inhibition of NADPH-dependent microsomal lipid peroxidation (Table 18). The data indicates that drug substrates of the microsomal mixed-function oxidase system do not inhibit NADPH-dependent lipid peroxidation by competing for reducing equivalents as others have proposed [156,157]. If the inhibitory effect was a results 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 P450 and lipid hydroperoxides [168,169] and that CHP can support enzymatic oxidation of microsomal mixed-function oxidase drug substrates [152,158-160] indicate that drug substrates may inhibit lipid peroxidation by reducing LOOH via a cytochrome P450 peroxidase type mechanism. Such a peroxidase reaction would inhibit lipid peroxidation by competing with LOOH-dependent initiation for LOOH produced during perferryl ion-dependent initiation. These results also indicate that cytochrome P450 may function in a variety of roles during lipid peroxidation in addition to its participation of LOOH-dependent initiation.

The ability of ferric cytochrome P450 to promote LOOH-dependent initiation of lipd peroxidation is clearly demonstrated in Tables 19 and 20. Addition of 0.3 nmoles/ml ferric cytochrome P450 to a reaction mixture in which LOOH are being generated in situ by soybean lipoxygenase, resulted in an 11-fold increase in MDA formation and a 3-fold increase in LOOH formation (Table 19). The results in Table 20 demonstrate the role that ferric cytochrome P450 plays in LOOH-dependent initiation of NADPH-dependent lipid peroxidation. In the reaction mixture, LOOH are generated by the ADP-perferryl ion initiation complex. Addition of 0.3 nmoles/ml ferric cytochrome P450, which cannot be reduced by the protease solubilized NADPH-cytochrome P450 reductase [80] results in an 8-fold increase in MDA formation and 15-fold increase in LOOH formation. Thus, ferric cytochrome P450 is apparently an excellent promoter of LOOH-dependent initiation of lipid peroxidation.

As summarized in Figure 8, the results presented in this paper clearly demonstrate that NADPH-dependent lipid peroxidation occurs in two sequential steps. The first step, perferryl ion-dependent initiation, is promoted by the ADP-perferryl ion. The second step, LOOH-dependent initiation, is dependent upon the LOOH formed during perferryl ion-dependent initiation, and results in the rapid formation of reactive intermediates and products of lipid peroxidation. Lipid hydroperoxide-dependent initiation is the EDTA-Fe^{+2}, DTPA-Fe^{+2} or ferric cytochrome P450 promoted breakdown of LOOH to form reactive intermediates of lipid peroxidation. Lipid hydroperoxide-dependent initiation accounts for more than 90% of the products formed during lipid peroxidation and is radical in nature. Lipid hydroperoxide-dependent initiation 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 $^1O_2$.

Figure 8. Summary of the Proposed Scheme of NADPH-Dependent Lipid Peroxidation.


ADP - Fe+3
NADPH- Cyt. P450 Reductase
NADPH + H+
NADP+
LIPID HYDROPEROXIDE- INDEPENDENT INITIATION
ADP-Fe+2
O2
[ADP- Fe+2-O2]
[ADP-Fe+3-O2-]
SOD
BHT
LH
ADP- Fe+3
LOOH
LIPID HYDROPEROXIDE-DEPENDENT INITIATION
Cytochrome P450 Fe+3
Degraded Cytochrome P450
NADP+
EDTA - Fe+2 (DTPA - Fe+2)
NADPH- Cyt. P450 Reductase
EDTA - Fe+2 (DTPA - Fe+2)
NADPH + H+
LOO . LO: L: O2-
BHT
LH
Intermediate Reactions of Propagation
END PRODUCTS OF LIPID PEROXIDATION
1O2
LH
LOOH
DPF

CHAPTER 4

LIPID HYDROPEROXIDE-DEPENDENT INITIATION OF $O_2^{\cdot}$-PROMOTED PEROXIDATION OF MICROSOMAL PHOSPHOLIPIDS

SUMMARY

Superoxide-dependent peroxidation of extracted microsomal lipid occurs in two distinct radical steps. The first step, lipid hydroperoxide-independent initiation, results in the formation of low levels of lipid hydroperoxides. Lipid hydroperoxide-independent initation, which is superoxide and ADP-iron dependent, may be promoted by the ADP-perferryl ion. Lipid hydroperoxide-independent initiation does not involve singlet oxygen or hydroxyl radical. The second step, lipid hydroperoxide dependent initiation, is the promotion of the breakdown of lipid hydroperoxides formed during lipid hydroperoxide-independent initiation resulting in the generation of reactive intermediates and the rapid formation of thiobarbituric acid reactive material and additional lipid hydroperoxides. Lipid hydroperoxide-dependent initiation is dependent upon lipid hydroperoxides, superoxide and chelated iron. The lipid hydroperoxide-dependent initiation reaction can be promoted by ethylenediamine tetraacetic acid chelated ferrous ion or by diethylenetriamine pentaacetic acid chelated ferrous ion. Superoxide-dependent peroxidation of intact microsomal lipid also occurs in two distinct free radical steps. In microsomes lipid hydroperoxide-dependent initiation is facilitated by ferric cytochrome P450, which is destroyed during promotion of the reaction.

INTRODUCTION

Interest in biological superoxide ($O_2^{\cdot}$) began with the demonstration that erythrocuperin catalyzed the dismutation of $O_2^{\cdot}$. Studies have since

demonstrated that erythrocuperin, now called superoxide dismutase, is ubiquitous throughout respiring organisms [170]. Such a prevalence of superoxide dismutase in aerobic organisms would indicate that the generation of $O_2^{\cdot}$ in biological tissue is a common event. Interest in the biological formation of $O_2^{\cdot}$ intensified with the demonstration that $O_2^{\cdot}$ was produced in many common biochemical reactions, including the autoxidation of reduced flavins [171,172], ferrodoxins [173] and the enzymatic reactions catalyzed by xanthine oxidase [174] and tryptophan dioxygenase [175]. The results of investigations by Bus et al. [132, 133] gave the first indication of the direct participation of $O_2^{\cdot}$ in aerobic tissue damage. They suggested that the toxicity of paraquat (methyl viologen) was at least in part mediated by $O_2^{\cdot}$-dependent lipid peroxidation. Since then $O_2^{\cdot}$-mediated lipid peroxidation has been implicated in the toxicity of alloxan [176], 6-hydroxydopamine [177], dialuric acid [178] and adriamycin [134,179]. Investigations into the in vitro mechanisms of $O_2^{\cdot}$-dependent lipid peroxidation were a natural outgrowth of the implication of its in vivo significance.

Investigations into the mechanism of $O_2^{\cdot}$-dependent lipid peroxidation have been directed in part by the relative chemical unreactivity of $O_2^{\cdot}$. It has been demonstrated that $O_2^{\cdot}$ cannot directly react with the diene bonds of polyunsaturated fatty acids to initiate lipid peroxidation [39,63,64,72]. However, activated oxygen intermediates, such as $^1O_2$ and HO$\cdot$ have been proposed to be formed from $O_2^{\cdot}$ by various reactions [65,135,146]. If formed, these reactive intermediates can directly react with polyunsaturated fatty acids to initiate lipid peroxidation

[30,180]. The involvement of $^1O_2$ and HO• in $O_2^{\bar{\cdot}}$-dependent lipid peroxidation has been proposed by several investigators [39,62,63,64,72,145, 154,170].

Pederson and Aust [62] proposed that $^1O_2$ was the initiating agent in $O_2^{\bar{\cdot}}$-dependent lipid peroxidation. They proposed that $^1O_2$ was formed by the nonenzymatic dismutation of $O_2^{\bar{\cdot}}$ [135]. McCay et al. [39,62,170] proposed that $O_2^{\bar{\cdot}}$-dependent lipid peroxidation was initiated by HO• formed via an iron catalyzed Haber-Weiss reaction [65]. Finally, Fridovich et al. [136,145] have proposed that $O_2^{\bar{\cdot}}$-dependent lipid peroxidation is initiated by $^1O_2$ formed via the uncatalyzed Haber-Weiss reaction.

There are several drawbacks to all of the mechanisms proposed, all of which require the dismutation of $O_2^{\bar{\cdot}}$ in a neutral aqueous solution to form either $^1O_2$ or H_2O_2. The H_2O_2 formed reacts further to produce HO• or $^1O_2$. First, the rate of nonenzymatic dismutation of $O_2^{\bar{\cdot}}$ in neutral aqeuous solutions is slow compared to other $O_2^{\bar{\cdot}}$ reactions especially at $O_2^{\bar{\cdot}}$ concentrations that would be generated by enzymatic activity in the reaction mixtures of interest [68,95,149]. Second, Nilsson and Kearns [137] and others [63,181] have recently questioned the formation of $^1O_2$ in an aqeuous reaction mixture containing a $O_2^{\bar{\cdot}}$ generator in the absence of enzymatic lipid peroxidation. Third, it is doubtful that the Haber-Weiss reaction, either catalyzed or uncatalyzed, participates in enzymatic lipid peroxidation since the levels of H_2O_2 that would accumulate in microsomes, which are contaminated with catalae, would be insufficient to account for the rates of initiation of lipid peroxidation observed [73,74]. Fourth, it is doubtful that HO•, if formed by an iron catalyzed Haber-Weiss reaction, would diffuse away from the site of formation before reacting [114]. It would more likely

react with the metal chelate at the site of formation before diffusing through the reaction media and into the interior of biological membranes. Fifth, it is doubtful that the uncatalyzed Haber-Weiss reaction occurs at rates sufficient to account for the rates of initiation of peroxidation observed by Fridovich et al. [67,68,70,182]. Finally, whether or not $^1O_2$ is formed by the uncatalyzed Haber-Weiss reaction has been questioned [71].

Previous investigations into the mechanism of lipid peroxidation led us to propose that lipid peroxidation occurred in two successive steps, initiation and propagation (Chapters 1 and 3). It was proposed that in $O_2^{\cdot-}$-dependent lipid peroxidation LOOH-independent initiation was catalyzed by the ADP-perferryl ion ($ADP\text{-}Fe^{+2}\text{-}O_2 \rightleftarrows ADP\text{-}Fe^{+3}\text{-}O_2^{\cdot-}$). Lipid hydroperoxide-independent initiation resulted in formation of low concentrations of lipid hydroxperoxides. It apparently did not involve $^1O_2$. In this chapter the LOOH-dependent initiation reaction of $O_2^{\cdot-}$-dependent lipid peroxidation will be demonstrated.

MATERIALS AND METHODS

Chemicals: The materials used in these investigations were obtained from the following sources: 2,5-diphenylfuran, Eastman Organic Company; bovine serum albumin, Pentex Chemical Company; ADP, butylated hydroxytoluene, cytochrome c (Type IV), xanthine and DTPA, Sigman Chemical Company; SKF 525-A, Smith, Kline and French Laboratories. All other chemicals used were of analytical grade. All chemicals were used without further purification.

Microsomes and Microsomal Lipid: Rat hepatic microsomes were isolated from 175-225 g male Sprague-Dawley rats (Spartan Research Animals, Haslett, Michigan) by the method of Pederson et al. [55]. Microsomes

were stored at -20°C under argon in argon saturated 0.05 M Tris-HCl -20% glycerol, pH 7.5 at 37°C. Total microsomal lipid was extracted from freshly prepared microsomes by the method of Folch et al. [97]. All solvents used for extraction procedures were purged with argon prior to use, and all operations were performed under argon at 4°C to minimize autoxidation of unsaturated lipids. The extracted lipid was stored at -20°C under argon in argon saturated $CHCl_2$:CH_3OH (2:1). Extracted lipids could be stored for several weeks without autoxidation as assayed by lipid hydroxperoxide content [33].

Enzyme Sources: Bovine erythrocyte superoxide dismutase (E.C.1.15.1), soybean liposygenase (Type I) (E.C.1.13.1.13) and xanthine oxidase (E.C.1.2.3.2) were obtained from Sigma Chemical Company. Superoxide dismutase activity was measured by the method of McCord and Fridovich [138]. Prior to use xanthine oxidase was desalted by column chromatography using Sephadex G-50. Xanthine oxidase activity was measured by aerobic cytochrome c reduction. Cytochrome P450 was purified by the method of Guengerich [150] to a specific content of 13.2 nmoles per mg protein.

Reaction Mixtures: Lipid hydroperoxides were generated in extracted microsomal lipid by the action of soybean lipoxygenase on detergent treated liposomes. Liposomes were prepared by sonication in distilled-deionized water at 4°C by the method of Pederson et al. [55] and diluted to a final concentration of 1.0 μmole lipid phosphate per ml in oxygenated 0.05 M Tris-HCl, pH 9.0 at 25°C. Sodium deoxycholate, 0.04% w/v, was added to the reaction mixture to accelerate the rate of lipid hydroperoxide formation [151]. Incubations were carried out in oxygen saturated buffers under an oxygen atmosphere in a metabolic shaking

water bath at 25°C. Reactions were initiated by the addition of soybean lipoxygenase (100 µg per ml). At the end of a 45 minute incubation period, lipids were extracted from the aqueous medium by the method of Folch et al. [97]. Lipid hydroperoxide content was measured as described by Buege and Aust [33]. Lipid hydroperoxide containing lipids were stored at -20°C under argon in argon saturated $CHCl_3$:CH_3OH (2:1). Under these conditions, the lipid hydroperoxide containing lipid could be stored for up to 2 months without further degradation as assayed by the formation of TBA reactive material [33].

Xanthine oxidase-dependent microsomal lipid peroxidation reaction mixtures contained 0.5 mg microsomal protein per ml, 1.7 mM ADP, 0.1 mM $FeCl_3$, 0.33 mM xanthine and 0.1 unit per ml xanthine oxidase in 0.05 M Tris-HCl, pH 7.5 at 37°C. Reactions were initiated by the addition of xanthine oxidase. Incubations were carried out under an air atmosphere at 37°C in a metabolic shaking water bath. Lipid peroxidation was measured by the formation of TBA-reactive material and lipid hydroperoxides [33]. Just prior to use of the TBA-TCA-HCl reagent, 0.03 volumes of a 2% butylated hydroxytoluene (BHT) ethanolic solution was added to prevent nonspecific chromophore formation during the assay procedure. Additions were made to the reaction mixtures as indicated in the tables. Rates of malondialdehyde and lipid hydroperoxide formation reported are initial rates.

Xanthine oxidase-promoted liposomal peroxidation reaction mixtures contained 1.0 µmole lipid phosphate per ml, 0.1 unit xanthine oxidase per ml, 1.7 mM ADP, 0.1 mM $FeCl_3$ and 0.33 mM xanthine in 0.05 M Tris-HCl, pH 7.5 at 37°C. Reactions were initiated by the addition of xanthine oxidase. Incubations and assays were performed as for xanthine

oxidase-dependent microsomal lipid peroxidation. Additions to the control reaction mixtures are as indicated in the legends to the tables.

The promotion of LOOH-dependent initiation of lipid peroxidation from lipid hydroperoxides was assessed by incubating lipid hydroperoxide containing liposomes with various propagating agents in 0.05 M Tris-HCl, pH 7.5 at 37°C. Additions to reaction mixtures are as specified in the legends to the appropriate tables. Incubations and assays were performed as for xanthine oxidase-dependent microsomal lipid peroxidation.

Other Methods: ADP, EDTA and DTPA chelated iron solutions were prepared in the following molar ratios: ADP:$FeCl_3$, 17:1; EDTA:$FeCl_3$, 1.1:1 and DTPA:$FeCl_3$, 1.1:1. Ferrous chelates were prepared using $FeCl_2$ in the same molar ratios as for ferric chelats. Buffers used in the preparation of ferrous ion chelates were purged with argon prior to use.

RESULTS

It has been prevously proposed that lipid peroxidaton occurs in two distinct sequential reactions, initiation and propagation (Chapters 1 and 3). In both NADPH-dependent and $O_2^{\cdot}$-dependent lipid peroxidation, initiation was defined as the formation of lipid hydroperoxides accompanied by minimal formation of malondialdehyde. Lipid hydroperoxide-independent initiation was proposed to be promoted by the ADP-perferryl ion. Investigation into the promotion of LOOH-dependent initiation during NADPH-dependent lipid peroxidation indicated that this form of initation was the reductive activation of initially formed lipid hydroperoxides generating reactive intermediates and products of lipid peroxidation. Lipid hydroperoxide-dependent initiation resulted in the rapid formation of malondialdehyde and additional lipid hydroperoxides.

The ability of a proposed agent to promote the LOOH-dependent initiation of lipid peroxidation should be reflected by its ability to promote the breakdown of lipid hydroperoxides. However, it would be difficult to asess lipid hydroperoxide breakdown in a polyunsaturated lipid matrix where lipid hydroperoxides could also be formed. Thus, cumene hydroperoxide was used to assess the ability of possible promoters of reductive activation to facilitate hydroperoxide breakdown. The choice of cumene hydroperoxide was based on its ability to support nonenzymatic microsomal lipid peroxidation [152] apparently derived from its ability to replace the initially formed lipid hydroperoxides in the mechanism of lipid peroxidation. In xanthine oxidase-dependent liposomal peroxidation, ADP-Fe^{+3} and EDTA-Fe^{+3} are required for maximal activity [62]. In the enzymatic reaction mixture, the $O_2^{\cdot}$ produced may reduce either of the metal complexes and thus a mixture of ferric and ferrous complexes exists [68,72,83,183]. To assess the participation of the ferric and ferrous forms of these iron chelates in LOOH-dependent initiation, these chelates were incubated with cumene hydroperoxide and their effect on the breakdown of cumene hydroperoxide was determined (Table 21). The ferrous ion chelates were formed by reduction by xanthine oxidase generated $O_2^{\cdot}$. As shown in Table 21 ADP-Fe^{+3}, EDTA-Fe^{+3}, ADP-Fe^{+2} and $O_2^{\cdot}$ were unable to promote the breakdown of cumene hydroperoxide. Only EDTA-Fe^{+2} can promote the rapid breakdown of cumene hydroperoxide. From these results, it appears that of the 4 iron chelates only EDTA-Fe^{+2} is likely to function as the promoter of LOOH-dependent initiation in $O_2^{\cdot}$-dependent lipid peroxidation.

The ability of these iron chelates to promote LOOH-dependent initiation of lipid peroxidation from lipid hydroperoxides was assessed by

TABLE 21

THE $O_2^{\cdot}$-DEPENDENT DECOMPOSITION OF CUMENE HYDROPEROXIDE IN THE PRESENCE OF CHELATED IRON

Reaction mixtures contained 200 nmoles of cumene hydroperoxide/ml, 0.33 mM xanthine and 0.1 unit xanthine oxidase/ml in 0.05 M Tris-HCl pH 7.5 at 37°C. Control reaction mixtures lacked xanthine oxidase. The following additions were made where indicated: 0.11 mM EDTA and 0.1 mM $FeCl_3$; 1.7 mM ADP and 0.1 mM $FeCl_3$. Reactions were initiated by the addition of the appropriate iron form for control reaction mixtures and by addition of xanthine oxidase to other reaction mixtures.

	nmoles cumene hydroperoxide lost $min\ ml^{-1}$
Control	
+ADP-Fe^{+3}	0.0
+EDTA-Fe^{+3}	0.0
+ADP-Fe^{+3} +EDTA-Fe^{+3}	0.0
+xanthine oxidase	0.0
+ADP-Fe^{+3}	0.3
+EDTA-Fe^{+3}	1.33
+ADP-Fe^{+3} +EDTA-Fe^{+3}	1.82

their addition to liposomes containing lipid hydroperoxides and measuring the formation of both TBA-reactive material (malondialdehyde) and lipid hydroperoxide (Table 22). Only EDTA-Fe^{+2} can promote LOOH-dependent initiation of lipid peroxidation. The initial rate of malondialdehyde formation, 1.2 nmoles/min/ml, reflects significant LOOH-dependent initiation since only 10% or less of total peroxidation is reflected in malondialdehyde formation (31,153). While the amount of malondialdehyde formed is a small percentage of total peroxidation products, it appears to be formed at a constant ratio to total products in a well defined reaction system as dealt with here. The sensitivity of the spectrophotometric assay makes it a most useful method for the determination of rate and extent of lipid peroxidation. The rate of lipid hydroperoxide formation, 9.3 nmoles/min/ml, also reflects significant LOOH-dependent initiation of lipid peroxidation. It must be realized that during LOOH-dependent initiation lipid hydroperoxides are both reactants and products. If lipid hydroperoxide breakdown occurs at a rate similar to cumene hydroperoxide breakdown catalyzed by EDTA-Fe^{+2}, 2.5 nmoles/min/ml (Table 21), an increase in lipid hydroperoxide content of 9.3 nmoles/min/ml actually reflects a formation rate of 11.8 nmoles/min/ml for lipid hydroperoxides.

The ability of ADP-Fe^{+2} to promote LOOH-dependent initiation of lipid peroxidation is actually considerably less than that indicated in Table 22 since ADP-Fe^{+2} can catalyze the LOOH-independent initiation of lipid peroxidation to the extent of 0.3 nmoles malondialdehyde/min/ml and 1.3 nmoles lipid hydroperoxide/min/ml (63). If these rates of LOOH-independent initiation are subtracted from the rates of ADP-Fe^{+2} promoted LOOH-dependent initiation in Table 22, it is apparent that

TABLE 22

IRON CHELATE PROMOTION OF LOOH-DEPENDENT INITIATION OF LIPID PEROXIDATION

Reaction mixtures contained 1.0 μmole lipid phosphate/ml (containing 0.1 μmole LOOH/μmole lipid phosphate) in 0.05 M Tris-HCl, pH 7.5 at 37°C. The following additions were made to the reaction mixtures as indicated: 1.7 mM ADP and 0.1 mM $FeCl_3$; 1.7 mM ADP and 0.1 mM $FeCl_2$; 0.11 mM EDTA and 0.1 mM $FeCl_3$; 0.11 mM EDTA and 0.1 mM $FeCl_2$; 1.0 unit SOD[a]; 0.2 mM DPF; 1.0 mM BHT and 40 mM benzoate. Reactions were initiated by addition of the appropriate iron form. Incubations and assays were performed as described under "Methods."

		nmoles MDA $min\ ml^{-1}$	nmoles LOOH $min\ ml^{-1}$
Control (No Additions)		0.01	0.6
+ADP-Fe^{+3}		0.02	0.6
+EDTA-Fe^{+3}		0.01	1.1
+ADP-Fe^{+2}		0.60	1.3
+EDTA-Fe^{+2}		1.16	9.3
+EDTA-Fe^{+2}	+SOD	1.11	6.2
+EDTA-Fe^{+2}	+DPF	1.03	6.0
+EDTA-Fe^{+2}	+BHT	0.14	1.0
+EDTA-Fe^{+2}	+Benzoate	1.22	8.8

[a]superoxide dismutase

ADP-Fe^{+2} promotion of LOOH-dependent initiation is minimal compared to EDTA-Fe^{+2} promotion of LOOH-dependent initiation.

The radical nature of the reactive intermediates formed during EDTA-Fe^{+2} catalyzed breakdown of lipid hydroperoxides is shown by the inhibition of LOOH-dependent initiation of lipid peroxidation upon addition of butylated hydroxytoluene (Table 22). Radicals formed may include $O_2^{\bullet-}$, lipid alkyl, lipid alkoxy and lipid hydroperoxy radicals. Lipid hydroperoxy radicals are a likely source of secondary $^1O_2$ formation during active lipid peroxidation (29,163). The secondary nature of $^1O_2$ is indicated by the minimal inhibition of lipid peroxidation in the presence of 2,5-diphenylfuran. The lack of inhibition upon the addition of benzoate indicates that HO• does not participate in propagation.

The ability of EDTA-Fe^{+3} to promote LOOH-dependent initiation of lipid peroxidation shown in Table 23. Lipid hydroperoxide-dependent initiation, as detected by both malondialdehyde and lipid hydroperoxide formation, is significant only in the presence of xanthine oxidase. It is apparent that the participation of EDTA-Fe^{+3} in $O_2^{\bullet-}$-dependent lipid peroxidation is dependent upon its reduction.

Investigation into the nature of the reactive intermediates formed during enzymatically promoted LOOH-dependent initiation (Table 23) gave results similar to those obtained during nonenzymatic promotion of LOOH-dependent initiation (Table 22) except for the greatly increased inhibition of LOOH-dependent initiation by superoxide dismutase. In $O_2^{\bullet-}$-promotion of LOOH-dependent initiation superoxide dismutase inhibits the formation of EDTA-Fe^{+2} and thus inhibits LOOH-dependent initiation of lipid peroxidation. Superoxide dismutase inhibits malondialdehyde formation by 86% and lipid hydroperoxide formation by 75%. The addition

TABLE 23

SUPEROXIDE-PROMOTED LOOH-DEPENDENT INITIATION

Reaction mixtures contained 1.0 μmole lipid phosphate/ml (containing 0.1 μmole LOOH/μmole lipid phosphate), 0.33 mM xanthine, 0.11 mM EDTA and 0.1 mM $FeCl_3$ and 0.1 unit xanthine oxidase/ml in 0.05 M Tris-HCl, pH 7.5 at 37°C. The following additions were made as indicated: 1.0 unit SOD/ml; 0.2 mM DPF; 1.0 mM BHT; 40 mM benzoate. Reactions were initiated by the addition of xanthine oxidase. Incubations and assays were performed as described under "Methods."

	nmoles MDA $min\ ml^{-1}$	nmoles LOOH $min\ ml^{-1}$
Control (- xanthine oxidase)	0.01	0.1
+ xanthine oxidase	0.77	4.4
+ xanthine oxidase + SOD	0.02	1.1
+ xanthine oxidase + DPF	0.68	2.8
+ xanthine oxidase + BHT	0.01	0.1
+ xanthine oxidase + Benzoate	0.77	5.0

of 2,5-diphenylfuran to the reaction mixture inhibited malondialdehyde and lipid hydroperoxide formation by 25% and 38%, respectively. (While there is some question as to the activity of 2,5-diphenylfuran as a radical trapping agent, we have been unable to show significant inhibition of ascorbate-iron dependent lipid peroxidation by 2,5-diphenylfuran at the concentration used in these experiments.) Addition of butylated hydroxytoluene completely inhibited LOOH-dependent initiation demonstrating the radical nature of the reaction. However, addition of benzoate did not inhibit $O_2^{\cdot}$-promotion of LOOH-dependent initiation of lipid peroxidation indicating that HO· does not participate in the reaction.

In xanthine oxidase-promoted lipid peroxidation of liposomes, LOOH-independent initiation and LOOH-dependent initiation occur simultanously. Lipid hydroperoxide-dependent initiation accounts for a significant portion of the malondialdehyde and lipid peroxidation formed during $O_2^{\cdot}$-dependent lipid peroxidation (Table 24). In the presence of ADP-iron alone, the rate of malondialdehyde and LOOH formation in $O_2^{\cdot}$-dependent lipid peroxidation is 0.18 nmoles/min/ml and 1.6 nmoles/min/ml, respectively. When the EDTA-Fe^{+3}, a promoter of LOOH-dependent initiation is included in the reaction mixture malondialdehyde and lipid hydroperoxide formation rates are increased by 11- and 14-fold, respectively. Superoxide dismutase, which can inhibit both LOOH-independent [63] and LOOH-dependent initiation (Table 23) of $O_2^{\cdot}$-dependent lipid peroxidation, almost totally inhibits $O_2^{\cdot}$-dependent lipid peroxidation. In this reaction mixture superoxide dismutase inhibits malondialdehyde and lipid hydroperoxide formation by 30% and 13%, respectively. The inhibition observed is consistent with that observed during the

$O_2^{\cdot}$-dependent propagation of lipid peroxidation (Table 23) emphasizing that $^1O_2$ is produced during LOOH-dependent initiation of lipid peroxidation. These results also emphasize the dependence of LOOH-dependent initiation upon LOOH-independent initiation. The addition of butylated hydroxytoluene to the reaction mixture completely inhibits both malondialdehyde and lipid hydroperoxide formation demonstrating the radical nature of LOOH-independent initiation. The radical nature of LOOH-dependent initiatiion was previously shown (Table 22). Addition of benzoate to the reaction mixture dd not inhibit $O_2^{\cdot}$-dependent lipid peroxidation indicating HO• participates in neither LOOH-independent or -dependent initiation (Tables 22 and 23).

The iron chelate DTPA has been used to inhibit the metal promoted breakdown of lipid hydroperoxides during $O_2^{\cdot}$-dependent lipid peroxidation [154]. Considering that the structure of DTPA is similar to that of a EDTA dimer, we examined the ability of DTPA-Fe^{+3} to replace EDTA-Fe^{+3} in $O_2^{\cdot}$-dependent lipid peroxidation (Table 25). The data indicates that DTPA-Fe^{+3} in the absence of ADP-Fe^{+3} could not function in the LOOH-independent initiation of $O_2^{\cdot}$-dependent lipid peroxidation.

The involvement of cytochrome P450 in $O_2^{\cdot}$-dependent lipid peroxidation of microsomes was investigated utilizing SKF 525-A. The drug SKF 525-A has been shown to inhibit NADPH-dependent microsomal lipid peroxidation by interacting with cytochrome P450 [155]. In an analogous manner, SKF 525-A was found to inhibit $O_2^{\cdot}$-dependent peroxidation of microsomes by 70% (Table 26). The addition of EDTA-Fe^{+3} to an SKF 525-A inhibited reaction mxture completely reversed the inhibition. The data indicate that SKF 525-A inhibits microsomal lipid peroxidation by

TABLE 24

SUPEROXIDE-DEPENDENT LIPID PEROXIDATION

Reaction mixtures contained 1.0 μmole lipid phosphate/ml, 1.7 mM ADP and 0.1 mM $FeCl_3$, 0.33 mM xanthine and 0.1 unit xanthine oxidase/ml in 0.05 M Tris-HCl, pH 7.5 at 37°C. The following additions were made as indicated: 0.11 mM EDTA and 0.1 mM $FeCl_3$; 1.0 unit SOD/ml; 0.2 mM DPF; 1.0 mM BHT and 40 mM benzoate. Reactions were initiated by the addition of xanthine oxidase. Incubations and assays were performed as described under "Methods."

	nmoles MDA $min\ ml^{-1}$	nmoles LOOH $min\ ml^{-1}$
Control (- xanthine oxidase)	0.02	0.8
+ EDTA-Fe^{+3}	0.01	1.0
+ xanthine oxidase	0.18	1.6
+ xanthine oxidase + EDTA-Fe^{+3}	2.36	22.8
+ xanthine oxidase + EDTA-Fe^{+3} + SOD	0.03	5.0
+ xanthine oxidase + EDTA-Fe^{+3} + DPF	1.67	19.9
+ xanthine oxidase + EDTA-Fe^{+3} + BHT	0.01	0.1
+ xanthine oxidase + EDTA-Fe^{+3} + Benzoate	2.64	23.9

TABLE 25

SUPEROXIDE-DEPENDENT LOOH-DEPENDENT INITIATION OF LIPID PEROXIDATION PROMOTED BY EDTA-Fe^{+3} AND DTPA-Fe^{+3}

Reaction mixtures contained 1.0 μmole lipid phosphate/ml and 0.33 mM xanthine in 0.55 Tris-HCl, pH 7.5 at 37°C. The following additions were made as indicated: 0.1 unit xanthine oxidase/ml; 1.7 mM ADP and 0.1 mM $FeCl_3$; 0.11 mM EDTA and 0.1 mM $FeCl_3$; 0.11 mM DTPA and 0.1 mM $FeCl_3$. Reactions were initiated by the addition of xanthine oxidase. Incubations and assays were performed as described under "Methods."

	nmoles MDA min ml^{-1}	nmoles LOOH min ml^{-1}
Control (- xanthine oxidase)	0.01	0.1
+ EDTA-Fe^{+3}	0.01	0.2
+ DTPA-Fe^{+3}	0.01	0.2
+ xanthine oxidase	0.20	0.8
+ xanthine oxidase + EDTA-Fe^{+3}	0.02	0.9
+ xanthine oxidase + DTPA-Fe^{+3}	0.02	0.8
+ xanthine oxidase + ADP-Fe^{+3}	0.71	5.2
+ xanthine oxidase + ADP-Fe^{+3} + EDTA-Fe^{+3}	4.17	13.6
+ xanthine oxidase + ADP-Fe^{+3} + DTPA-Fe^{+3}	3.24	14.8

TABLE 26

THE EFFECT OF SKF 525-A ON SUPEROXIDE-DEPENDENT MICROSOMAL LIPID PEROXIDATION

Reaction mixtures contained 0.5 mg microsomal protein/ml, 0.33 mM xanthine, 0.1 unit xanthine oxidase/ml and 1.7 mM ADP-0.1 mM $FeCl_3$ in 0.05 M Tris-HCl, pH 7.5 at 37°C. The following additions were made as indicated: 100 μM SKF 525-A and 0.11 mM EDTA-0.1 mM $FeCl_3$. Reactions were initiated by the addition of xanthine oxidase. Incubations and assays were performed as described under "Methods."

	nmoles MDA $min\ ml^{-1}$
Control (- xanthine oxidase)	0.59
+ xanthine oxidase	5.23
+ xanthine oxidase + SKF 525-A	1.58
+ xanthine oxidase + SKF 525-A + EDTA-Fe^{+3}	5.26

interacting with an endogenous microsomal promoter of LOOH-dependent initiation. The specificity of the SKF 525-A reaction [155] indicates that the endogenous microsomal promoter of LOOH-dependent initiation agent may be cytochrome P450.

The ability of ferric cytochrome P450 to function as a promoter of LOOH-dependent initiation of lipid peroxidation in $O_2^{\cdot}$-dependent peroxidation of liposomes is clearly shown in Table 27. In the presence of ADP-Fe^{+3}, xanthine oxidase promotes only LOOH-independent initiation of lipid peroxidation as indicated by the low rae of lipid hydroperoxide and malondialdehyde formation. The addition of 0.3 nmoles ferric cytochrome P450/ml as a promoter of LOOH-dependent initiation increases malondialdehyde and lipid hydroperoxides formation rates by 7- and 5-fold, respectively. These results indicate the key role played by cytochrome P450 in the $O_2^{\cdot}$-dependent peroxidation of microsomal lipid.

DISCUSSION

These results preesnted here suggest a mechanism of $O_2^{\cdot}$-dependent lipid peroxidation. Previously, it was shown that lipid peroxidation occurred in two sequential radical reactions (Chapters 1 and 3). Investigation of the first reaction, LOOH-independent initiation, led to the proposal that the reaction was promoted by the ADP-perferryl ion and resulted in the formation of mainly lipid hydroperoxides. The lipid hydroperoxides formed during this reaction were proposed to be the reactants in a second reaction, LOOH-dependent initiation. In this chapter it is shown that LOOH-dependent initiation is the metal promoted breakdown of initially formed lipid hydroperoxides generating reactive intermediates of lipid peroxidation that continue the oxidative degradation of PUFA. Lipid hydroperoxide-dependent initiation of lipid peroxidation

TABLE 27

PROMOTION OF LOOH-DEPENDENT INITIATION OF O_2^--DEPENDENT LIPID PEROXIDATION BY FERRIC CYTOCHROME P450

Reaction mixtures contained 1.0 μmole lipid phosphate/ml, 0.33 mM xanthine, and 1.7 mM ADP-0.1 mM $FeCl_3$. The following additions were made as indicated: 0.1 unit xanthine/ml oxidase and 0.3 nmole ferric cytochrome P450/ml. Reactions were initiated by the addition of xanthine oxidase. Incubations and assays were performed as described under "Methods."

	nmoles MDA $min\ ml^{-1}$	nmoles LOOH $min\ ml^{-1}$
Control (No additions)	0.03	0.1
+ ferric cytochrome P450	0.05	0.3
+ xanthine oxidase	0.71	5.2
+ xanthine oxidase + ferric cytochrome P450	5.1	24.1

can be promoted by EDTA-Fe^{+2}, DTPA-Fe^{+2} or by ferric cytochrome P450. In $O_2^{\cdot}$-dependent lipid peroxidation LOOH-dependent initiation accounts for approximately 80% of the total peroxidation.

Xanthine oxidase-promoted liposomal peroxidation requires both ADP-Fe^{+3} and EDTA-Fe^{+3} for maximal activity [62]. In a reaction mixture where $O_2^{\cdot}$ is being produced these iron chelates may exist in either their ferric or ferrous form, the presence of the ferrous form being $O_2^{\cdot}$-dependent. We have previously shown that only the ADP-perferryl ion can promote LOOH-independent initiation as reflected by the formation of lipid hydroperoxides in unperoxidized lipid [63]. The reaction promoted by the ADP-perferryl ion accounts for 15-20% of the total peroxidative products formed during xanthine oxidase-promoted liposomal peroxidation [63]. Addition of EDTA-Fe^{+3} to the xanthine oxidase-dependent reaction mixture was necessary for maximal activity. The EDTA-iron complex apparently expands upon ADP-perferryl ion-dependent LOOH-independent initiation of lipid peroxidation. In LOOH-dependent initiation, the promoting agent must be able to efficiently promote the formation of reactive intermediates of lipid peroxidation. In iron-mediated xanthine oxidase-catalyzed liposomal peroxidaton reactive intermediates are likely to be formed by iron-mediated breakdown of initially formed lipid hydroperoxides. Reactive intermediates are likely to include a variety of lipid derived radicals, such as the lipid alkoxy radical. Using cumene hydroperoxide as a model organic hydroperoxide to assay the promotional activity of the four possible iron complexes present during enzymatic promotion of lipid peroxidation, it was found that only EDTA-Fe^{+2} could promote the efficient breakdown of the organic hydroperoxide. In like manner, it was observed that only EDTA-Fe^{+2}

could promote the LOOH-dependent initiation of lipid peroxidation. Although the formation of malondialdehyde and lipid hydroperoxides by ADP-Fe^{+2} promoted LOOH-dependent initiation at first appears significant, it must be recognized that ADP-Fe^{+2} can promote LOOH-independent initiation of lipid peroxidation in unperoxidized fatty acids (Chapters 1 and 3). Considering the ability of ADP-Fe^{+2} to promote LOOH-independent initiation of lipid peroxidation at rates similar to those observed in the investigation of LOOH-dependent initiation of lipid peroxidation, it is evident that ADP-Fe^{+2} promoted LOOH-dependent initiation is minimal. Thus, although it has been previously shown that EDTA-Fe^{+2} cannot promote the LOOH-independent initiation of lipid peroxidation (Chapters 1 and 3) it does promote in the LOOH-dependent initiation of lipid peroxidation.

The nature of some of the intermediates of EDTA-Fe^{+2} promoted LOOH-dependent initiation was demonstrated by the effects of addition of various reported inhibitors of lipid peroxidation. The observed inhibition of LOOH-dependent initiation upon the addition of superoxide dismutase and 2,5-diphenylfuran indicate that both $O_2^{\cdot}$ and $^1O_2$ may play relatively minor roles as reactive intermediates formed during the reaction. The presence of $^1O_2$ perhaps indicates that lipid hydroperoxide radicals are generated during propagation. It has been shown that $^1O_2$ is generated by the bimolecular reaction of organic hydroperoxide radicals [29,163] and that $^1O_2$ is present only during LOOH-dependent initiation of lipid peroxidation and does not participate in LOOH-independent [63,72]. The addition of butylated hydroxytoluene totally inhibits the EDTA-Fe^{+2} promoted reaction indicating the radical nature of LOOH-dependent initiation and strengthening the proposal that $^1O_2$ is a secondary

product of LOOH-dependent initiation arising from the formation of some primary radical species. However, as evidenced by the lack of inhibition upon addition of benzoate, HO• is not one of the radical intermediates formed during the LOOH-dependent initiation of lipid peroxidation.

Using xanthine oxidase to generate $O_2^{\bullet}$ and thus reduce EDTA-Fe^{+3} *in situ* it was demonstrated that the enzymatic promotion of LOOH-dependent initiation was essentally equivalent to the nonenzymatic promotion of LOOH-dependent initiation except for the expected sensitivity to superoxide dismutase. The enzymatic reaction shows increased sensitivity to superoxide dismutase since the formation of the promoting complex, EDTA-Fe^{+2}, is dependent upon the reduction of EDTA-Fe^{+3} by $O_2^{\bullet}$. Superoxide dismutase does not completely inhibit LOOH-dependent initiation since EDTA-Fe^{+3} can compete with superoxide dismutase for $O_2^{\bullet}$ [83]. The inhibition of LOOH-dependent initiation upon the addition of 2,5-diphenylfuran was found to be equivalent to that observed in the nonenzymatic reaction mixture. The LOOH-dependent initiation reaction was not inhibited by benzoate but was completley inhibited by butylated hydroxytoluene. Thus, the overall enzymatic reaction is dependent upon the $O_2^{\bullet}$ reduction of EDTA-Fe^{+3}. The EDTA-Fe^{+2} promotion of lipid hydroperoxide breakdown generates mainly radical intermediates of lipid peroxidation and produces $^1O_2$ as a secondary product of radical generation. The hydroxyl radical does not appear to be generated by the LOOH-dependent initiation reaction.

The effect of various reported inhibitors of $O_2^{\bullet}$-dependent liposomal peroxidation are consistent with both the mechanism of LOOH-independent initiation proposed previously (Chapter 1) and the mechanism of

LOOH-dependent initiation being proposed here. Superoxide dismutase almost completely inhibits enzymatic lipid peroxidation because it inhibits both $O_2^{\cdot}$ promoted LOOH-independent and -dependent initiation of lipid peroxidation. The results also indicate that $^1O_2$ is a minor participant in $O_2^{\cdot}$-dependent lipid peroxidation. It was previously shown that $^1O_2$ does not particpate in the LOOH-independent initiation of $O_2^{\cdot}$-dependent lipid peroxidation (Chapter 1). The minimal inhibition evidenced here indicates that $^1O_2$ participates only in the LOOH-dependent initiation of lipid peroxidation. This is consistent with the observed participation of $^1O_2$ in the LOOH-dependent initiation of NADPH-dependent lipid peroxidation. Addition of butylated hydroxytoluene to the reaction mixture completely inhibits $O_2^{\cdot}$-dependent lipid peroxidation indicating the radical nature of the LOOH-independent initiation reaction. The lack of benzoate inhibition of $O_2^{\cdot}$-dependent lipid peroxidation indicates that HO$\cdot$ does not participate in either the LOOH-independent initiation of $O_2^{\cdot}$ promoted lipid peroxidation.

Ethylenediamine tetraacetate is not unique among the chelators of iron in its ability to facilitate the iron promotion of LOOH-dependent initiation of lipid peroxidation. Diethylenetriamine pentaacetate-Fe^{+3} can also promote LOOH-dependent initiation of $O_2^{\cdot}$-dependent lipid peroxidation. This effect has been previously demonstrated in NADPH-dependent lipid peroxidation (Chapter 3). The effect of chelation upon the reduction potential of iron is perhaps the single most important factor in the formation of a promoter of LOOH-dependent initiation of lipid peroxidation. Free iron in aqueous solution has a reduction potential of approximately 0.77 v [164,165]. Adenosine 5'-diphosphate chelated-Fe^{+2} is an efficient initiator of lipid peroxidation (Chapter

1) but a very poor promoter of the breakdown of organic hydroperoxides, or the LOOH-dependent initiation of lipid peroxidation. Chelation of iron by EDTA lowers the reduction potential of iron to approximately 0.25 v [164,165] making the ferrous complex a strong reducing agent. If LOOH-dependent initiation is a Fentons type reaction, producing the alkoxy radicals and OH^-, then the promotion of the reaction would increase with the reducing strength of the iron complex. Such an effect of iron chelation has been shown for the Fentons-type reaction with H_2O_2 [65]. Since the chelation of iron by DTPA is similar to that by EDTA [166,167], the change in the reduction potential of the iron upon chelation appears to determine the complexes' effectiveness as a promoter of LOOH-dependent initiation of lipid peroxidation.

As previously discussed (Chapter 3), but more directly shown here, the ability of DTPA-Fe^{+2} to promote the LOOH-dependent initiation of lipid peroxidation may offer an alternate explanation to the experimental data on $O_2^{\cdot}$-dependent lipid peroxidation reported by Thomas et al. [154]. They proposed that $O_2^{\cdot}$-dependent lipid peroxidation was dependent upon the presence of contaminating levels of lipid hydroperoxides formed by autoxidation in the lipid matrix. The mechanism of $O_2^{\cdot}$-dependent lipid peroxidation proposed involved the reaction of $O_2^{\cdot}$ with LOOH as follows:

$$O_2^{\cdot} + LOOH \rightarrow LO\cdot + O_2 + OH^- \qquad (38)$$

The reaction proposed is equivalent to an uncatalyzed Haber-Weiss reaction [65]. The alkoxy radical (LO·) formed was proposed to initiate lipid peroxidation by hydrogen abstraction and formation of lipid radicals from adjacent polyunsaturated fatty acids. The experimental procedure of Thomas et al. (the use of DTPA to chelate contaminating iron in

incubation buffers), the dependence of peroxidation on lipid hydroperoxide concentration, the kinetics of the observed reaction and the correlation of the data of Thomas et al. with that data which is reported here for the NADPH-dependent (Chapter 1) and $O_2^{\cdot}$-dependent LOOH-dependent initiation of lipid peroxidation (Table 25) indicates that Thomas et al. may have observed an iron-dependent LOOH-dependent initiation of lipid peroxidation promoted by DPTA-Fe^{+2} as outlined below.

$$DTPA\text{-}Fe^{+3} + O_2^{\cdot} \rightarrow DTPA\text{-}Fe^{+2} + O_2 \quad (39)$$

$$DTPA\text{-}Fe^{+2} + LOOH \rightarrow DTPA\text{-}Fe^{+3} + LO\cdot + OH^- \quad (40)$$

As in NADPH-dependent microsomal lipid peroxidation, addition of EDTA-Fe^{+3} enhances $O_2^{\cdot}$-dependent microsomal lipid peroxidation but is not required. In a manner analogous to the investigations into the identity of an endogenous microsomal promoter of LOOH-dependent initiation in NADPH-dependent microsomal lipid peroxidation (Chapter 3) SKF 525-A, a specific inhibitor of cytochrome P450 mediated reactions [155], was used to determine if ferric cytochrome P450 participated in $O_2^{\cdot}$-dependent microsomal lipid peroxidation. It was observed that SKF 525-A inhibits $O_2^{\cdot}$-dependent microsomal lipid peroxidation in a similar manner to NADPH-dependent microsomal lipid peroxidation (Chapter 3). The addition of EDTA-Fe^{+3} completely reverses the SKF 525-A inhibition of $O_2^{\cdot}$-dependent microsomal lipid peroxidation. The specificity of the interaction of SKF 525-A with cytochrome P450 [155] and the complete reversal of inhibition upon addition of EDTA-Fe^{+3}, a promoter of LOOH-dependent initiation, indicates that cytochrome P450 is an endogenous microsomal promoter of LOOH-dependent initiation in $O_2^{\cdot}$-dependent microsomal lipid peroxidation.

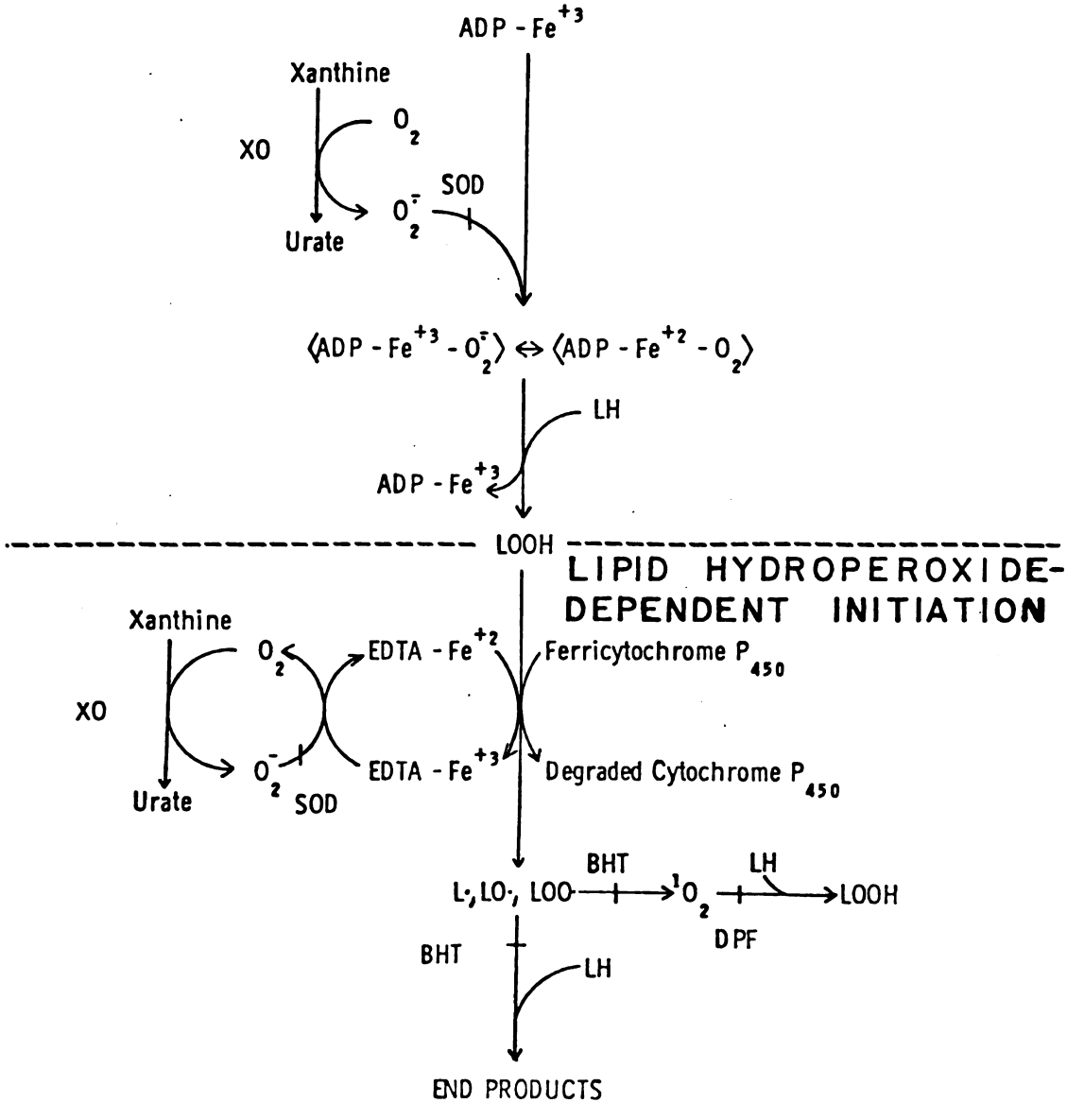
These results also indicate the complexity of the interrelationship of the reactions that constitute lipid peroxidation. The dependence of the LOOH-dependent initiation reaction(s) upon the products of the LOOH-independent initiation reaction(s) is clearly demonstrated and the data indicate that the overall rate of peroxidation, under these experimental conditions, is dependent upon the rate of LOOH-independent initiation of lipid peroxidation and not LOOH-dependent initiation of lipid peroxidation. This is shown by the results of the experiment in which cytochrome P450 participation in LOOH-dependent initiation is blocked by SKF 525-A and the inhibition is reversed by the addition of EDTA-Fe^{+3}. The addition of EDTA-Fe^{+3}, a promoter of LOOH-dependent initiation which can be redox cycled, in more than a thousand fold excess of microsomal cytochrome P450 concentration restores the rate of peroxidation but does not stimulate the rate above that inthe uninhibited reaction mixture. Since the huge excess of added promoter does not stimulate peroxidation it is apparent that LOOH-independent and not LOOH-dependent initiation is the rate limiting reaction in lipid peroxidation under these experimental conditions.

The ability of ferric cytochrome P450 to promote the LOOH-dependent initiation of lipid peroxidation has been previously demonstrated (Chapter 1). The ability of ferric cytochrome P450 to function in the LOOH-dependent initiation of $O_2^{\cdot-}$-dependent peroxidation of liposomes where initial lipid hydroperoxides are generated <u>in situ</u> by the ADP-perferryl ion was shown. The addition of 0.3 nmole ferric cytochrome P450/ml to an incubation mixture promoting the LOOH-independent initiation of lipid peroxidation results in a 7-fold and 5-fold increase in malondialdehyde and lipid hydroperoxide formation, respectively. Thus,

ferric cytochrome P450 is apparently an excellent promoter of LOOH-dependent initiation of $O_2^{\cdot}$-dependent lipid peroxidation.

The data presented in this chapter indicates that $O_2^{\cdot}$-dependent lipid peroxidation ocurs in two sequential free radical steps. Lipid hydroperoxide-independent initiation is promoted by the ADP-perferryl ion and leads to the formation of lipid hydroperoxides (Chapter 1). Lipid hydroperoxide-dependent initiation of $O_2^{\cdot}$-promoted lipid peroxidation can be promoted by EDTA-Fe^{+2}, DTPA-Fe^{+2} and by ferric cytochrome P450. Lipid hydroperoxide-dependent initiation of lipid peroxidation results in the formation of high levels of malondialdehyde and lipid hydroperoxides from originally formed lipid hydroperoxides. These findings are schematically presented in Figure 9.

Figure 8. Schematic of the Reactions of $O_2^{\cdot -}$-Dependent Lipid Peroxidation.


PERFERRYL ION-DEPENDENT INITIATION
ADP - Fe+3
Xanthine
XO
O2
O2-
SOD
Urate
⟨ADP - Fe+3 - O2-⟩ ⇔ ⟨ADP - Fe+2 - O2⟩
LH
ADP - Fe+3
LOOH
LIPID HYDROPEROXIDE-DEPENDENT INITIATION
Xanthine
XO
O2
O2-
EDTA - Fe+2
EDTA - Fe+3
SOD
Urate
Ferricytochrome P450
Degraded Cytochrome P450
L·, LO·, LOO·
BHT
1O2
LH
LOOH
DPF
BHT
LH
END PRODUCTS

SUMMARY

The results presented in this thesis demonstrate that NADPH-dependent and $O_2^{\cdot}$-dependent peroxidation of microsomal phospholipid occur by similar if not the same mechanism. Lipid peroxidation is proposed to consist of two sequential steps. The first step, LOOH-independent initiation, involves the formation of low levels of LOOH in previously unperoxidized lipid. The second step, LOOH-dependent initiation is the breakdown of initially formed LOOH generating reactive intermediates of lipid peroxidation.

LOOH-independent initiation is promoted by an ADP-ferrous ion dependent reaction. Nonenzymatic initiation of lipid peroxidation promoted by ADP-Fe^{+2} is SOD sensitive. Since $O_2^{\cdot}$ cannot directly promote LOOH-independent initiation of lipid peroxidation, the possible involvement of reactive oxygen intermediates, $^1O_2$ and HO$\cdot$ formed by the nonenzymatic dismutation of $O_2^{\cdot}$ and subsequent reactions in initiation was investigated. The ADP-Fe^{+2} LOOH-independent initiation reaction was only slightly inhibited by addition of DPF indicating that $^1O_2$ was not involved in the reaction to any great extent. The NADPH-dependent LOOH-independent initiation of lipid peroxidation was found to be dependent upon ADP-Fe^{+3} indicating that initiation likely occurred by an ADP-Fe^{+2} mediated reaction. The NADPH-dependent LOOH-independent initiation of lipid peroxidation demonstrated SOD sensitivity but little participation by $^1O_2$. NADPH-dependent LOOH-independent initiation was found to be radical in nature but not to involve HO$\cdot$. Thus the LOOH-independent initiation of NADPH-dependent lipid peroxidation did not require the nonenzymatic dismutation of $O_2^{\cdot}$ to give rise to $^1O_2$ or further reactions of the dismutation products to generate HO$\cdot$ as has been proposed by

others. The requirement for ADP-iron in the LOOH-independent initiation and the sensitivity of their initiation to SOD suggest that NADPH-dependent initiation of lipid peroxidation may occur via an iron bound $O_2^{\cdot}$ complex or the ADP-perferryl ion. Superoxide dismutase dismutation of iron bound $O_2^{\cdot}$ has been suggested by others [144]. Superoxide-dependent LOOH-independent initiation of lipid peroxidation is also SOD sensitive and radical in nature. The $O_2^{\cdot}$-dependent LOOH-independent initiation of lipid peroxidation does not involve $^1O_2$ or HO·. Again, since $O_2^{\cdot}$ is not reactive enough to directly promote LOOH-independent initiation of lipid peroxidation [39,64,72] and because possible reactive intermediates formed during and subsequent to the nonenzymatic dismutation of $O_2^{\cdot}$ do not participate in LOOH-independent initiation of $O_2^{\cdot}$-dependent lipid peroxidation, it is here proposed that the ADP-perferryl ion catalyzes the LOOH-independent initiation of $O_2^{\cdot}$-dependent lipid peroxidation. Thus, it appears that LOOH-independent initiation of lipid peroxidation in both NADPH-dependent and $O_2^{\cdot}$-dependent lipid peroxidation may occur via the ADP-perferryl ion. In NADPH-dependent lipid peroxidation the ADP-perferryl ion is likely formed by direct reduction of the ferric chelate and its subsequent reaction with $O_2^{\cdot}$. In $O_2^{\cdot}$-dependent lipid peroxidation the ADP-perferryl ion is formed by the reaction of $O_2^{\cdot}$ with the ferric chelate.

Lipid hydroperoxide-dependent initiation of lipid peroxidation can be promoted by EDTA-Fe^{+2}, DTPA-Fe^{+2}, and ferric cytochrome P450. The EDTA-Fe^{+2} promotion of LOOH-dependent initiation of lipid peroxidation showed only minor inhibition by SOD or DPF. These results indicate that $O_2^{\cdot}$ and $^1O_2$ may participate in LOOH-dependent initiation to a limited extent. The NADPH-dependent LOOH-dependent initiation of lipid

peroxidation requires EDTA-Fe^{+3}. The NADPH-dependent LOOH-dependent iniiation reaction does not demonstrate SOD or benzoate sensitivity. Thus the enzymatic reduction of EDTA-Fe^{+3} does not occur via NADPH-cytochrome P450 reductase generated $O_2^{\cdot}$ and $O_2^{\cdot}$ is not a major participant in the LOOH-dependent initiation of NADPH-dependent lipid peroxidation. Also, the data indicates that HO• does not participate in LOOH-dependent initiation. The NADPH-dependent LOOH-dependent initiation reaction does show minor inhibition by DPF and significant inhibition by BHT. Thus the NADPH-dependent LOOH-dependent initiation of lipid peroxidation appears to generate minor amounts of $^1O_2$ and to be essentially radical mediated. The minor amount of $^1O_2$ generated during peroxidation could perhaps arise from the reaction of two lipid hydroperoxy radicals [29,163]. The $O_2^{\cdot}$-dependent LOOH-dependent initiation of liid peroxidation demonstrated many similarities to the NADPH-dependent reaction. The only significant difference being the expected SOD sensitivity. Superoxide-dependent LOOH-dependent initiation requires EDTA-Fe^{+3}. Superoxide-dependent LOOH-dependent initiation does not show benzoate sensitivity and thus does not involve HO•. Again, as for NADPH-dependent LOOH-dependent initiation, $O_2^{\cdot}$-dependent initiation demonstrates minor inhibition upon the addition of DPF to the reaction mixture and total inhibition upon the addition of BHT. These results indicate that LOOH-dependent initiation is essentially radical in nature but may perhaps involve the secondary generation $^1O_2$ as discussed above. The EDTA-Fe^{+2} promoted LOOH-dependent initiation of lipid peroxidation may be a Fentons-type reaction. Such a reaction would require the redox cycling of EDTA-iron. Thus the observed enzymatic dependence of LOOH-dependent initiation of lipid peroxidation. The EDTA-ferric complex can

be reduced either directly by NADPH-cytochrome P450 reductase or by reaction with $O_2^{\cdot}$. Since HO• apparently is not formed during LOOH-dependent initiation the most significant product generated during this reaction may be the lipid alkoxy radical (LO•).

$$LOOH + EDTA\text{-}Fe^{+2} \leftarrow LO\cdot + OH^{-} + EDTA\text{-}Fe^{+3} \qquad (41)$$

Apparently the lipid hydroperoxy radical (LOO•) is also formed as is evidenced by the participation of $^1O_2$ in LOOH-dependent initiation.

The investigation of NADPH-dependent and $O_2^{\cdot}$-dependent liposomal peroxidation, where LOOH-independent and -dependent initiation occurs simultaneously, yielded results consistent with the proposed two step mechanism of lipid peroxidation. Superoxide dismutase, which can inhibit LOOH-independent initiation of both $O_2^{\cdot}$-dependent and NADPH-dependent lipid peroxidation, almost completely inhibited $O_2^{\cdot}$-dependent and NADPH-dependent liposomal peroxidation. These results also demonstrate the dependence of LOOH-dependent initiation upon LOOH-independent initiation. Butylated hydroxytoluene, which inhibits both LOOH-dependent and -dependent initiation, totally inhibits the completely reconstituted liposomal reaction mixtures. Benzoate, which does not inhibit either LOOH-independent or -dependent initiation, does not inhibit either NADPH-dependent or $O_2^{\cdot}$-dependent liposomal peroxidation. Diphenylfuran, which only inhibits LOOH-dependent initiation to a minor extent, inhibits NADPH-dependent and $O_2^{\cdot}$-dependent liposomal peroxidation to a similar extent.

The ability to promote O_2^{-}-dependent and NADPH-dependent LOOH-dependent initiation of lipid peroxidation is not unique to the EDTA-iron complex but is also shown by the DTPA-iron complex. These multidentate chelators, EDTA and DTPA, form similar strong complexes with

iron. The complexes formed, stabilize the ferric ion relative to the ferrous ion and lead to a lowering of the complexes reduction potential in respect to that of free iron. The complexation of iron by EDTA changes the redox potential of iron from 0.77 for free iron to 0.25 for EDTA-complexed iron [164,165]. The EDTA- and DTPA-ferrous complexes are quite strong reducing agents. It may be this alteration in reduction potential that makes an iron complex an efficient promoter of LOOH-dependent initiation. The chelation of iron by phosphates, such as ADP, does not stabilize the ferric ion relative to the ferrous ion to as great an extent as does EDTA or DTPA and as we have shown ADP-Fe^{+2} does not promote the LOOH-dependent initiation of lipid peroxidation. The effect of chelation on the spin state of iron may also be a consideration in the formation of an effective propagating agent. The ability of DTPA-iron to promote the LOOH-dependent initiation of both NADPH-dependent and $O_2^{\cdot}$-dependent lipid peroxidation may offer an alternate explanation of the recently reported results of Thomas et al. [154]. These authors found that $O_2^{\cdot}$-dependent lipid peroxidation was dependent on contaminating levels of LOOH in the lipid matrix being peroxidized. They proposed that $O_2^{\cdot}$-dependent lipid peroxidation was initiated by LO$\cdot$ formed via the following reaction, which is essentially an uncatalyzed Haber-Weiss reaction.

$$O_2^{\cdot} + LOOH \rightarrow LO\cdot + OH^- + O_2 \qquad (37)$$

The authors added DTPA to their reaction mixtures to chelate contaminating iron and presumably inhibit iron-promoted lipid peroxidation. In light of the results presented here, we feel that Thomas et al. [154] may have been observing the $O_2^{\cdot}$-dependent LOOH-dependent initiation of

lipid peroxidation promoted by DTPA-Fe^{+2}. The rate of the $O_2^{\cdot}$-dependent peroxidation reaction reported by Thomas et al. is in agreement with the rate of DTPA-Fe^{+2} promoted propagation of lipid peroxidation reported here.

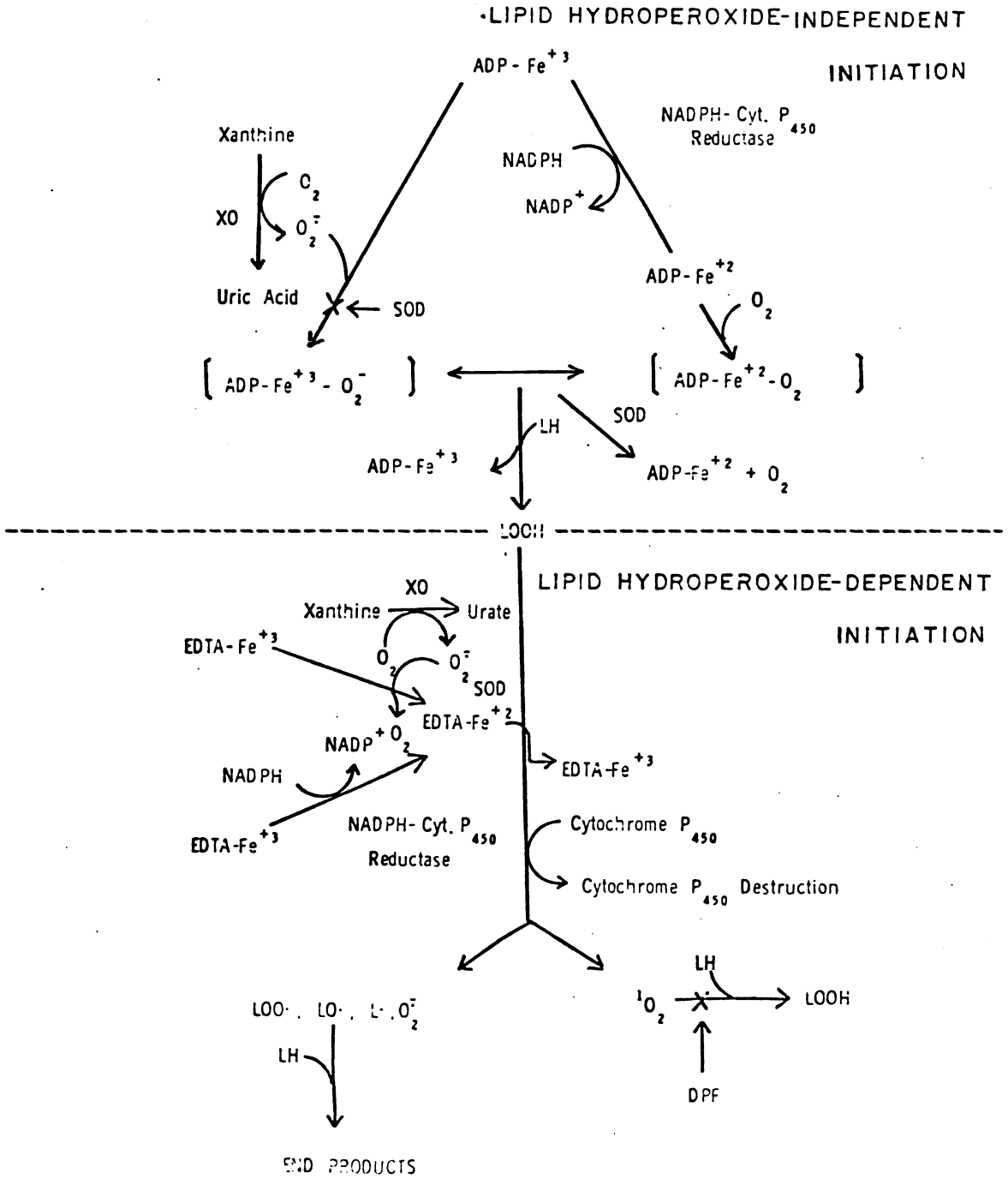
NADPH-dependent and $O_2^{\cdot}$-dependent microsomal lipid peroxidation also occur by the proposed two step mechanism of lipid peroxidation. Both NADPH-dependent and $O_2^{\cdot}$-dependent microsomal lipid peroxidation require ADP-Fe^{+3}. By analogy to the previous investigation on NADPH-dependent and $O_2^{\cdot}$-dependent liposomal peroxidation it is likely that ADP-Fe^{+3} participates in the LOOH-independent initiation of microsomal lipid peroxidation. The SOD sensitivity of NADPH-dependent microsomal lipid peroxidation observed by others [39,72] indicates the possible involvement of the ADP-perferryl ion in the LOOH-independent initiation of microsomal lipid peroxidation. Lipid hydroperoxide-dependent initiation of both NADPH-dependent and $O_2^{\cdot}$-dependent microsomal lipid peroxidation is apparently facilitated by cytochrome P450. NADPH-dependent microsomal lipid peroxidation can be inhibited by both SKF 525-A and AP SKF 525-A, at the concentration used in these experiments, is proposed to inhibit only cytochrome P450 mediated reactions without having significant effect on other microsomal activities or constituents [155]. The addition of a promoter of LOOH-dependent initiation, EDTA-Fe^{+3}, to SKF 525-A inhibited NADPH-dependent microsomal lipid peroxidation completely reversed the observed inhibition. This data indicates the significant role that may be played by cytochrome P450 in microsomal lipid peroxidation. The inhibition of NADPH-dependent microsomal lipid peroxidation by drug substrates of the microsomal mixed-function oxidase system has classically been considered to be mediated by competition for

reducing equivalents between drug metabolism and lipid peroxidation [156,157]. However, we have shown that the addtion of a promoter of LOOH-dependent initiation, EDTA-Fe^{+3}, to an AP inhibited NADPH-dependent microsomal lipid peroxidation reaction mixture completely reverses the observed inhibition. This data indicates that AP inhibition of NADPH-dependent lipid peroxidation is not mediated by the competition for reducing equivalents but rather upon the interaction of AP with an endogenous microsomal promoter of LOOH-dependent initiation of lipid peroxidation. The ability of EDTA-Fe^{+3} to reverse both SKF 525-A and AP inhibition of NADPH-dependent microsomal lipid peroxidation indicates that the endogenous promoter may be cytochrome P450. That cytochrome P450 is the endogenous microsomal promoter of LOOH-dependent initiation of lipid peroxidation is further substantiated by the ability of EDTA-Fe^{+3} to reverse the SKF 525-A mediated inhibition of $O_2^{\cdot}$-dependent microsomal lipid peroxidation. The ability of ferric cytochrome P450 to function as a promoter of LOOH-dependent initiation is shown by its promotion of lipid peroxidation from LOOH and by its ability to promote lipid peroxidation in a reaction mixture capable of initiating NADPH-dependent lipid peroxidation.

The unified mechanisms of NADPH-dependent and $O_2^{\cdot}$-dependent lipid peroxidation we are proposing is presented schematically in Figure 10.

The mechanism of lipid peroxidation proposed here offers a plausable guide for initial investigations into the mechanism <u>in vivo</u> lipid peroxidation. Considering the concentation of nucleotides [184] and "free" iron [185] in the hepatic cell, the participation of nucleotide-iron complexes, such as ADP-iron, in the LOOH-independent initiation of

Figure 10. A Schematic of the Reactions of NADPH-Dependent and $O_2^{\bar{\cdot}}$-Dependent Lipid Peroxidation.


LIPID HYDROPEROXIDE-INDEPENDENT
INITIATION
ADP-Fe+3
NADPH-Cyt. P450 Reductase
Xanthine
NADPH
XO
O2
O2-
NADP+
Uric Acid
SOD
ADP-Fe+2
O2
[ADP-Fe+3-O2-]
[ADP-Fe+2-O2]
LH
SOD
ADP-Fe+3
ADP-Fe+2 + O2
LOOH
LIPID HYDROPEROXIDE-DEPENDENT
INITIATION
XO
Xanthine
Urate
EDTA-Fe+3
O2
O2-
SOD
EDTA-Fe+2
NADP+ O2
NADPH
EDTA-Fe+3
NADPH-Cyt. P450 Reductase
EDTA-Fe+3
Cytochrome P450
Cytochrome P450 Destruction
LOO·, LO·, L·, O2-
LH
END PRODUCTS
LH
1O2
LOOH
DPF

in vivo lipid peroxidation is reasonable. Under normal cellular conditions the concentration of such nucleotide-iron complexes may be quite significant. The involvement of promoters of LOOH-dependent initiation, such as hemoproteins like cytochrome P450, in *in vivo* lipid peroxidation is also a reasonable asumption because of their integration into the lipid matrix of cellular membranes where lipid peroxidation occurs. The relative contribution of LOOH-independent and -dependent initiation to the total peroxidative event, as demonstrated here, also agrees with what is known about *in vivo* lipid peroxidation. Several investigators have demonstrated that lipid peroxidation is a constitutive phenomenon even in the unstressed animal [186-188]. Because of the innate detrimental nature of lipid peroxidation, such an occurrance would imply that under normal conditions *in vivo* lipid peroxidation must be a closely controlled reaction. The control of lipid peroxidation at the level of LOOH-independent initiation would be a relatively simple function for which the cell is excellently equipped. Defense mechanisms may include SOD, which is ubiquitous in aerobic life and can inhibit both $O_2^{\cdot-}$-dependent and NADPH-dependent initiation, membrane antioxidants such as α-tocopherol and cholesterol, which can act to efficiently control initial radical formation, and a membrane repair mechanism involving phospholipases to excise LOOH and acyltransferases to replace the damaged fatty acid with an unperoxidized fatty acid. The toxicity of the newly formed free fatty acid hydroperoxides may be limited by the ability of glutathione peroxidase to reduce the LOOH to lipid alcohols (LOH). An additional defense mechanism, that is currently being researched, may be the peroxidase action of cytochrome P450 with membrane LOOH and various electron donors [158,160,168,169,189]. The deleterious effects of *in*

vivo lipid peroxidation may become apparent only after the second step of lipid peroxidation, LOOH-dependent initiation enters into the scheme of in vivo lipid peroxidation. The LOOH-dependent initiation of lipid peroxidation may become significant only under stressed conditions such as observed during the toxicity of paraquat, alloxan, CCl_4, adriamycin and similar agents. Under stressed conditions initiation overwhelms normal cellular control mechanisms and what initial damage does occur is magnified several fold during LOOH-dependent initiation of lipid peroxidation. The type of damage considered characteristic of in vivo lipid peroxidation may only be observed after lipid peroxidation has entered into LOOH-dependent initiation and becomes rampant. Lipid peroxidation undergoing LOOH-dependent initiation may result in the lose of membrane integrity and function of membrane proteins. Such damage can result in lysosomal lysis, loss of mitochondrial function, loss of cellular reduction power (NADPH, NADH and GSH) and even eventual cell lysis and death [165].

REFERENCES

1. L. Bateman, Quart. Rev. (London) 8, 147 (1954).

2. L. Bateman and J.L. Bolland, Proc. Int. Cong. Pure and Appl. Chem., 11th Congress (London) 1, 325 (1947).

3. L. Bateman, J.L. Bolland and G. Gee, Trans. Faraday Soc., 47, 274 (1951).

4. L. Bateman and G. Gee, Proc. Roy. Soc. (London) A195, 391 (1948).

5. L. Bateman and G. Gee, Trans. Faraday Soc., 47, 155 (1951).

6. L. Bateman, G. Gee, A.L. Morris and W.F. Watson, Disc. Faraday Soc., 10, 250 (1951).

7. L. Bateman and H. Hughes, J. Chem. Soc., 1594 (1952).

8. L. Bateman, H. Hughes and A.L. Morris, Disc. Faraday Soc., 14, 190 (1953).

9. J.L. Bolland, Quart. Rev. (London) 3, 1 (1949).

10. J.L. Bolland and G. Gee, Trans. Faraday Soc., 42, 236 (1946).

11. J.L. Bolland and G. Gee, Trans. Faraday Soc., 42, 244 (1946).

12. J.L. Bolland and H.P. Koch, J. Chem. Soc., 445 (1945).

13. J.F. Mead, in "Free Radicals in Biology" (W.A. Pryor, ed.) Vol. I, p. 51, Academic Press, New York, 1976.

14. W.O. Lundberg, in "Autoxidations and Antioxidants" (W.O. Lundberg, ed.), Vol. I, p. 31, Wiley (Interscience), New York, 1961.

15. S.W. Benson, in "Thermochemical Kinetics" Wiley, New York, 1968.

16. S.W. Benson, Adv. Chem. Ser., 76, 143 (1968).

17. P.J. O'Brien, Can. J. Biochem., 47, 485 (1969).

18. W.A. Pryor, Fed. Proc., 32, 1862 (1973).

19. G. McLendon and A.E. Martell, Coord. Chem. Revs., 19, 1 (1979).

20. V.P. Maier and A.L. Tappel, J. Am. Oil Chemists Soc., 36, 893 (1954).

21. A.L. Tappel, W.D. Brown, H. Zodkin and V.P. Maier, J. Am. Oil Chemists Soc., 38, 5 (1961).

22. A.L. Tappel, in "Autoxidation and Antioxidants" (W.O. Lundberg, ed.) Vol. I, p. 325, Wiley (Interscience), New York, 1961.

23. A.L. Tappel, in "Lipids and Their Oxidation" (H.W. Schultz, E.A. Day and R.O. Sinnbaur, eds.), p. 122, Avi. Publishing Co., Inc., Westport, Connecticut, 1962.

24. P.J. O'Brien and C. Little, Can. J. Biochem., 47, 493 (1969).

25. N. Uri, in "Autoxidation and Antioxidants" (W.O. Lundberg, ed.), Vol. I, p. 55, Wiley (Interscience), New York, 1961.

26. K.U. Ingold, in "Lipids and Their Oxidation" (H.W. Schultz, E.A. Day and R.O. Sinnbaur, eds.), p. 93, Avi. Publishing Co., Inc., Westport, Connecticut, 1962.

27. B.A. Svingen and S.D. Aust, in "Molecular Basis of Environmental Toxicity," (R.S. Bhatnagar, ed.), p. 69, Ann Arbor Science Publishers, 1980.

28. G.S. Wu, R.A. Stein and J.F. Mead, Lipids, 12, 971 (1978).

29. M. Nakano, K. Takayama, Y. Shimizu, Y. Tsuji, H. Inaba and T. Migita, J. Am. Chem. Soc., 98, 1974 (1976).

30. C.S. Foote, Accts. Chem. Res., 1, 104 (1963).

31. L.K. Dahle, E.G. Hill and R.T. Holman, Arch. Biochem. Biophys., 98, 253 (1962).

32. N.A. Porter, J. Nixon and R. Isaac, Biochem. Biophys. Acta, 441, 506 (1976).

33. J.A. Buege and S.D. Aust, in "Methods in Enzymology," Vol. II (S. Fleischer and L. Packer, eds.), p. 302, Academic Press, New York, 1978.

34. W.A. Pryor, J.P. Stanley and E. Blair, Lipids, 11, 370 (1977).

35. W.A. Pryor and J.P. Stanley, J. Org. Chem., 40, 3615 (1975).

36. N.A. Porter, M.O. Funk, D. Gilmore, R. Isaac and J. Nixon, J. Am. Chem. Soc., 98, 6000 (1976).

37. P. Hochstein and L. Ernster, Biochem. Biophys. Res. Commun., 12, 388 (1963).

38. E.D. Wills, Biochem. J., 113, 315 (1969).

39. K.L. Fong, P.B. McCay, J.L. Poyer, B.B. Keele and H. Misra, J. Biol. Chem., 248, 7792 (1973).

40. E.D. Wills, Biochem. J., 113, 325 (1969).

41. W.O. Lundberg, in "Lipids and Their Oxidation" (H.W. Schultz, E.A. Day and R.O. Sinnbaur, eds.), p. 31, Avi. Publishing Co., Inc., Westport, Connecticut, 1962.

42. R.T. Holman, in "Autoxidation of Fats and Related Substances; Progress in the Chemistry of Fats and Other Lipids" (R.T. Holman, W.O. Lundberg and T. Vaangaard, eds.), Vol. II, p. 51, Pergamon Press, London, 1954.

43. P. Hochstein, K. Nordenbrand and L. Ernster, Biochem. Biophys. Res. Commun., 14, 323 (1964).

44. L. Ernster and K. Nordenbrand, in "Methods in Enzymology," Vol. X (R.W. Estabrook and M.E. Pullman, eds.), p. 574, Academic Press, New York, 1967.

45. P. Hochstein and L. Ernster, in "Ciba Foundation Symp. on Cellular Injury" (A.V.S. deReuck and J. Knight, eds.), p. 123, Churchill (London), 1964.

46. A. Beloff-Chain, R. Catanzaro, G. Serlupi-Crescenzi, Nature, 198, 351 (1963).

47. A. Beloff-Chain, G. Serlupi-Crescenzi, R. Catanzaro, D. Ventacci and M. Balliano, Biochem. Biophys. Acta, 97, 416 (1965).

48. H.E. May and P.B. McCay, J. Biol. Chem., 243, 2288 (1968).

49. J.L. Poyer and P.B. McCay, J. Biol. Chem., 246, 263 (1971).

50. Y.A. Vladimirov and A.I. Archakav, in "Lipid Peroxidation in Biological Membranes," Nauka Press, Moscow, 1972.

51. V.V. Lyakhovich, L.N. Pospelova, V.M. Mishin and A.G. Pokrovsky, FEBS Letts., 71, 303 (1976).

52. B.K. Tam and P.B. McCay, J. Biol. Chem., 245, 2295 (1970).

53. M. Nakano, Y. Tsutsumi and Y. Ushijima, Biochem. Biophys. Acta, 252, 335 (1971).

54. K. Kameda, J. Ono and Y. Imai, Biochem. Biophys. Acta, 572, 77 (1979).

55. T.C. Pederson, J.A. Buege and S.D. Aust, J. Biol. Chem., 248, 7134 (1973).

56. C. Lai and L.H. Piette, Biochem. Biophys. Res. Commun., 78, 51 (1977).

57. C. Lai and L.H. Piette, Arch. Biochem. Biophys., 190, 27 (1978).

58. T.C. Pederson and S.D. Aust, Biochem. Biophys. Res. Commun., 48, 789 (1972).

59. K. Sugioko and M. Kakano, Biochem. Biophys. Acta, 423, 203 (1976).

60. T. Noguchi and M. Nakano, Biochem. Biophys. Acta, 368, 446 (1974).

61. L.N. Pospelova, A.G. Pokrovsky, V.M. Michin and L.L. Lyakovich, FEBS Letts., 74, 225 (1974).

62. T.C. Pederson and S.D. Aust, Biochem. Biophys. Res. Commun., 52, 1071 (1973).

63. B.A. Svingen, F.O. O'Neal and S.D. Aust, Photochem. Photobiol., 28, 803 (1978).

64. D.D. Tyler, FEBS Letts., 51, 180 (1975).

65. F. Haber and J. Weiss, Proc. Roy. Soc. Edinburgh, Sect. A., 147, 332 (1934).

66. P. George, Disc. Faraday Soc., 2, 196 (1947).

67. G.J. McClune and J.A. Fee, FEBS Letts., 67, 294 (1976).

68. B. Halliwell, FEBS Letts., 72, 8 (1976).

69. C. Ferradini, J. Foos, C. Houee and J. Pucheault, Photochem. Photobiol., 28, 697 (1978).

70. G. Czapski and Y.A. Ilan, Photochem. Photobiol., 28, 651 (1978).

71. W.H. Koppenol, J. Butler and J.W. van Leeuwen, Photochem. Photobiol., 28, 655 (1978).

72. M.M. King, E.K. Lai and P.B. McCay, J. Biol. Chem., 250, 6496 (1975).

73. A.G. Hildebrandt and I. Roots, Arch. Biochem. Biophys., 171, 385 (1975).

74. A.G. Hildebrandt, L. Lehns, I. Roots and M. Tjoe, in "Microsomes and Drug Oxidations," (V. Ullrich, I. Roots, A. Hildebrandt, R.W. Estabrook and A.H. Conney, eds.), p. 492, Pergamon Press, London, 1977.

75. T.C. Pederson and S.D. Aust, Biochem. Biophys. Acta, 385, 232 (1975).

76. C. Lai, T.A. Grover and L.H. Piette, Arch. Biochem. Biophys., 193, 373 (1979).

77. E.G. Janzen, Accts. Chem. Res., 4, 31 (1971).

78. B.A. Svingen, J.A. Buege, F.O. O'Neal and S.D. Aust, J. Biol. Chem., 254, 5892 (1979).

79. M.H. Bilimoria and H. Kamin, Ann. N.Y. Acad. Sci., 212, 428 (1973).

80. M.J. Coon, H.W. Strobel and R.F. Boyer, Drug Metab. Disp., 1, 92 (1973).

81. Y.A. Ilan and G. Czapski, Biochem. Biophys. Acta, 498, 386 (1977).

82. J.A. Fee and J.S. Valentine, in "Superoxide and Superoxide Dismutases" (A.M. Michelson, J.M. McCord and I. Fridovich, eds.), p. 20, Academic Press, New York, 1977.

83. G.J. McClune, J.A. Fee, G.A. McCluskey and J.T. Groves, J. Am. Chem. Soc., 99, 5220 (1977).

84. E. Finkelstein, F.J. Cohen and E.L. Raukman, Arch. Biochem. Biophys. (1980), in press.

85. J.F. Elsworth and J.S. Milamcen, African Chem. Inst., 23, 61 (1970).

86. J.F. Elsworth and J.S. Milamchen, African Chem. Inst., 24, 196 (1974).

87. H.S. Mason, Ann. Rev. Biochem., 34, 595 (1965).

88. H.S. Mason, Adv. Enzym., 19, 79 (1957).

89. F.W. Heaton and N. Uri, J. Lipid Res., 2, 152 (1961).

90. E.D. Wills, Biochem. Biophys. Acta, 98, 238 (1965).

91. F. Bernheim, Radiat. Res. Suppl, 3, 17 (1963).

92. S. Udenfriend, C. Clark, J. Axelrod and B.B. Brodie, J. Biol. Chem., 208, 741 (1954).

93. C. Nofre, A. Cier and A. Lefier, Bull. Chem. Soc. Fr., 430 (1961).

94. S.A. Goscin and I. Fridovich, Arch. Biochem. Biophys., 153, 778 (1972).

95. D. Behar, G. Czapski, J. Rabani, L.M. Dorfman and H.A. Schwarz, J. Phys. Chem., 74, 3209 (1920).

96. A.M. Michelson, Biochemie, 55, 365 (1973).

97. J. Folch, M. Lees and G.H. Sloane-Stanley, J. Biol. Chem., 226, 497 (1957).

98. J. Fergusson, W.T. Robinson and G.A. Rodley, Aust. J. Biol. Sci., 25, 1365 (1972).

99. A.J. Chalk and A.H. Smith, Trans. Faraday Soc., 53, 1214 (1957).

100. A.J. Chalk and A.H. Smith, Trans. Faraday Soc., 53, 1235 (1957).

101. J.P. Collman, R. Gagne, T. Halbert, J. Marchon and C.A. Reed, J. Am. Chem. Soc., 95, 7868 (1973).

102. J.R. Collman, R. Gagne and C.A. Reed, J. Am. Chem. Soc., 96, 2629 (1974).

103. J. Almog, J.E. Baldwin, R. Dyer and M. Peters, J. Am. Chem. Soc., 97, 226 (1975).

104. O. Leal, D.L. Anderson, R.G. Bowman, F. Basolo and R.L. Burwell, J. Am. Chem. Soc., 97, 5125 (1975).

105. D.A. Peterson, J.M. Gerrand, G.H.R. Raa, T.R. Krick and J.G. White, Prostaglandins and Medicine, 1, 304 (1978).

106. M.S. Kharasch, F.S. Arimoto and W. Nudenberg, J. Org. Chem., 16, 1556 (1951).

107. J.K. Kochi, Science, 155, 415 (1967).

108. W.A. Pryor, in "Free Radicals," McGraw-Hill, New York (1966).

109. N. Uri, Chem. Rev., 50, 375 (1952).

110. J.W. Fordham and H.L. Williams, J. Am. Chem. Soc., 72, 4465 (1950).

111. M. Nakano and K. Sugioka, Arch. Biochem. Biophys., 181, 371 (1977).

112. B.A. Svingen, S.R. Tonsager, T.D. Lindstrom and S.D. Aust, Fed. Proc., 37, (1979).

113. J.J.M.C. Degroot, G.J. Garssen, J.F.G. Vliegenthart and J. Boldingh, Biochem. Biophys. Acta, 326, 279 (1973).

114. C. Walling, Accts. Chem. Res., 8, 125 (1975).

115. J.T. Groves and W.W. Swanson, Tett. Letts., 24, 1953 (1975).

116. J.T. Groves and G.A. McClusky, J. Am. Chem. Soc., 98, 859 (1976).

117. J.T. Groves and M. VanderPuy, J. Am. Chem. Soc., 98, 5290 (1976).

118. W.G. Bray and M. Gorin, J. Am. Chem. Soc., 54, 2124 (1932).

119. J.H. Wang, J. Am. Chem. Soc., 77, 822 (1955).

120. P. George, in "Advances in Catalysis" (W.G. Frankenberg, V.I. Komarewsky and E.K. Rideal, eds.), Vol. 4, p. 367, Academic Press, New York, 1952.

121. I.M. Kolthoff and A.I. Medalia, J. Am. Chem. Soc., 71, 3777 (1949).

122. P. George, J. Chem. Soc., 4349 (1959).

123. G.S. Hammond and C.S. Wu, Adv. Chem. Ser., 77, 186 (1968).

124. H.B. Demopoulos, Fed. Proc., 32, 1903 (1973).

125. G. Sosnovsky and D.J. Rawlinson, in "Organic Peroxides" (D. Swern, ed.), Vol. 2, p. 153, Wiley-Interscience, New York, 1971.

126. J.A. Sharp, J. Chem. Soc., 2030 (1957).

127. W.G. Barb, J.H. Baxendale, P. George and K.R. Hargrave, Trans. Faraday Soc., 47, 591 (1951).

128. J. Weiss, Faraday Soc. Disc., 2, 188 (1947).

129. A.L. Tappel and H. Zalkin, Arch. Biochem. Biophys., 80, 326 (1959).

130. A.L. Tappel, Arch. Biochem. Biophys., 44, 378 (1953).

131. A.L. Tappel, Food Res., 18, 560 (1953).

132. J.S. Bus, S.D. Aust and J.E. Gibson, Biochem. Biophys. Res. Commun., 58, 749 (1974).

133. J.S. Bus, S.D. Aust and J.E. Gibson, Res. Commun. Chem. Path. Pharmacol., 11, 31-38 (1975).

134. C.E. Myers, W.P. McGuire, R.H. Liss, H. Ifrim, K. Grotzinger and R.C. Young, Science, 197, 165 (1977).

135. A.U. Khan, Science, 168, 476 (1970).

136. E.W. Kellog and I. Fridovich, J. Biol. Chem., 250, 8812 (1975).

137. R. Nilsson and D.R. Kearns, J. Phys. Chem., 78, 168 (1974).

138. J.M. McCord and I. Fridovich, J. Biol. Chem., 243, 5753 (1968).

139. F. Bernheim, M.L.C. Bernheim and K.M.J. Wilbur, J. Biol. Chem., 174, 257 (1948).

140. R.E. Lutz and F.N. Wilder, J. Am. Chem. Soc., 56, 978 (1934).

141. G.R. Bartlett, J. Biol. Chem., 234, 466 (1959).

142. O.H. Lowry, N.T. Rosebrough, A.L. Far and R.J. Randall, J. Biol. Chem., 193, 265 (1951).

143. F.J. Howco, C.B. O'Brien and P.J. O'Brien, Biochem. Biophys. Res. Commun., 76, 354 (1977).

144. C. Richter, A. Azzi, U. Wesser and A. Wendel, J. Biol. Chem., 252, 5061 (1971).

145. E.W. Kellog and I. Fridovich, J. Biol. Chem., 252, 6728 (1977).

146. B. Zak, E.S. Baginski, E. Epstein and L.M. Weiner, Clin. Tox., 4, 621 (1971).

147. A.I. Cederbaum, E. Dicker, E. Rubin and G. Cohen, Biochem. Biophys. Res. Commun., 78, 1254 (1977).

148. C. Walling, R.E. Partch and T. Weid, Proc. Natl. Acad. Sci., USA, 72, 140 (1975).

149. C. Czupski, Ann. Revs. Phys. Chem., 22, 171 (1971).

150. F.P. Guengerich, J. Biol. Chem., 252, 3970 (1977).

151. J.L. Haining and B. Axelrod, J. Biol. Chem., 232, 193 (1958).

152. P.J. O'Brien and A. Rahimtula, J. Agr. Food Chem., 23, 154 (1975).

153. C.B. Kenaston, K.M. Wilbur, A. Ottolenghi and F. Bernheim, J. Am. Oil Chem. Soc., 32, 33 (1955).

154. M.J. Thomas, K.S. Mehl and W.A. Pryor, Biochem. Biophys. Res. Commun., 83, 927 (1978).

155. I.P. Lee, H.I. Yamamma and R.L. Dixon, Biochem. Pharm., 17, 1671 (1968).

156. E.D. Wills, Biochem. J., 113, 333 (1969).

157. S. Orrenius, G. Dallner and L. Ernster, Biochem. Biophys. Res. Commun., 14, 329 (1964).

158. A.D. Rahimtula and P.J. O'Brien, Biochem. Biophys. Res. Commun., 60, 440 (1974).

159. F.F. Kadlubar, K.C. Morton and D.M. Ziegler, Biochem. Biophys. Res. Commun., 54, 1255 (1973).

160. A.D. Rahimtula and P.J. O'Brien, Biochem. Biophys. Res. Commun., 62, 268 (1975).

161. H. Nishibayashi, T. Omura, R. Sata and R.W. Estabrook in Structure and Function of Cytochromes (K. Okumuki, H.D. Kanin and I. Sekuzi, eds.), University Park Press, Baltimore, MD (1968).

162. W. Levin, A.Y.H. Lu, M. Jacobson, M. Kuntzman, J.L. Poyer and P.B. McCay, Arch. Biochem. Biophys., 158, 872 (1973).

163. J.A. Howard and K.U. Ingold, J. Am. Chem. Soc., 90, 1056 (1968).

164. I.M. Kolthoff and C. Auerbach, J. Am. Chem. Soc., 72, 1452 (1952).

165. R.W. Schmidt and C.N. Reilly, Anal. Chem., 28, 520 (1956).

166. J. Bond and T.I. Jones, J. Chem. Soc. Lond., 1310 (1959).

167. L.H. Hall, J.J. Spijkerman and J.K. Lambert, J. Am. Chem. Soc., 83, 2770 (1961).

168. E.G. Hrycay and P.J. O'Brien, Arch. Biochem. Biophys., 147, 14 (1971).

169. E.G. Hrycay and P.J. O'Brien, Arch. Biochem. Biophys., 147, 28 (1971).

170. J.M. McCord, B.B. Keele and I. Fridovich, Proc. Natl. Acad. Sci., USA, 68, 1024 (1971).

171. J.M. McCord and I. Fridovich, J. Biol. Chem., 245, 1374 (1970).

172. W.H. Orme-Johnson and H. Beinert, Biochem. Biophys. Res. Commun., 36, 905 (1969).

173. H.P. Misra and I. Fridovich, J. Biol.Chem., 246, 6886 (1971).

174. I. Fridovich and P. Handler, J. Biol. Chem., 233, 1581 (1968).

175. F.O. Brady, H.J. Forman and P. Feigelson, J. Biol. Chem., 246, 7119 (1971).

176. G. Cohen and R.K. Heikkila, J. Biol. Chem., 249, 2447 (1974).

177. R.E. Heikkila and G. Cohen, Science, 181, 456 (1973).

178. R.E. Heikkila and G. Cohen, Ann. N.Y. Acad. Sci., 258, 221 (1975).

179. K. Handa and S. Sato, Gann., 66, 43 (1974).

180. W. Fenical, D.R. Kearns and P. Radlick, J. Am. Chem. Soc., 91, 3396 (1969).

181. R. Poupho and I. Rosenthal, J. Phys. Chem., 77, 1722 (1973).

182. A. Rigo, R. Stevanato, A. Finazzi-Agro and G. Rotilio, FEBS Lett., 80, 130 (1977).

183. P.B. McCay, P.M. Pfiefer and W.H. Stripe, Ann. N.Y. Acad. Sci., 203, 62 (1972).

184. R.W. Guynn, D. Veloso, J.W.R. Lawson and R.L. Veech, Biochem. J., 140, 369 (1974).

185. M.C. Linder and H.N. Munro, Fed. Proc., 36, 2017 (1977).

186. C.J. Dillard and A.L. Tappel, Lipids, 6, 715 (1971).

187. C.J. Dillard, E.E. Dumelin and A.L. Tappel, Lipids, 12, 109 (1977).

188. K. Reddy, B. Fletcher, A. Tappel and A.L. Tappel, J. Nutr., 103, 908 (1973).

189. F.F. Kadlubar, K.C. Morton and D.M. Ziegler, Biochem. Biophys. Res. Commun., 54, 1255 (1973).

190. J. Högberg, P. Moldéus, B. Arborgh, P.J. O'Brien and S. Orrenius, Eur. J. Biochem., 59, 457 (1975).

APPENDIX

LIST OF PUBLICATIONS

Brody, M.S., Steinberg, J.R., Svingen, B.A. and Leuke, R.W.: In creased purine nucleotide cycle activity associated with dietary zinc deficiency. Biochem. Biophys. Res. Comm. 78, 144-150 (1977).

Svingen, B.A., O'Neal, F.O. and Aust, S.D.: The role of superoxide and singlet oxygen in lipid peroxidation. Photochem. Photobiol. 28, 803-810 (1978).

Svingen, B.A., Buege, J.A., O'Neal, F.O. and Aust, S.D.: The mechanism of NADPH-dependent lipid peroxidation: The propagation of lipid peroxidation. J. Biol. Chem. 254, 5892-5894 (1979).

Svingen, B.A. and Aust, S.D.: The mechanism of NADPH-dependent and O_2-dependent lipid peroxidation. Molecular Basis of Environmental Toxicity. ACS, Ann Arbour Science, Ann Arbour, MI, pp. 69-110 (1980).

Tien, M., Svingen, B.A. and Aust, S.D.: Superoxide dependent lipid peroxidation. Fed. Proc. 40, 179-182 (1981).

Aust, S.D. and Svingen, B.A.: The role of iron in Lipid Peroxidation in Free Radicals in Biology, Vol. V (W.A. Pryor, ed.) (in press).

ABSTRACTS

Svingen, B.A., Buege, J.A., O'Neal, F.O. and Aust, S.D.: Relationship between liver microsomal P450 dependent drug metabolism and lipid peroxidation. Fed. Proc. 36, 998 (1977).

Buege, J.A., Svingen, B.A., O'Neal, F.O. and Aust, S.D.: The mechanism of microsomal NADPH-dependent lipid peroxidation. Fed. Proc. 36, 843 (1977).

Svingen, B.A. and Aust, S.D.: Propagation of lipid peroxidation EDTA chelated iron. Fed. Proc. 37, 1706 (1978).

Tien, M., Svingen, B.A. and Aust, S.D.: Rabbit liver microsomal lipid peroxidation. Pharmacologist 20, 183 (1978).

Svingen, B.A. and Aust, S.D.: The mechanism of NADPH-dependent and superoxide-dependent lipid peroxidation. 1978 ACS Annual Meetings Abstracts, Division of Environmental Chemistry and Biological Chemistry 176, 156.

Svingen, B.A., Tonsager, S.R., Lindstrom, T.D. and Aust, S.D.: The demonstration of the specific generation of the alkyl, alkoxy, and hydroperoxy radicals of linoleic acid by EPR spin-trapping techniques. Fed. Proc. 38, 648 (1979).

Tien, M., Svingen, B.A. and Aust, S.D.: The mechanism of initiation of lipid peroxidation. Fed. Proc. 39, 1727 (1980).

Aust, S.D., Tien, M. and Svingen, B.A.: Ferric ion dependent lipid peroxidation as a function of pH. J. Am. Oil Chem. 57, A186 (1980).