THE INVOLVEMENT OF CHELATED IRON AND ACTIVATED OXYGEN IN RADICAL-MEDIATED PEROXIDATION OF LIPID

By

Ming Tien

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1981

ABSTRACT

THE INVOLVEMENT OF CHELATED IRON AND ACTIVATED OXYGEN IN RADICAL-MEDIATED PEROXIDATION OF LIPID

By

Ming Tien

Comparative aspects of several lipid peroxidation (LP) systems were examined to investigate factors which affect the rate of peroxidation. The rate of LP in these systems is sensitive to the content of unsaturated fatty acids in the lipid, the structure of the lipid, iron concentration and the chelation of iron.

Microsomes catalyze a NADPH-cytochrome P450 reductase-dependent LP in the presence of NADPH and ADP-Fe^{3+}. Liver microsomes from rat and rabbit contain equal levels of reductase, yet the rate of peroxidation in rabbit microsomes is 40% of that in rat liver microsomes. By reconstituting lipid peroxidation with purified microsomal components, the lower rates of LP in rabbit microsomes was found not to be due to the reductase, but rather to lower content of unsaturated fatty acids. Gas chromatographic analysis of fatty acids lost during LP revealed that the degree of fatty acid unsaturation correlated with rates of LP.

The mechanism of NADPH-dependent LP was studied by reconstituting LP with rat microsomal components. LP was assessed by malondialdehyde (MDA) formation in liposomes made from extracted microsomal lipid and diene conjugation in linoleate. Numerous workers have proposed that NADPH-dependent LP is a consequence of superoxide ($O_2^{\cdot-}$) generation by the

reductase. Our results are in discord with this proposal since NADPH-dependent LP is not inhibited by superoxide dismutase (SOD). The rate and mechanism of peroxidation is dependent on the chelation of iron. With detergent-dispersed linoleate, NADPH-dependent LP requires only EDTA-Fe^{3+} for activity while both EDTA-Fe^{3+} and ADP-Fe^{3+} are required for peroxidation of liposomes. NADPH-dependent LP in the presence of EDTA-Fe^{3+} alone occurs via hydroxyl radical (•OH) formation. In the presence of ADP-Fe^{3+}, the participation of the •OH is minimal.

In contrast to NADPH-dependent LP, $O_2^{\cdot-}$-dependent LP was inhibited by SOD. The generation of $O_2^{\cdot-}$ by xanthine oxidase in the presence of EDTA-Fe^{3+} initiated peroxidation of linoleate by an iron-catalyzed Haber-Weiss reaction. The $O_2^{\cdot-}$-dependent peroxidation of linoleate in the presence of ADP-Fe^{3+} alone or EDTA-Fe^{3+} plus ADP-Fe^{3+} was not sensitive to catalase or •OH scavengers. In $O_2^{\cdot-}$-dependent peroxidation of liposomes, both EDTA-Fe^{3+} and ADP-Fe^{3+} are required for activity. Again, the involvement of the •OH or the iron-catalyzed Haber-Weiss reaction is minimal.

The mechanism of enzyme-catalyzed LP was also investigated by initiating LP via the direct addition of ADP-iron and EDTA-iron (ferrous and ferric) to liposomes. Again, the participation of the •OH appears minimal in systems containing ADP-chelated iron. It is proposed that initiation of LP in systems containing ADP-chelated iron occurs via an iron-oxygen complex.

If the liposomes contained lipid hydroperoxides, the direct addition of unchelated Fe^{3+} resulted in a pH sensitive initiation of LP. Initiation of LP occurred through the Fe^{3+}-catalyzed decomposition of

the lipid hydroperoxides to yield free radical products. The initial radical formed was demonstrated by EPR spin trapping experiments to be the lipid peroxy radical.

To My Parents

ACKNOWLEDGMENTS

I wish to thank my thesis advisor, Steven D. Aust, for his financial and moral support throughout my graduate studies. Without his patience and tolerance, this dissertation would not be in existance.

I would also like to thank other members of the lab for their collaboration in numerous studies. Some of these collaborations have resulted in publications which are listed in the Appendix.

Although this volume marks the end of a chapter in my life, the friendships I've developed with the people in the Department will hopefully continue. Their support and companionship has been appreciated.

I would like to express my appreciation for the continued faith my family has provided me.

Finally, I wish to acknowledge my friend Mary for her crucial support when life seemed most confusing.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ADP	adenosine-5'-diphosphate
BHT	butylated hydroxyltoluene
DMPO	5,5-dimethyl-1-pyrroline-N-oxide
EDTA	ethylenediaminetetracetate
EPR	electron paramagnetic resonance
HPLC	high pressure liquid chromatography
hps	hyperfine splitting
LAHPO	linoleic acid 13-hydroperoxide
LP	lipid peroxidation
LOOH	lipid hydroperoxide
MDA	malondialdehyde
NADPH	reduced nicotinamide adenine dinucleotide phosphate
POBN	α-(4-pyridyl-1-oxide)-N-tert-butylnitrone
SOD	superoxide dismutase
Tris	Tris(hydroxymethyl)aminomethane

INTRODUCTION

A possible factor in oxygen or other radical-mediated toxicity is the peroxidative damage to cellular membranes. The free radical process of lipid peroxidation has been implicated in a wide variety of pathological states. Both enzymatic and non-enzymatic initiators of lipid peroxidation have been discovered.

Enzyme catalyzed lipid peroxidation was first demonstrated in rat liver microsomes. The rate of microsomal lipid peroxidation was enhanced by NADPH and nucleotide-chelated iron. These studies were performed before the realization that superoxides, hydroxyl radical, and other oxgyen centered radicals can exist in biological systems. The initiation of microsomal NADPH-dependent lipid peroxidation was proposed to occur through iron-oxygen complexes. The discovery that certain enzymes liberate superoxide and that erythrocuperin (renamed superoxide dismutase) catalyzed the dismutation of superoxide expanded the traditional view of oxygen toxicity. Superoxide was soon seen to be a product of dioxygen reduction from a variety of reactions and an important agent of oxygen toxicity. Superoxide involvement has been suggested in microsomal NADPH-dependent lipid peroxidation in addition to numerous other enzyme catalyzed lipid peroxidation systems. The generation of superoxide by the action of xanthine oxidase on xanthine in the presence of iron readily initiates lipid peroxidation. Superoxide is not reactive with a large number of organic substrates including polyunsaturated fatty

acids. Thus the mechanism by which superoxide initiates lipid peroxidation has been the subject of intense research. It is now recognized that initiation of lipid peroxidation by superoxide requires the catalysis by iron. A growing number of investigators have proposed that the role of iron in lipid peroxidation systems mediated by superoxide is to catalyze the Haber-Weiss reaction which generates the hydroxyl radical. The hydroxyl radical can then react with polyunsaturated fatty acids to initiate lipid peroxidation.

Two enzyme-catalyzed lipid peroxidation systems, microsomal NADPH-dependent and xanthine oxidase-dependent, have been studied in Dr. Aust's lab. This work started in the late sixties with Tom Pederson and no evidence for hydroxyl radical involvement has been found in either lipid peroxidation system. My research in lipid peroxidation started with an investigation into factors that affect the rate of microsomal NADPH-dependent lipid peroxidation (Chapter I). Microsomal NADPH-dependent lipid peroxidation is catalyzed by NADPH-cytochrome P450 reductase. Rat and rabbit liver microsomes contain comparable levels of this enzyme, yet the rate of lipid peroxidation in rabbit liver microsomes is 40% of that in rat liver microsomes.

Chapter II is an investigation on hydroxyl radical involvement in NADPH-dependent lipid peroxidation. Initiation of NADPH-dependent lipid peroxidation has been proposed to occur through hydroxyl radical formation via an iron-catalyzed Haber-Weiss reaction. This proposal was re-examined by reconstituting lipid peroxidation with purified microsomal components. Hydroxyl radical involvement was assessed by examining the effects of iron chelating agents, catalase, and hydroxyl radical trapping agents on the rate of peroxidation catalyzed by the reconstituted system.

The radicals generated by this reconstituted system are identified by EPR spin trapping experiments.

Chapter III examines the involvement of hydroxyl radical in a lipid peroxidation system dependent on superoxide generated by the action of xanthine oxidase. This study utilizes Fe^{2+} and hydrogen peroxide to generate the hydroxyl radical for the purpose of initiating lipid peroxidation. The response of this hydroxyl radical-dependent lipid peroxidation system to iron chelating agents, and to hydroxyl radical trapping agents are characterized and compared to the results of a similar study with xanthine oxidase-dependent lipid peroxidation.

Chapter IV summarizes the results of Chapters II and III with further investigations into the mechanism of initiation of lipid peroxidation. A model system for enzymatic lipid peroxidation is developed where peroxidation is initiated by the addition of iron chelates (ferrous and ferric ion chelated by ADP or EDTA). The results obtained with this non-enzymatic system are correlated to the enzymatic system. Chapter V characterized lipid peroxidation initiated by ferric ion and lipid hydroperoxides at low pH.

The chapters of this dissertation are written in a format similar to that used in many scientific journals. The chapters are preceded by a Literature Review which presents a broad overview of lipid peroxidation. Since this study is focused on lipid peroxidation initiated by activated oxygen, a broad overview on the chemistry of oxygen, oxygen-centered radicals and free radicals is also presented. The introduction to each chapter is designed to provide a more specific background to that aspect of the research. All references cited in the chapters and Literature Review have been combined at the end of the dissertation.

LITERATURE REVIEW

Oxygen and Oxidations

The extensive oxidation of most biological macromolecules is a thermodynamically favored process. This is because the sum of the bond energies of the carbon-carbon, carbon-hydrogen and oxygen-oxygen bonds which are broken are less than the sum of those carbon-oxygen and oxygen-hydrogen bonds which are formed (George, 1965; March, 1968). Given the exothermicity nature of these reactions it is surprising that organic compounds can exist in an atmosphere of dioxygen. A property which decreases the reactivity of dioxygen is that it has a triplet ground state possessing two unpaired electrons in its outer orbital with parallel spin (Taube, 1965; Ogryzlo, 1978). Since all stable organic compounds are singlets in the ground state (i.e., have all their electrons paired), the reaction with dioxygen by an ionic mechanism would require that one electron spin in dioxygen be inverted in order to avoid the placement of two parallel spins in one orbital. Since the spin inversion process is slow compared to the lifetime of the collisional complex, dioxygen is kinetically unreactive. A more detailed discussion of spin restriction is available (Taube, 1965).

The oxidation of organic compounds is the energy source of aerobic life. This process is kinetically regulated by the action of enzymes. But under certain conditions, the oxidation of biological matter becomes autocatalytic or uncontrolled resulting in a toxic response. One class

of macromolecules that show a strong propensity to autoxidation is polyunsaturated fatty acids (PUFA) (May and McCay, 1968a; Khon and Liversedge, 1944; Bernheim et al., 1948; Tappel and Zalkin, 1959). Membranes are particularly susceptable to peroxidative attack due to their high content of PUFA and dioxygen (McCay, 1966; Slater, 1972). The reaction of PUFA (singlet) with dioxygen (triplet) to form lipid hydroperoxide (singlet) is a spin forbidden process. To overcome this restriction, lipid peroxidation proceeds by a free radical mechanism.

Free Radical Chemistry

A free radical is defined as any molecule with one unpaired electron occupying an outer orbital. This endows it with very unusual chemical reactivity and physical characteristics. The reactivity of a free radical is dependent on the stability of the unpaired electron. In cases where the radical is unreactive, the unpaired electron is usually part of a conjugated system and is highly stabilized by resonance delocalization (Massey et al., 1969; Misra and Fridovich, 1972). However, most free radicals have a strong tendency to react with other electrons to form an electron pair and thus a chemical bond. Their reactivity vary greatly with some, like the hydroxyl radical, being so energetic that it reacts with the first molecule with which it collides (Dorfman and Adams, 1973; Pryor, 1976).

Radical reactions can be divided into three types; initiation, propagation, and termination reactions. Initiation reactions are reactions which produce free radical products from non-radical reactants:

$$R{:}R' \longrightarrow R\cdot + R'\cdot$$

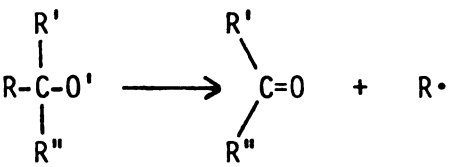
Initiation reactions may occur in living tissue by radiolysis of water, homolysis of peroxides and by enzymatic redox reactions (Pryor, 1966, 1976).

Propagation reactions conserve the net number of free radicals, but the site of the free radical is altered. The reactions can be divided into the following groups (Pryor, 1966, 1971, 1973).

Atom Transfer: $R\cdot + R'H \longrightarrow RH + R'\cdot$

Additions: $R\cdot + C{=}C \longrightarrow R\text{-}C\text{-}C\cdot$

β-scission:

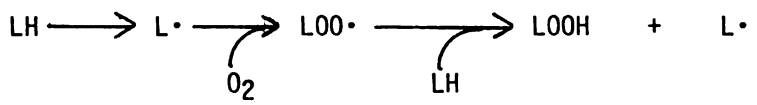


Termination reactions are reactions which result in the destruction of free radicals to yield non-radical products.

$$R\cdot + R'\cdot \longrightarrow R{:}R'$$

Free Radical Reactions of Lipid Peroxidation

The initiation reactions of lipid peroxidation will be discussed in greater detail in the latter part of the literature review and in Chapters II, III, and IV of this thesis. Once initiated, lipid peroxidation is an autocatalytic radical chain reaction as illustrated below.



If initiation occurs by the abstraction of a hydrogen atom to form the PUFA alkyl radical, the reaction then enters the propagation stage of lipid peroxidation with the addition of dioxygen. The reaction with dioxygen proceeds with a rate limited by diffusion ($k=10^9$-10^{10} $M^{-1}s^{-1}$) (Pryor, 1973). Hydrogen abstraction by a lipid peroxy radical (atom transfer) is an important part of chain propagation where a lipid hydroperoxide is formed and a lipid alkyl radical is regenerated to continue the chain reaction (Uri, 1961). Under conditions of abundant oxygen (>100 mm partial pressure), the concentration of lipid peroxy radicals is high and only termination reactions involving two lipid peroxy radicals occur (Pryor, 1976). However, in the autoxidation of methyl linoleate, the rate of hydrogen abstraction by the methyl linoleate peroxy radical is seven orders of magnitude greater than the termination reaction of two lipid peroxy radicals (Howard, 1973). The site of hydrogen abstraction is most favored at the divinylmethane portion of the PUFA. These hydrogens are especially susceptable to abstraction due to the lower carbon-hydrogen bond energy of 85 kcal/mole compared to 94 kcal/mole for an unactivated secondary carbon-hydrogen bond (Mead, 1976; Dahle et al., 1962). The resultant free radical is resonance stabilized by the neighboring double bonds:

In a phospholipid bilayer with the PUFA hydrocarbon chains parallel, the possibilities for chain reactions are maximized since the unsaturated centers are in close proximity to each other.

Termination of lipid peroxidation can occur by several mechanisms. Termination reactions entail any recombination of two free radicals to form a non-radical product:

$$L\cdot + L\cdot \longrightarrow L{:}L$$

$$LOO\cdot + L\cdot \longrightarrow LOOL$$

$$LOO\cdot + LOO\cdot \longrightarrow LOOL + O_2\ (^1O_2)$$

It should be pointed out that the first two termination reactions shown involving alkyl radicals need to be considered only at low dioxygen partial pressures (<100 mm) where the PUFA alkyl radical has an extended half-life (Pryor, 1973). Although the products of termination reactions are non-radical, this should not be construed as non-reactive. The peroxide products are unstable and readily decompose to radical products in the presence of transition metals (Demopoulos, 1973; Sosnovsky and Rawlinson, 1971). There is evidence for singlet oxygen formation from the decomposition of two lipid peroxy radicals (Nakano et al., 1976). Singlet oxygen and olefins readily react to form dioxetenes and peroxides (Foote, 1963). It seems evident that the rate of termination reactions is influenced by the relative stability of the radical. The probability of two radicals recombining increases with increasing concentration of free radicals. The rate of termination can also be enhanced by the action of antioxidants. Antioxidants are by definition, compounds that form relatively stable free radicals and are not reactive with the

methylene hydrogens of PUFA. Thus, they increase the rate of termination reaction by decreasing the rate of propagation reactions (Boguth and Niemann, 1971).

$$LOO\cdot + AH \longrightarrow LOOH + A\cdot$$

In the case of tocopherol, the tocopherol free radical may dimerize (Csallany, 1971), reduce another radical or form the tocopherol quinone (Boguth and Niemann, 1971). Thio compounds are also well known scavengers of free radicals (Slater, 1972). Compounds like glutathione terminate radical processes by acting as hydrogen donors, followed by dimerization:

$$2GSH + 2L\cdot \longrightarrow L{:}L + GSSG$$

Free Radicals in Biology

Numerous processes have been demonstrated to generate free radicals in the cell. Among these, radiolysis (Ausloos, 1968; Bacq and Alexander, 1966; Errera and Forssberg, 1960; Altman et al., 1970), photolysis (Turro, 1965; Foote, 1976), exposure to pollutants such as ozone (Pryor, 1971; Diaper, 1973), NO_2 (Roehn et al., 1971; Thomas et al., 1968), and singlet oxygen (Foote, 1976) have been reviewed elsewhere and are not within the scope of this thesis.

Free radicals in biology can also be generated by enzymatic reactions that proceed by sequential one-electron steps. It is known that some enzymes do produce radicals which can diffuse from the enzyme surface before they are oxidized or reduced to an even electron species

(Yamazaki, 1971). The oxidant in many one-electron reactions is dioxygen and any such leakage must be rare or life would be impossible since the destructive power of dioxygen would overwhelm its usefulness as a thermodynamically desirable electron sink (Geroge, 1965; Yamazaki et al., 1968). Enzymes that have been demonstrated to generate free radicals and catalyze lipid peroxidation in vitro include NADPH-cytochrome P450 reductase (Pederson and Aust, 1972; Sugioka and Nakano, 1975; King et al., 1975), NADH-cytochrome b_5 reductase (Pederson et al., 1973), xanthine oxidase (King et al., 1975; Pederson and Aust, 1973; Fong et al., 1973; Kellogg and Fridovich, 1975) lipoxygenases (Funk et al., 1976; Dolev et al., 1967) and lactoperoxidase (Welton and Aust, 1972). However, the in vivo significance of radicals "leaking out" from these enzymatic processes and initiating autoxidation reactions is yet to be delineated.

One process where free radical oxidative damage occurs is in the phagocytosic killing of ingested microorganisms by polymorphonuclear leukocytes. During ingestion of the particulate material, leukocytes display a sudden burst in oxygen consumption (Baldrige and Gerard, 1933; Sbarra and Karnovsky, 1959) which is not associated with mitochondrial oxidative phosphorylation (Sbarra and Karnovsky, 1959). Large amounts of hydrogen peroxide (Iyer et al., 1961; Paul and Sbarra, 1968; Root et al., 1975) and superoxide (Babior et al., 1973; Weening et al., 1975) are produced, both of which are precursors for the hydroxyl radical (Haber and Weiss, 1934).

Free Radical Formation from Metabolism of Xenobiotics

The metabolism of chlorinated hydrocarbons such as chloroform and carbon tetrachloride causes toxic reactions in vivo that are mediated by

radical formation (Slater, 1972; Rechnagel, 1967). Although these compounds are relatively stable in respect to unimolecular homolysis, they are strong one-electron acceptors and are known to undergo reactions of this type (Biaselle and Miller, 1974):

$$\text{Donor}^{n+} + CCl_4 \longrightarrow \text{Donor}^{(n+1)+} + \cdot CCl_3 + Cl^-$$

Recknagel (1967) suggested that the microsomal NADPH-dependent drug metabolism system forms the $\cdot CCl_3$ radical via reduction of carbon tetrachloride. These radicals subsequently attack lipid molecules and initiate lipid peroxidation (Ugazio et al., 1973). The metabolism of ethanol may also result in free radical production. Ethanol intoxication results in increased peroxide content in liver extracts (Di Luzio and Costales, 1965; Kalish and Di Luzio, 1966). Concomitant administration of antioxidants with ethanol protected rats against fatty livers (Di Luzio, 1963, 1964).

Paraquat, a non-selective herbicide (Calderbank 1968), can be enzymatically reduced to form a free radical in aqueous solutions in the absence of dioxygen (Michaelis and Hill, 1933). The reduction is catalyzed by the microsomal NADPH-dependent mixed function oxidase system (Bus et al., 1974). Dioxygen rapidly reoxidizes this radical forming superoxide (Farrington et al., 1973). Bus et al. (1974, 1975) suggested the cyclic reduction and oxidation of paraquat with the concomitant formation of superoxide results in cell death. Lipid peroxidation has been suggested to result from excess superoxide production (Bus et al., 1974; Heikkila and Cohen, 1973). Other xenobiotics which undergo this cyclic reduction and oxidation are 6-hydroxydopamine (Heikkila and Cohen, 1973),

adriamycin (Goodman and Hochstein, 1977; Myers et al., 1977), alloxan (Cohen and Heikkila, 1974) and dialuric acid (Heikkila and Cohen, 1975).

Biological Defense Against Free Radical-Dependent Reactions

The reduction of dioxygen to water by a series of one-electron transfers generates exceedingly reactive intermediates which can initiate lipid peroxidation. It is these intermediates, superoxide, hydrogen peroxide, and hydroxyl radical that are thought to cause oxygen toxicity.

$$O_2 \xrightarrow{e^-} O_2^{\cdot-} \xrightarrow[H^+]{e^-} H_2O_2 \xrightarrow{e^-} {\cdot}OH \xrightarrow{e^-} H_2O$$

Organisms which utilize dioxygen as an electron sink must minimize the production of these intermediates and also efficiently scavenge those whose production cannot be avoided. Cytochrome oxidase, which accounts for most of the dioxygen consumed by aerobes, produces H_2O as the first detectable product of dioxygen reduction (Malkin and Malmstrom, 1970). However, as previously mentioned, numerous biological oxidations do generate free superoxide (Fridovich, 1974). The aqueous production of superoxide in the presence of transition metals rapidly leads to the production of hydrogen peroxide and hydroxyl radical (Haber and Weiss, 1934). To scavenge superoxide, most aerobes contain superoxide dismutase which rapidly dismutases superoxide to hydrogen peroxide and dioxygen (McCord and Fridovich, 1968). Hydrogen peroxide is, in turn, metabolized by catalase and glutathione peroxidase (Sies et al., 1972, 1974). The biological significance of superoxide dismutase is exemplified by its ubiquity in aerobic organisms. A large number of aerobes, aerotolerant anaerobes, and strict anaerobes were surveyed for their content of

superoxide dismutase (McCord et al., 1971). All the microorganisms which could grow in the presence of dioxygen (aerobes and aerotolerant anaerobes) contained superoxide dismutase and those which could not did not contain the enzyme. Catalase was found to be ubiquitous in all aerobes screened but not present in aerotolerant anaerobes or strict anaerobes. Superoxide dismutase activity could also be induced by increased partial pressure of dioxygen in numerous aerotolerant anaerobes (Gregory and Fridovich, 1973). Induction of superoxide dismutase activity permitted the cells to be much more resistant toward hyperbaric oxygen than uninduced cells (Gregory and Fridovich, 1973).

In situations where the rate of superoxide and hydrogen peroxide formation exceeds the rate of their metabolism, lipid peroxidation may occur. The highly reactive hydroxyl radical is formed by the reaction of superoxide and hydrogen peroxide with transition metal catalyst (Haber and Weiss, 1934). In such cases, a large number of biological antioxidants act as radical scavengers. Vitamin E is perhaps the most studied lipid-soluble antioxidant (Tappel, 1962; Roels, 1967). It inhibits in vitro lipid peroxidation and vitamin E deficiency in vivo increases lipid peroxidation in erythrocytes, adipose and adrenals exposed to hyperoxia (Mengel and Kann, 1966).

Water-soluble antioxidants include vitamin C and thio-containing compounds. Vitamin C is often used as a food preservative and scavenges radicals by a sequential two-electron oxidation to form dehydroascorbate (Levandoski et al., 1964; Kluge et al., 1967). Cysteinamine, glutathione, ergothionine and related sulfhydryl-containing compounds all impart radical scavenging activity (Slater, 1972).

Once lipid hydroperoxides are formed by lipid peroxidation, secondary initiation can arise from the decomposition of lipid hydroperoxides. Enzymatic removal of the hydroperoxides would minimize secondary initiation reactions. This is the function of the selenium-containing glutathione peroxidase (Christophersen, 1968). Glutathione, in addition to having antioxidant properties, also functions as a reductant for glutathione peroxidase (Swern, 1970). Removal of lipid hydroperoxides also minimizes the formation of malondialdehyde (MDA) (Pryor et al., 1976). MDA, a minor decomposition product of lipid hydroperoxides, has been demonstrated to cause tissue damage (Reddy et al., 1973). Glutathione peroxidase is of particular importance in erythrocytes. Protection against oxidative damage or lysis of erythrocytes induced by hyperbaric oxygen is afforded by both antioxidants and glutathione peroxidase (Kosower et al., 1969; Rotruck et al., 1972). Dietary studies demonstrated that erythrocytes from selenium-deficient animals were more sensitive to oxidative damage. Those animals were practically devoid of glutathione peroxidase activity and were not protected by added glutathione (Rotruck et al., 1973).

Biological Consequence of Lipid Peroxidation

The major sites within a cell that are susceptable to lipid peroxidation are the membranes, especially those of subcellular organelles. The endoplasmic reticulum is particularly susceptible due to its high concentration of unsaturated fatty acids (May and McCay 1968a, 1968b). Mitochondria are also susceptible to lipid peroxidation as evidenced by mitochondrial swelling and eventual lysis during oxidative stress (Ottolenghi et al., 1955). Membrane associated enzymes and proteins are also apt to suffer from lipid peroxidation and undergo loss of enzyme

activity (Chio and Tappel, 1969), loss of solubility due to aggregation (Pokorny and Janicek, 1968), peptide chain scission (Zirlin and Karel, 1969) as well as loss of amino acids (Roubal and Tappel, 1966). Loss of sulfhydryl containing amino acids usually occurs through the formation of disulfide crosslinks (Wills, 1961). Lipid peroxidation can also cause extensive damage to nucleic acids (Roubal, 1971) and hemoproteins (Tappel, 1955). Hemoproteins have been demonstrated to promote lipid peroxidation when they are partially denatured, presumably by exposing the heme group (Eriksson et al., 1971). Ultimately, lipid peroxidation can result in cell lysis and death. Excellent reviews on the biological consequences of lipid peroxidation are available (Plaa and Witschi, 1976; Lewis and Del Maestro, 1980).

Detection of Radical Reaction Products of Lipid Peroxidation

Lipid peroxidation produces a variety of breakdown products including alcohols, ketones, aldehydes and ethers (Gardner, 1975). The peroxidation of linoleic acid alone results in the formation of at least 20 degradation products (Gardner et al., 1974). One of the most frequently used assays for lipid peroxidation is measuring MDA formation by the thiobarbituric acid (TBA) test (Buege and Aust, 1978). The formation of lipid endoperoxides in PUFA containing at least 3 methylene interrupted double bonds can lead to the formation of MDA as a breakdown product (Dahle et al., 1962). MDA reacts with TBA to form a 1:2 adduct which absorbs light of 535 nm (Niehaus and Samuelsson, 1968).

Lipid hydroperoxide determination by iodide titration has also been used to assess the extent of peroxidation (Buege and Aust, 1978). The procedure is based on the ability of I^- to reduce hydroperoxides by the following reaction (Mair and Hall, 1971).

$$2H^+ + LOOH + 3\ I^- \longrightarrow H_2O + ROH + I_3^-$$

The triiodide content can be determined spectrophotometrically (Buege and Aust, 1978). Measurement of the lipid hydroperoxide content is advantageous over the TBA test since it permits a more accurate comparison of lipid peroxide levels in dissimilar lipid membranes.

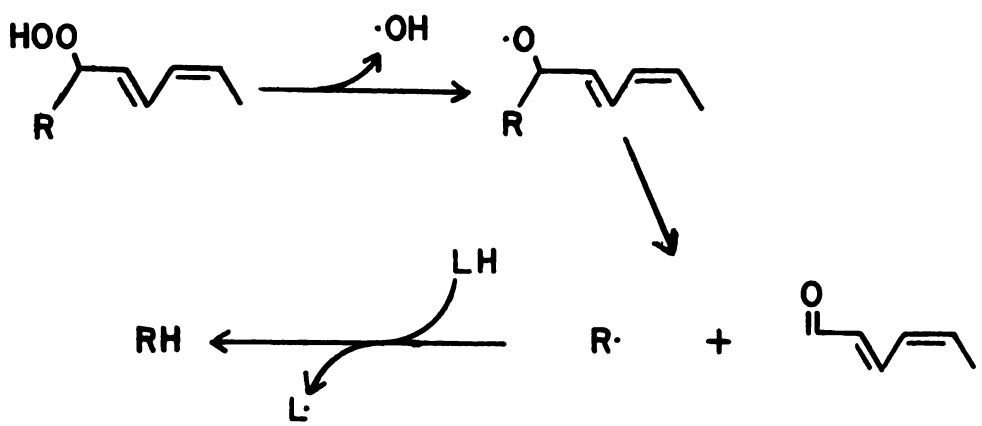
Lipid peroxidation is accompanied by a rearrangement of the PUFA double bonds, leading to the formation of conjugated dienes (Bolland, 1949). The diene chromophore absorbs at 233 nm and is easily measured to follow the course of lipid peroxidation (Buege and Aust, 1978). The detection of conjugated dienes is a sensitive assay for both *in vivo* and *in vitro* lipid peroxidation (Pryor, 1980). Neither TBA nor peroxide determinations are reliable assays for *in vivo* lipid peroxidation. Quantitation of hydroperoxides in living tissue is difficult because the are metabolized by glutathione peroxidase (Lawrence and Burk, 1976) and they readily decompose in the presence of hemoproteins (Eriksson *et al.*, 1971) and transition metals (O'Brien and Little, 1969). The quantitation of MDA in living tissue is also difficult due to Schiff base condensation of MDA with amino groups (Dillard and Tappel, 1971).

$$RNH_2 + CHO\text{-}CH_2\text{-}CHO + H_2N\text{-}R' \longrightarrow R\text{-}N{=}CH\text{-}CH{=}CH\text{-}NR\text{-}R'$$

Double bond rearrangement after condensation results in the formation of a material called liposfuscin. Liposfuscin is known to accumulate with age in virtually all animal cells (Porta and Hartroft, 1969). Quantitation of this fluorescent chromophore is a reliable assay for *in vivo* lipid peroxidation (Dillard and Tappel, 1971; Chio and Tappel, 1968).

The widespread occurrence of this pigment and its clear dependence upon oxidative stress is good evidence that lipid peroxidation occurs *in vitro* (Tappel *et al.*, 1973).

The decomposition of lipid hydroperoxides produces a variety of volatile products (Pryor, 1978). Two volatile products, ethane and pentane, arise from the peroxidative decomposition of ω-3 and ω-6 fatty acids, respectively, as shown below (Pryor, 1980).



R=C_2H_5 or C_5H_{11}

The formation of the alkoxy radical requires the catalysis of a metal ion (O'Brien and Little, 1969). The resultant alkoxy radical can then undergo β-scission to form the alkyl radical. Hydrogen abstraction by the alkyl radical gives either pentane or ethane gas. The quantitation of these gases from expired animal breath has been used as an index of lipid peroxidation (Pryor, 1978; Riley *et al.*, 1974).

Detection of Radicals of Lipid Peroxidation

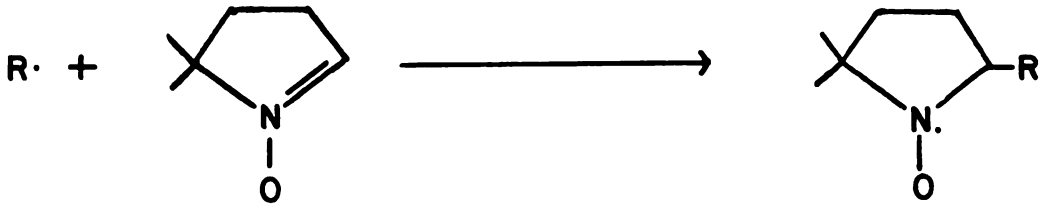
A free radical is, by definition, a species with unpaired electrons. Since a moving charge generates a magnetic field, the spin of the unpaired electron results in a magnetic dipole moment, not dissimilar to the

north and south poles of a magnet. The spin of the unpaired electron and thus the magnetic moment has only two allowed orientations relative to any axis of atomic or molecular reference. In the absence of an external magnetic field, the orientations are usually equal in energy (degenerate). However, in the presence of an applied magnetic field, the two spin orientations separate in energy. The spin state with its magnetic moment aligned parallel to the applied field will be lower in energy than the antiparallel orientation. It is this separation in energy which allows for detection of free radicals by electron paramagnetic (EPR) spectroscopy. (See Wertz and Bolton, 1972 for detailed discussion of theory and application of EPR spectroscopy).

The lower limits of free radical detection by EPR spectroscopy with existing instruments is about 10^{-6} to 10^{-8} M (Borg, 1976). Thus it is only possible to detect stable free radicals or radicals which accumulate to measurable concentration. However, many radicals of biological interests are highly reactive and never reach concentration high enough for detection. Of interest to this thesis is the $\cdot OH$ which is so reactive (Dorfman and Adams, 1973) that direct detection by EPR spectroscopy in a biological system would be impossible.

The recent introduction of spin trapping by Janzen and Blackburn (1968) has offered an indirect method for the detection of these short-lived radicals by EPR spectroscopy. In theory, the short-lived radical of interest reacts with a nitrone (the spin trap) to yield the nitroxide radical (spin adduct). The nitroxide free radical is relatively stable and long-lived which permits it to be observed at room temperature using conventional EPR equipment. Since the spin adduct accumulates, spin trapping is an integrative method of detecting free radicals and is more

sensitive than procedures which measure steady state levels (Swartz, 1972). The hyperfine splitting (hps) of the adduct by neighboring nuclei can provide information for identification and quantitation of the original radical. Hyperfine splitting arises from neighboring nuclei that possess nuclear spin angular momenta (^{1}H,^{2}D,^{14}N,^{13}C). Spin traps possessing a β-hydrogen such as 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) (shown below), will yield adducts with hps due to both the β-hydrogen and the nitroxide nitrogen. The magnitude of nitrogen hps (A_N) and β-hydrogen hps (A_H) are very sensitive to the nature of the trapped radical. This can aid in identification of the original free radical. With DMPO, the bulkiness of the trapped radical gives rise to a relatively large variation in A_H (Janzen, 1980). The greater the variation in A_H, the better the spin trap is for purposes of identifying the original radical.



Initiation of Lipid Peroxidation Via Oxygen Activation

A significant finding in the field of lipid peroxidation and oxygen toxicity is that superoxide is formed in numerous biochemical reactions and that the generation of superoxide can result in lipid peroxidation. Central to the field of lipid peroxidation research is the mechanism of superoxide-dependent initiation. Superoxide is not exceptionally

reactive with a large number of organic substrates, including PUFA (Tyler, 1975; Fong et al., 1973). Thus, superoxide appears to be a precursor to a more reactive species which initiates lipid peroxidation. Singlet oxygen and the hydroxyl radical have been products proposed most often. Pederson and Aust (1973) proposed that initiation of superoxide-dependent lipid peroxidation occurred via singlet oxygen. The predominant reaction of superoxide in aqueous solutions is that of dismutation to hydrogen peroxide (Czapski, 1971).

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

The nonenzymatic dismutation of superoxide has been proposed to produce singlet oxygen (Khan, 1970). Singlet oxygen can then react with diene bonds of PUFA to form lipid hydroperoxides (Foote, 1968). Further reactions of the lipid hydroperoxides can give rise to free radicals and initiate lipid peroxidation (Pryor, 1976). The involvement of singlet oxygen in superoxide-dependent lipid peroxidation was predicated on the ability of diphenylisobenzylfuran, a singlet oxygen trap, to inhibit lipid peroxidation (Pederson and Aust, 1973). This interpretation of the experimental data was incorrect since diphenylisobenzylfuran was later shown to be a radical scavenger (King et al., 1975). Subsequent studies demonstrated that less than 0.008% of the dioxygen formed from non-enzymatic aqueous dismutation of superoxide is in the singlet state (Foote, et al, 1980).

In 1934, Haber and Weiss observed the generation of a strong oxidant during the aqueous generation of superoxide. These workers proposed

that the strong oxidant was hydroxyl radical, formed from the reduction of hydrogen peroxide by superoxide.

$$O_2^{\cdot-} + H_2O_2 \longrightarrow O_2 + {\cdot}OH + {}^-OH$$

This reaction is now called the Haber-Weiss reaction and has been the subject of controversy over the years. Numerous studies have failed to demonstrate such a reaction (McClure and Fee, 1976; Halliwell, 1976). Fee and Valentine (1977) have labelled the Haber-Weiss reaction a non-reaction. The generation of the hydroxyl radical observed by Haber and Weiss (1934) was most likely due to iron contamination.

Aside from dismutation, superoxide also reduces transition metals such as iron (Ilan and Czapski, 1977). The reductive cleavage of hydrogen peroxide by iron (II) to produce hydroxyl radical does occur (Walling, 1975).

$$O_2^{\cdot-} + Fe(III) \longrightarrow O_2 + Fe(II)$$
$$Fe(II) + H_2O_2 \longrightarrow Fe(III) + {\cdot}OH + {}^-OH$$

The reduction of Fe(III) by superoxide proceeds with a second order rate constant of 10^7 $M^{-1}s^{-1}$ (Ilan and Czapski, 1977). The rate constant for the reduction of hydrogen peroxide by iron(II) was measured to be 76 $M^{-1}s^{-1}$ (Hardwick, 1957). Thus, it appears that the iron- catalyzed Haber-Weiss reaction can kinetically account for the initiation of superoxide-dependent lipid peroxidation. This mechanism has been proposed by numerous investigators (Fong et al., 1973b; Kellogg and Fridovich, 1975, 1977; Fridovich and Porter, 1981).

Other workers have found little evidence for hydroxyl radical involvement in superoxide-dependent lipid peroxidation. Removal of hydrogen peroxide, an intermediate of the iron-catalyzed Haber-Weiss reaction, by catalase did not inhibit lipid peroxidation (Tyler, 1975; Svingen et al., 1978; Svingen and Aust, 1980). These workers also failed to inhibit superoxide-dependent lipid peroxidation by hydroxyl radical traps.

To account for the observed data, an alternate mechanism of initiation involving an activated dioxygen-transition metal complex has been proposed (Hochstein and Ernster, 1963; Svingen et al., 1978). This complex, $Fe^{3+}O_2 \quad Fe^{2+}O_2$, is termed the perferryl ion. Perferryl ion formation occurs by the complexation of superoxide with iron (III). The proposal that the perferryl ion is involved in the initiation of lipid peroxidation has relied on negative data indicating that hydrogen peroxide, superoxide alone or iron alone cannot initiate lipid peroxidation. No positive evidence for the involvement of the perferryl ion exist, and the mechanism by which the perferryl ion would initiate lipid peroxidation is unclear (Chapter IV addresses this topic).

Initiation of Lipid Peroxidation Via Peroxide Decomposition

Once lipid hydroperoxides are formed, secondary initiation reactions can occur via the decomposition of these lipid hydroperoxides to free radical products. Contrary to popular belief, lipid hydroperoxides are relatively stable. The calculated half-life for the uncatalyzed, unimolecular homolysis of tert-butyl hydroperoxide at 37°C is 10^9 years (Pryor, 1976).

$$LOOH \longrightarrow LO\cdot + \cdot OH$$

The half-life should be very similar for all simple alkyl hydroperoxides (Benson, 1968). Thus, it is doubtful that this mechanism is of any significance in biological systems. But in the presence of transition metals, peroxides readily undergo reductive activation reactions (O'Brien, 1969).

$$Fe(II) + LOOH \longrightarrow Fe(III) + LO\cdot + -OH$$

This reaction is analogous to the reductive cleavage of hydrogen peroxide. The rate of reductive activation is greatly affected by the chelation of iron (Chalk and Smith, 1957). Although a body of literature exists on the effect of iron chelation on reductive activation of lipid hydroperoxides (Svingen et al., 1978; Svingen and Aust, 1980; O'Brien and Little, 1969), in vivo reductive activation of lipid hydroperoxides probably occurs through the action of hemoproteins. Numerous hemoproteins have been demonstrated to accelerate the rate of lipid peroxidation, presumable by reacting with lipid hydroperoxides (Tappel and Zalkin, 1959; Tappel, 1953a, 1953b). Svingen et al., (1978) demonstrated that the microsomal hemoprotein cytochrome P450 enhances the rate of lipid peroxidation only in the presence of lipid hydroperoxides. Secondary initiation reactions involving lipid hydroperoxide decomposition can make a significant contribution to the extent of peroxidative damage. It has been suggested that over 90% of the peroxidation products formed in lipid peroxidation are due to peroxide-dependent initiation reactions (Svingen et al., 1978).

CHAPTER I

RABBIT LIVER MICROSOMAL LIPID PEROXIDATION

ABSTRACT

Rat and rabbit liver microsomes catalyze an NADPH-cytochrome P450 reductase-dependent peroxidation of endogenous lipid in the presence of ADP chelate Fe^{3+}. Although liver microsomes from both species contain comparable levels of NADPH-cytochrome P450 reductase and cytochrome P450, the rate of lipid peroxidation (assayed by malondialdehyde and lipid hydroperoxide formation) catalyzed by rabbit liver microsomes is only about 40% of that catalyzed by rat liver microsomes. Microsomal lipid peroxidation was reconstituted with liposomes made from extracted microsomal lipid and purified protease-solubilized NADPH-cytochrome P450 reductase from both rat and rabbit liver microsomes. The results demonstrated that the lower rates of lipid peroxidation catalyzed by rabbit liver microsomes could not be attributed to the reductase. Microsomal lipid from rabbit liver was found to be much less susceptable to lipid peroxidation. It was due to the lower polyunsaturated fatty acid content rather than the presence of antioxidants in rabbit liver microsomal lipid. Gas-liquid chromatographic analysis of fatty acids lost during microsomal lipid peroxidation revealed that the degree of fatty acid unsaturation correlated well with rates of lipid peroxidation.

INTRODUCTION

Upon the homogenation of tissues, the endoplasmic reticulum breaks up and reseals as small spheres (Palade and Siekevitz, 1956). These small spheres, microsomes, can be isolated by differential centrifugation (Palade and Siekevitz, 1956). Microsomes can be isolated from numerous mammalian tissue, but the vast majority of research has been with liver microsomes.

Microsomes contain an NADPH-dependent electron transport chain which catalyzed the hydroxylation of more than two hundred substrates, both endogenous and exogenous (Conney, 1967). The first component of this chain is NADPH-cytochrome P450 reductase, a flavoprotein which transfers reducing equivalents from NADPH to the terminal oxidase (Omura and Sato, 1964). The terminal oxidase, cytochrome P450, incorporates one atom of oxygen from dioxygen into the substrate as a hydroxyl group or as a epoxide. The other atom is incorporated into water (Gillette et al., 1972; Gunsalus et al., 1975).

Lipid peroxidation (LP) initiated by an enzymatic redox reaction was first demonstrated in rat liver microsomes. Lipid peroxidation, as evidenced by dioxygen uptake with the concomitant formation of malondialdehyde (MDA) and lipid hydroperoxides (LOOH), was enhanced by the addition of NADPH (Hochstein and Ernster, 1963; Beloff-Chain et al., 1965) and nucleotide-chelated iron (Beloff-Chain et al., 1963; Hochstein

et al., 1964; Wills, 1969a, 1969b; May and McCay 1968a; Poyer and McCay, 1971). The most efficient iron chelator for microsomal LP was ADP.

The involvement of NADPH-cytochrome P450 reductase in microsomal LP was initially established by the ability of an antibody to the reductase to inhibit NADPH-dependent microsomal LP (Pederson et al., 1973). Studies also demonstrated that microsomal LP could be reconstituted utilizing purified protease-solubilized NADPH-cytochrome P450 reductase (Pederson and Aust, 1972). Both NADPH and ADP-Fe^{3+} were required for activity in the reconstituted system.

A unique property of the reductase is its ability to catalyze the electron transfer from a two-electron donor (NADPH) to a one-electron acceptor (cytochrome P450) (Omura and Sato, 1964). Aside from its in vivo electron acceptor, the reductase can also reduce other one-electron acceptors such as cytochrome c (Vermilion and Coon, 1978), ferricyanide (Lyakhovich et al., 1977) and EDTA-Fe^{3+} (Kamin and Masters, 1968). The reductase has also been shown to reduce dioxygen to form superoxide (Mishin et al., 1976a; Gnosspelius et al., 1969-1970). With the facile transfer of one-electron to either iron (III) or possibly dioxygen, it is not surprising that the reductase can readily catalyze LP. In fact, enzyme catalyzed LP can be viewed as catalysis of one-electron transfer to either iron (III) or dioxygen from two-electron donors.

Cytochrome P450 involvement in microsomal LP has also been proposed. Compounds such as SKF525-A, which specifically bind to cytochrome P450 were found to inhibit microsomal lipid peroxidation (Svingen et al., 1979). The inhibition by drug substrates could not be attributed to electrons being diverted away from initiation reactions toward drug metabolism since SKF525-A is not readily metabolized (Lee et al., 1968).

EDTA-Fe^{3+} could reverse the inhibition of LP by SKF525-A (Svingen et al., 1979). These observations, in addition to the ability of purified cytochrome P450 to enhance reconstituted NADPH-dependent LP (Svingen et al., 1979), suggests that cytochrome P450 is involved in microsomal LP. It also suggests that EDTA-Fe^{3+} has similar catalytic activity. Svingen et al., (1979) proposed that both ferric cytochrome P450 and EDTA-Fe^{3+} (upon reduction to EDTA-Fe^{2+} by the reductase) enhance LP by decomposing LOOH to free radical products.

$$\text{LOOH} + \text{EDTA-Fe}^{2+} \longrightarrow \text{LO}\cdot + \text{-OH} + \text{EDTA-Fe}^{3}$$

$$\text{LOOH} + \text{cyto. P450} \longrightarrow \text{LO}\cdot + \text{-OH} + \text{denatured cyto. P450}$$

It has been reported that rabbit and rat liver microsomes contain comparable levels of NADPH-cytochrome P450 reductase and cytochrome P450 (Chhabra et al., 1974). The rate of LP catalyzed by rabbit liver microsomes is much lower than that of rat liver microsomes. Levin et al. (1973) found lower rates of LP catalyzed by rabbit liver microsomes than by rat liver microsomes as detected by cytochrome P450 destruction. Furthermore, Gram and Fouts (1966) observed that the microsomal supernatant fraction of rabbit livers catalyzed lower rates of LP than that of rat livers when assayed by MDA formation. If the rate of LP in rat and rabbit liver microsomes is dependent only on the reductase specific activity and cytochrome P450 specific content, then these results are inconsistent with previously proposed mechanisms of microsmal LP.

Mishin et al., (1976b) reported the lower rates of LP catalyzed by rabbit liver microsomes were not due to any distinct properties of the

reductase, but were due to either the increased content of endogenous antioxidants or structural peculiarities of the rabbit liver microsomal membrane. We have therefore reconfirmed the relatively slow rates of LP catalyzed by rabbit liver microsomes, and in addition have characterized the lipid of rat and rabbit liver microsomes to determine whether possible differences in the composition of the lipid could account for the observed rates of LP.

MATERIALS AND METHODS

Thiobarbituric acid, NADPH, cytochrome c (Type VI), ADP, bromelain and butylated hydroxytoluene (BHT) were obtained from Sigma Chemical Co. Bovine serum albumin was obtained from Pentex Chemical Co. Column packing for gas-liquid chromatography was a product of Supelco. All reagents were analytical grade and used without further purification.

Rat liver microsomes were isolated from 250-274 g male Sprague Dawley rats (Spartan Research Animals, Haslett, MI) by the method of Pederson et al. (1973). Rabbit liver microsomes were also isolated by this method from 2 kg male New Zealand white rabbits. Microsomes were washed by resuspension in argon purged distilled deionized water to a protein concentration of 5 mg/ml and centrifugation at 100,000 x g for 90 minutes. All microsomes were stored in argon purged 50 mM Tris-Cl at -20°C (pH 7.5 at 37°C). Microsomal lipid was extracted by the method of Folch et al. (1957) and stored at -20°C in $CHCl_3$:CH_3OH (2:1). All solvents used in microsomes and lipid isolation and storage were purged with argon and kept at 4°C to prevent autoxidation of microsomal lipid.

NADPH-cytochrome P450 reductase (EC 2.3.6.4) was purified from both rat and rabbit liver bromelain solubilized microsomes to a specific activity to 55 units/mg protein (Pederson et al., 1973). Cytochrome P450 was purified to a specific content of 13 nmol/mg protein (Guengerich, 1977).

Microsomal LP reaction mixtures contained ADP-Fe^{3+} (3 mM ADP, 0.15 mM $FeCl_3$), 0.5 mg/ml microsomal protein and 0.1 mM NADPH in 50 mM Tris-Cl, pH 7.5 at 37°C, unless otherwise indicated in the figure legends. Liposomal LP reaction mixtures contained 1 μmol lipid phosphate/ml, 0.1 units NADPH-cytochrome P450 reductase/ml, ADP-Fe^{3+} (3 mM ADP, 0.15 mM $FeCl_3$) and 0.1 mM NADPH in 50 mM Tris-Cl, pH 7.5 at 37°C. Reactions were initiated by the addition of NADPH. Iron chelate solutions were made by the addition of $FeCl_3$ to the appropriate chelate solutions and then pH was adjusted. All iron chelate concentrations are expressed as concentrations of $FeCl_3$. LP was assayed by MDA and LOOH formation (Buege and Aust, 1978). To prevent further peroxidation of lipids during the MDA assay, 0.02 volumes of ethanolic 2% BHT was added to the 2-thiobarbituric acid stock solution. All rates are expressed as initial rates and samples were assayed for MDA or LOOH within 5 minutes after the start of the reaction. All reactions were carried out at 37°C in metabolic shaking water baths under an air atmosphere.

The fatty acid composition of the microsomal phospholipids was determined by gas-liquid chromatographic analysis of the methyl esters. Methyl esters were prepared by the method of Morrison and Smith (1964). Chromatography was performed on a Varian model 3700 gas chromatograph equipped with a FID detector. The glass column (6 feet x 1/4 inch O.D.) was packed with 10% DEGS-PS on 80/100 Supelcoport.

Protein content was determined by the method of Lowry et al. (1951). Total lipid phosphate was assayed by the method of Bartlett (1959). NADPH-cytochrome P450 reductase activity was measured by cytochrome c reduction (Pederson et al., 1973). Cytochrome P450 content was calculated from its carbon monoxide difference spectrum (Omura and Sato, 1964).

RESULTS

Microsomal Enzymes and Lipid Peroxidation in Rat and Rabbit Liver Microsomes.

Table 1 compares the specific activity of NADPH-cytochrome P450 reductase (measured by cytochrome c reduction), the specific content of cytochrome P450, and the rates of lipid peroxidation in rat and rabbit liver microsomes. The specific activities of the reductase and the specific content of cytochrome P450 in microsomes from the two species were approximately equal. In contrast, the rates of NADPH and ADP-Fe^{3+}-dependent LP catalyzed by rabbit liver microsomes were only 41% and 38% of the activity of rat liver microsomes when assessed by MDA and LOOH formation, respectively. Extensive studies were performed to assure that the comparative rates of LP in the rat and rabbit systems were determined under optimal conditions for each microsomal preparation. LP incubation conditions were optimized in regards to pH, ionic strength, NADPH concentration, and protein concentrations (data not shown). The effect of iron and chelation of iron by ADP were also examined to optimize the rate of LP. Figure 1 plots the rate of MDA and LOOH formation in rabbit liver microsomes as a function of ADP concentration. The Fe^{3+} concentration was kept constant (0.15 mM). The results indicate that the rate of LP is exceedingly sensitive to ADP chelation of Fe^{3+}. A similar LP profile was seen with rat liver microsomes (not shown). Figure 2 shows the effect of Fe^{3+} (chelated by

Table 1. Microsomal Enzymes and LP in Rat and Rabbit Liver Microsomes

	Cytochrome P450 (nmol/mg protein)	NADPH-cytochrome P450 Reductase (units/mg protein)	Lipid Peroxidation MDA (nmol/min/mg protein)	LOOH
Rabbit liver microsomes n=6	1.06 ± 0.09 (96%)	0.25 ± 0.03 (89%)	1.15 ± 0.20 (41%)	6.2 ± 0.4 (38%)
Rat liver microsomes n=6	1.10 ± 0.06	0.28 ± 0.01	2.78 ± 0.08	16.5 ± 1.0

Rat and rabbit liver microsomal lipid peroxidation reaction mixtures contained 0.5 mg protein/ml, ADP-Fe^{3+} (1.7 mM ADP, 0.1 mM $FeCl_3$) and 0.1 mM NADPH in 50 mM Tris-Cl, pH 7.5 at 37°C. Incubation and assay conditions are described in Materials and Methods. Values are mean ± SEM. Values in parenthesis are percentage of the corresponding parameter of rat liver microsomes.

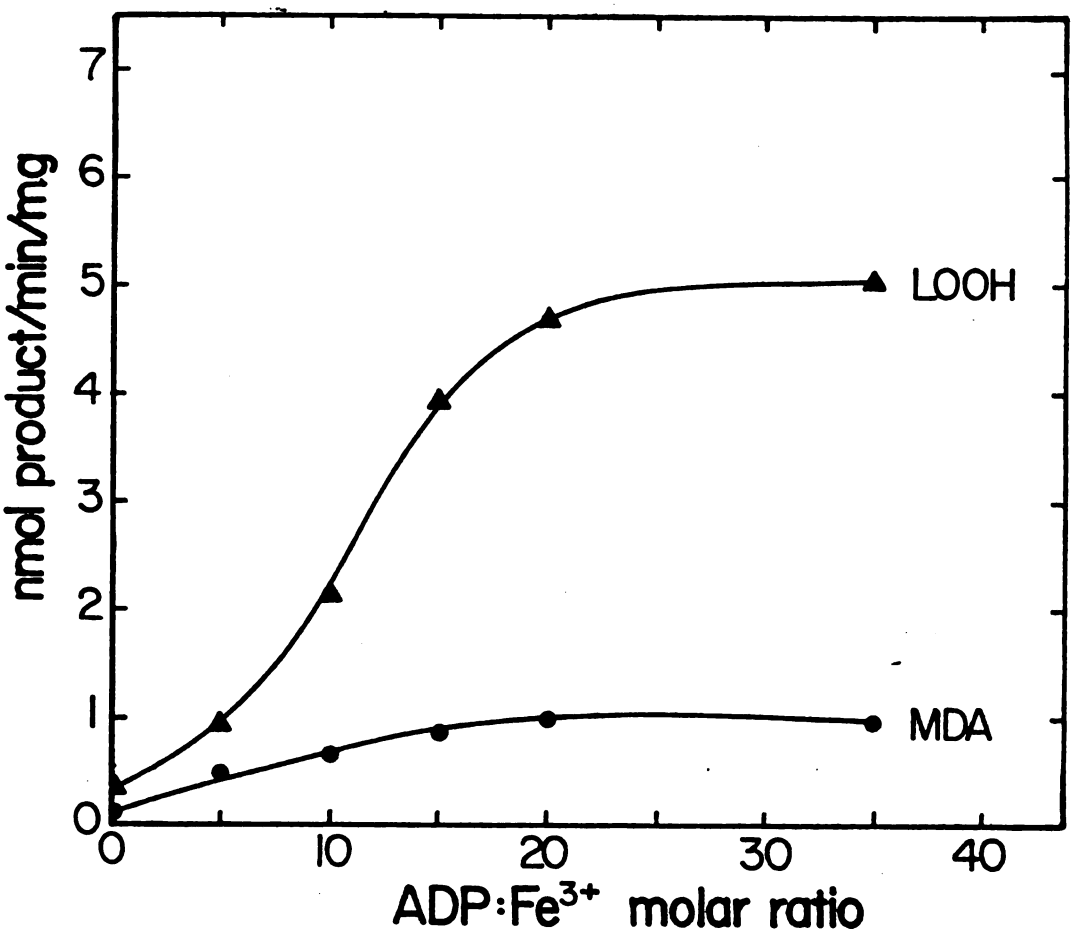


Figure 1. Rabbit Liver Microsomal LP as a Function of ADP to $FeCl_3$ Molar Ratio. Microsomal reaction mixtures contained 0.5 mg of microsomal protein/ml, 0.1 mM NADPH, 0.15 mM $FeCl_3$ and the specified amount of ADP in 50 mM Tris-Cl, pH 7.5 at 37°C. Incubation and assay conditions are described in Materials and Methods.

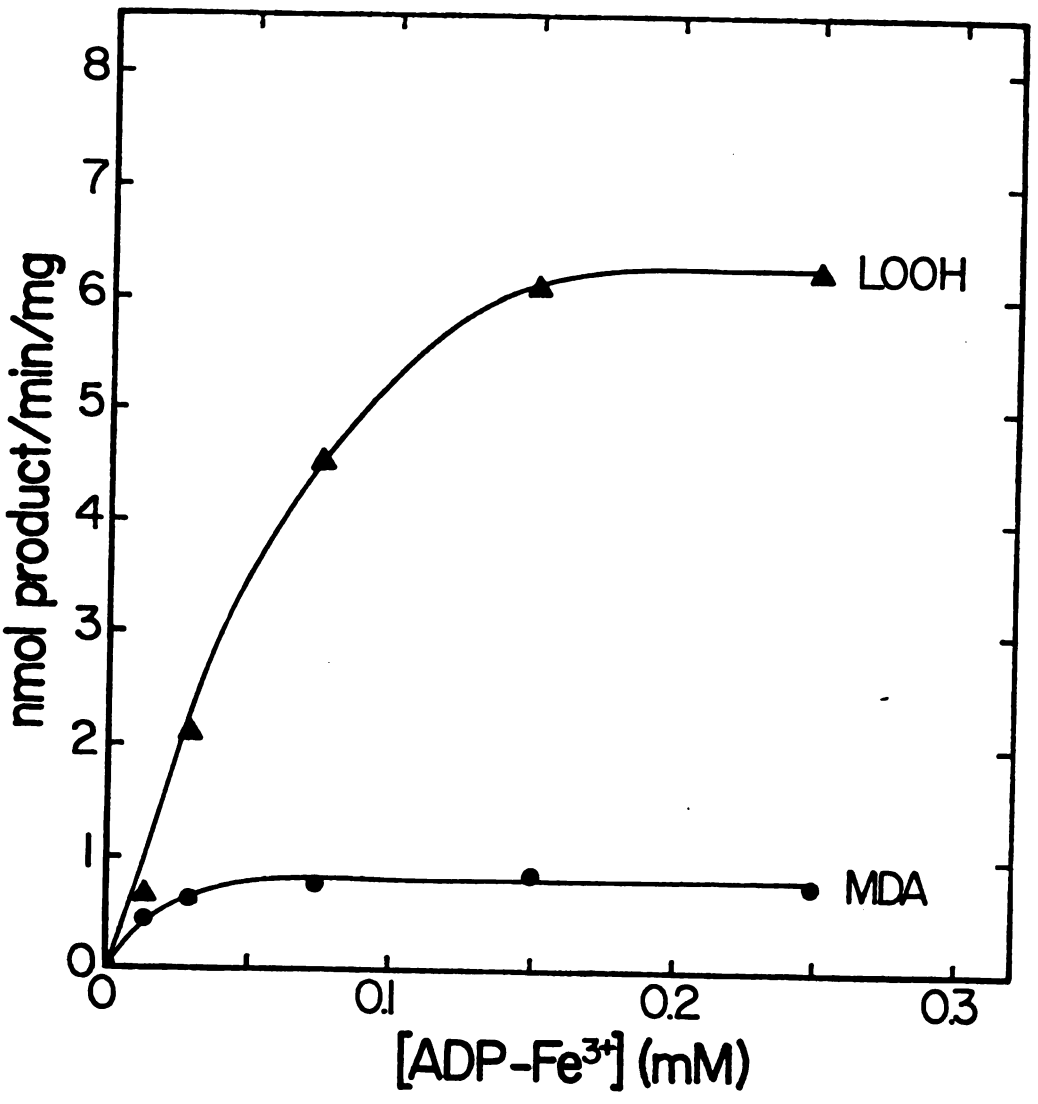


Figure 2. Rabbit Liver Microsomal LP as a Function of ADP-Fe^{3+} Concentration. Microsomal reaction mixtures contained 0.5 mg of microsomal protein/ml, 0.1 mM NADPH, and the specified amount of $FeCl_3$ in 50 mM Tris-Cl, pH 7.5 at 37°C. The ADP to $FeCl_3$ molar ratio was kept constant at 20 to 1. Incubation and assay conditions are described under Materials and Methods.

Table 2. Effect of Tris-Cl on Rabbit Liver Microsomal Lipid Peroxidation

Additions	nmol MDA/min/mg
-NADPH	0.12
50 mM Tris-Cl	1.03
50 mM NaCl	1.05

Reaction mixtures contained 0.5 mg microsomal protein/ml, ADP-Fe^{3+} (3 mM ADP, 0.15 mM $FeCl_3$), and 0.1 mM NADPH plus either 50 mM Tris-Cl or 50 mM NaCl, pH 7.5 at 37°C. Incubations and assay conditions are described in Materials and Methods.

Table 3. Reconstitution of Rabbit Liver Microsomal Lipid Peroxidation

Additions	MDA (nmol/min/ml)	LOOH (nmol/min/ml)
None	0.01	0.1
NADPH	0.05	0.4
ADP-Fe^{3+}	0.05	0.3
NADPH, reductase	0.05	0.4
ADP-Fe3+, reductase	0.05	0.3
NADPH, ADP-Fe^{3+}, reductase	0.23	0.9
NADPH, ADP-Fe^{3+}, reductase cytochrome P450	0.81	2.4
NADPH, ADP-Fe^{3+}, reductase EDTA-Fe^{3+}	0.83	7.5

Reaction mixtures contained liposomes (1 μmol lipid phosphate/ml) in 50 mM Tris-Cl, pH 7.5 at 37°C. The following additions were made as indicated: 0.1 mM NADPH, ADP-Fe^{3+} (1.7 mM ADP, 0.1 mM $FeCl_3$), EDTA-Fe^{3+} (0.11 mM EDTA, 0.1 mM $FeCl_3$), 0.3 nmol cytochrome P450/ml and 0.1 unit NADPH- cytochrome P450 reductase. Incubations and assay conditions are described in Materials and Methods.

ADP 20 to 1 molar ratio) concentration on the rate of rabbit liver microsomal LP. Very low rates of LP occurred in the absence of added Fe^{3+}. The rates of MDA and LOOH formation increased rapidly with increased ADP-Fe^{3+} concentration. Maximum rates of LP were obtained when the ADP-Fe^{3+} concentration was greater than 0.12 mM. Again, the rat microsome preps exhibited a similar LP profile.

Figure 3 shows the time course of MDA and LOOH formation during a rabbit liver microsomal LP reaction at optimal conditions. The rate of LP calculated from Figure 3 is still approximately 40% of that for rat liver microsomes. These microsomal LP studies have utilized Tris-Cl as a buffer. Tris-Cl has been shown to inhibit radical-dependent reactions (Tien, et al., 1981). Thus the effect of Tris-Cl on microsomal LP was investigated. Table 2 illustrates that 50 mM Tris-HCl had no effect of the rate of microsomal LP when compared to incubations containing 50 mM NaCl.

Reconstitution of Microsomal Lipid Peroxidation

LP experiments were performed using a reconstituted system containing liposomes made from extracted microsomal lipid, purified protease-solubilized reductase and cytochrome P450 from both rat and rabbit livers. Table 3 demonstrates that activity of reconstituted rabbit liver microsomal LP is dependent upon the reductase (0.1 units/ml), NADPH (0.1 mM), and ADP-Fe^{3+} (0.1 mM). The inclusion of cytochrome P450 (0.3 nmol/ml) in the reaction mixture resulted in a 3.5 and 2.6 fold increase in the rate of MDA and LOOH formation, respectively. The pro-oxidant activity observed for cytochrome P450 was also observed for EDTA-Fe^{3+}. EDTA-Fe^{3+} (0.11 mM EDTA, 0.1 mM $FeCl_3$) caused an 8

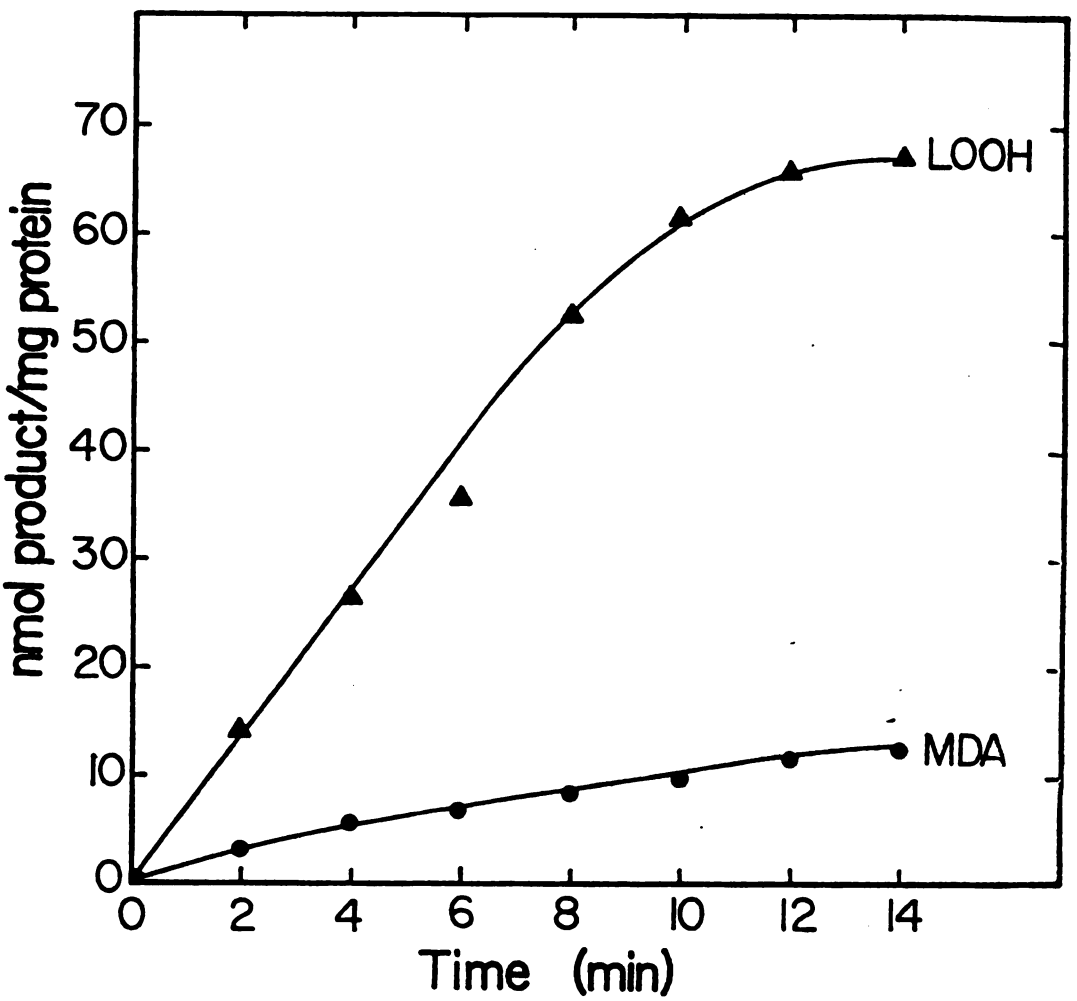


Figure 3. Time Course of Rabbit Liver Microsomal LP. Microsomal reaction mixtures contained 0.5 mg of microsomal protein/ml, ADP-Fe^{3+} (3 mM ADP, 0.15 mM $FeCl_3$), and 0.1 mM NADPH in 50 mM Tris-Cl, pH 7.5 at 37°C. Incubation and assay conditions are described under Materials and Methods.

Table 4. Reconstitution of Microsomal Lipid Peroxidation with Rat and Rabbit Liver Microsomal Components.

	MDA (nmol/min/ml	LOOH (nmol/min/ml)
Rabbit lipid, rabbit reductase	1.19	8.0
Rabbit lipid, rat reductase	1.12	8.2
Rat lipid, rabbit reductase	2.70	18.9
Rat lipid, rat reductase	3.18	18.9
Rat and rabbit lipid, rat reductase	2.90	20.5

Reaction mixtures contained ADP-Fe^{3+} (1.7 mM ADP, 0.1 mM $FeCl_3$), EDTA-Fe^{3+} (0.11 mM EDTA, 0.1 mM $FeCl_3$), 0.1 mM NADPH, liposomes (1 µmol lipid phosphate/ml) made from rat or rabbit microsomal lipid, as indicated, and 0.1 unit NADPH-cytochrome P450 reductase from rat or rabbit microsomes, as indicated, in 50 mM Tris-Cl, pH 7.5 at 37°C. The incubation containing liposomes made from both rat and rabbit microsomal lipid contained an equal molar ratio totalling 2 µmol lipid phosphate/ml. Incubation and assay conditions are described under Materials and Methods.

fold increase in the rate of LOOH formation and a 3.6 fold increase in the rate of MDA formation.

The relative rates of LP catalyzed by rat and rabbit liver microsomal reductases are shown in Table 4. As previously reported (Mishin et al., 1976b), rat and rabbit liver microsomal reductases are equally active in catalyzing LP. Irrespective of the reductase origin, the rates of MDA and LOOH formation in liposomes made from extracted rabbit liver microsomal lipid (1 µmol lipid phosphate/ml) were consistently 40% and 43% respectively, of that for liposomes made from extracted rat liver microsomal lipid (1 µmol lipid phosphate/ml). The same relative difference in rate was observed in microsomal reaction mixtures. No depression of the rate of LP was seen if liposomes (1 µmol lipid phosphate/ml) of

Figure 4. Effect of Lipid Phosphate Concentration on Reconstituted LP.
A. Reaction mixtures contained 0.1 units/ml of rat microsomal reductase, ADP-Fe^{3+} (1.7 mM ADP, 0.1 mM $FeCl_3$), EDTA-Fe^{3+} (0.11 mM EDTA, 0.1 mM $FeCl_3$); 0.1 mM NADPH, and liposomes (made from extracted rat microsomal lipid) at the specified lipid phosphate concentration in 50 mM Tris-Cl, pH 7.5 at 37°C.
B. Experimental conditions were as indicated in (A) except the liposomes were made from extracted rabbit microsomal lipid. Incubation and assay conditions are described in Materials and Methods.

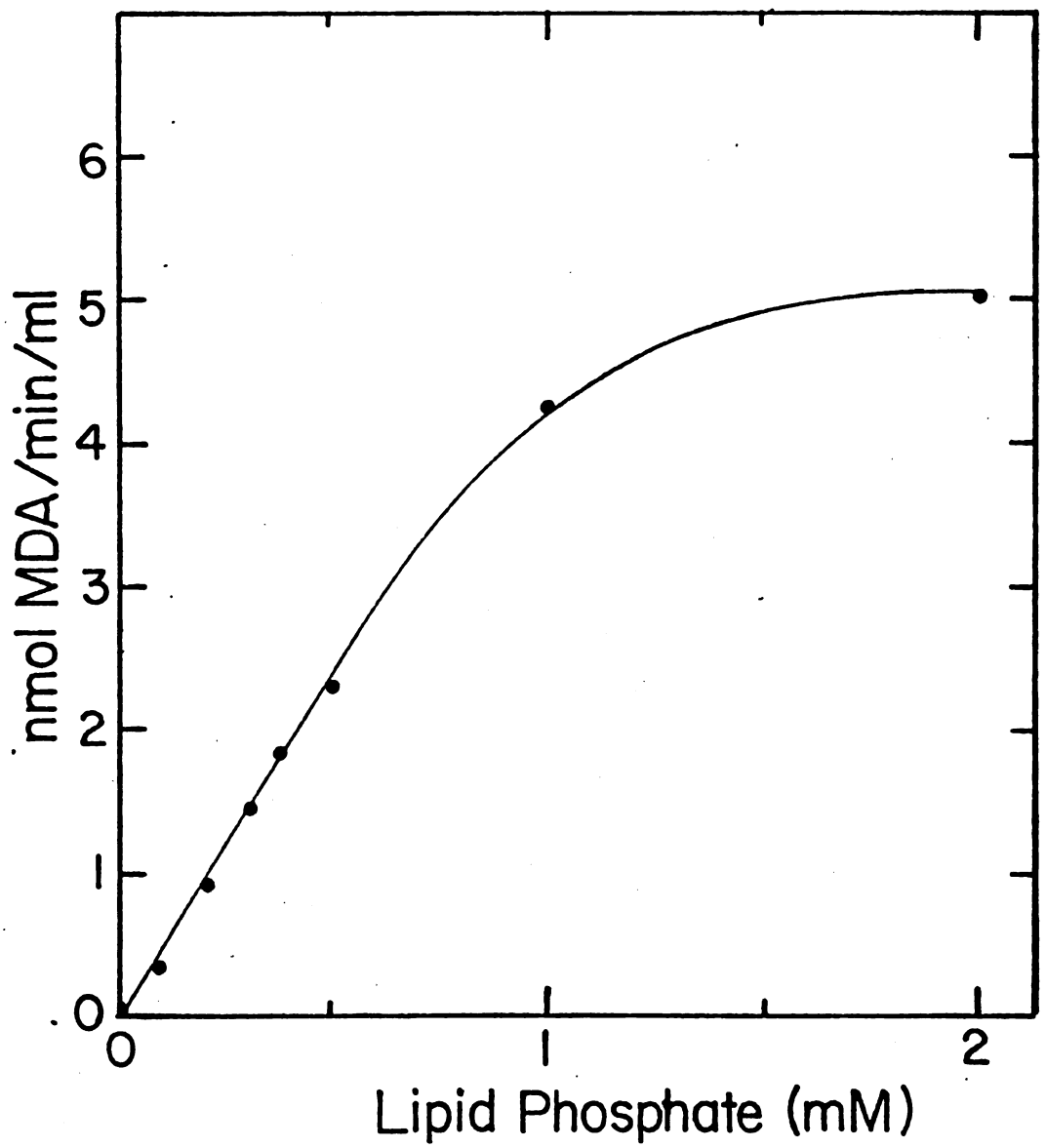


Figure 4A

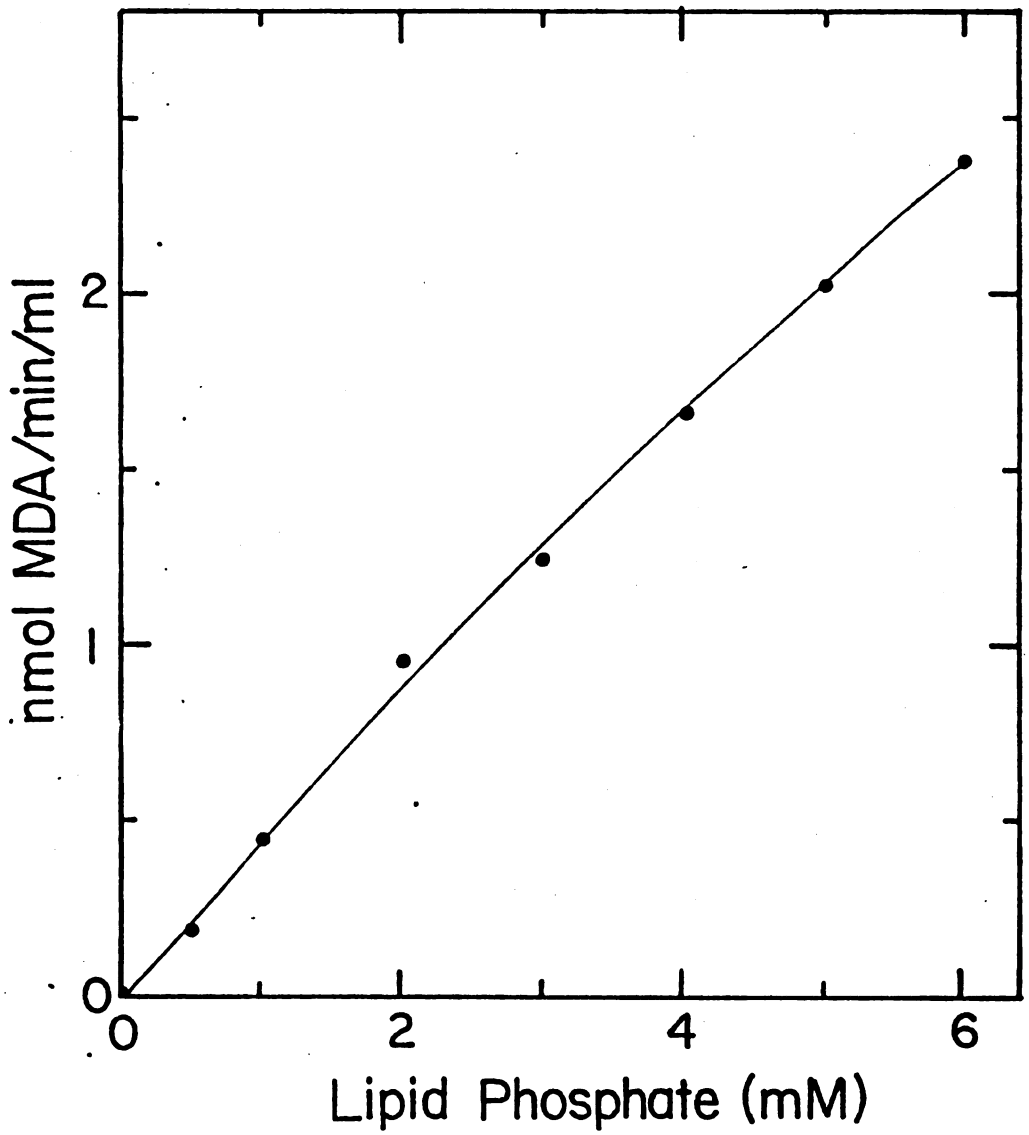


Figure 4B

rabbit origin were added to a reaction mixture with components isolated from rat liver microsomes (Table 4). An inhibition of activity would be expected if rabbit liver microsomal lipid contained high levels of antioxidants or other inhibitors.

The effect of lipid concentration (standardized by lipid phosphate content) on the rate of LP in the reconstituted system is shown in Figure 4. Rat liver microsomal lipid was near saturation at a concentration of 1 μmol lipid phosphate/ml (Figure 4A). However, at an identical concentration, rabbit liver microsomal lipid was still limiting (Figure 4B). The rates of MDA formation increased linearly with increased lipid content demonstrating no evidence of saturation even at 6 μmol lipid phosphate/ml.

Gas-Liquid Chromatography Analysis of Fatty Acids

Since the results shown in Table 4 suggest that lower rates of rabbit liver microsomal LP were not due to antioxidants or other inhibitors, the fatty acid composition of microsomal phospholipids was examined. The results of these analysis are shown in Table 5. The content of each fatty acid is expressed as percentage of palmitic acid (w/w). The data shows that rat liver microsomal lipid is enriched with arachidonic and docosahexaenoic acid. Docosahexaenoic acid was undetectable and arachidonic acid was present in much lower levels in the rabbit liver microsomal lipid. Linoleic acid accounted for 29% (w/w) of the total rabbit liver microsomal fatty acids. The fatty acid to protein ratio (w/w) of rat liver microsomes were approximately the same as rabbit liver microsomes.

In order to test whether fatty acid composition is correlated to the rate of LP, loss of certain fatty acids during microsomal LP was measured

Table 5. Fatty Acid Composition of Microsomal Phospholipids.

Fatty Acid	Rat Microsomes	Rabbit Microsomes
	(mg/100 mg palmitic acid)	
Palmitic	100	100 (21%)
Stearic	147	109
Oleic	39	65
Linoleic	70	132
Arachidonic	156	49
Docosahexaenoic	25	Not detected

Phospholipids were extracted from rat and rabbit liver microsomes (5 mg protein). The phospholipids were hydrolyzed and the methyl esters of the fatty acids were prepared for gas chromatographic analysis as described in Materials and Methods. The instrument conditions were: carrier gas flow rate of 30 ml/min, temperature program of 170° to 220°C at 3°C/minute. The fatty acid composition is expressed as the percentage (w/w) of palmitic acid in the specific sample. The values in parenthesis represent the percentage (w/w) of palmitic acid in each sample.

by gas-liquid chromatography. The results of the analysis show that the degree of unsaturation of a fatty acid correlated well with the rate of its disappearance (Figure 5). Although docosahexaenoic acid accounts for less than 5% (w/w) of the total rat liver microsomal fatty acids, the rate of disappearance was greatest (Figure 5A). The rates of arachidonic and linoleic acid disappearance were lower; the saturated fatty acids and oleic acid did not show any appreciable loss (Figure 5A). The profile of fatty acid loss in rabbit liver microsomes was similar to that of rat liver microsomes (Figure 5B). These results suggest that the lower polyunsaturated fatty acid content in microsomal phospholipid is the limiting factor in rabbit liver microsomal LP.

Figure 5. The rate of fatty acids lost during microsomal LP was determined by sampling 5 ml aliquots of reaction mixtures at the times indicated. The aliquots were analyzed for fatty acid content as described in the legend to Table IV and Materials and Methods. The content of the specified fatty acids are expressed as the percentage (w/w) of the original content. Number preceeding colon indicates the carbon chain length; number following colon indicates the number of double bonds present.
A. Reaction mixtures contained 0.5 mg/ml of rat liver microsomal protein, ADP-Fe^{3+} (3 mM ADP, 0.15 mM $FeCl_3$), and 0.1 mM NADPH in 50 mM Tris-Cl, pH 7.5 at 37°C.
B. Experimental conditions were as indicated in (A) except the microsomes were of rabbit liver origin.

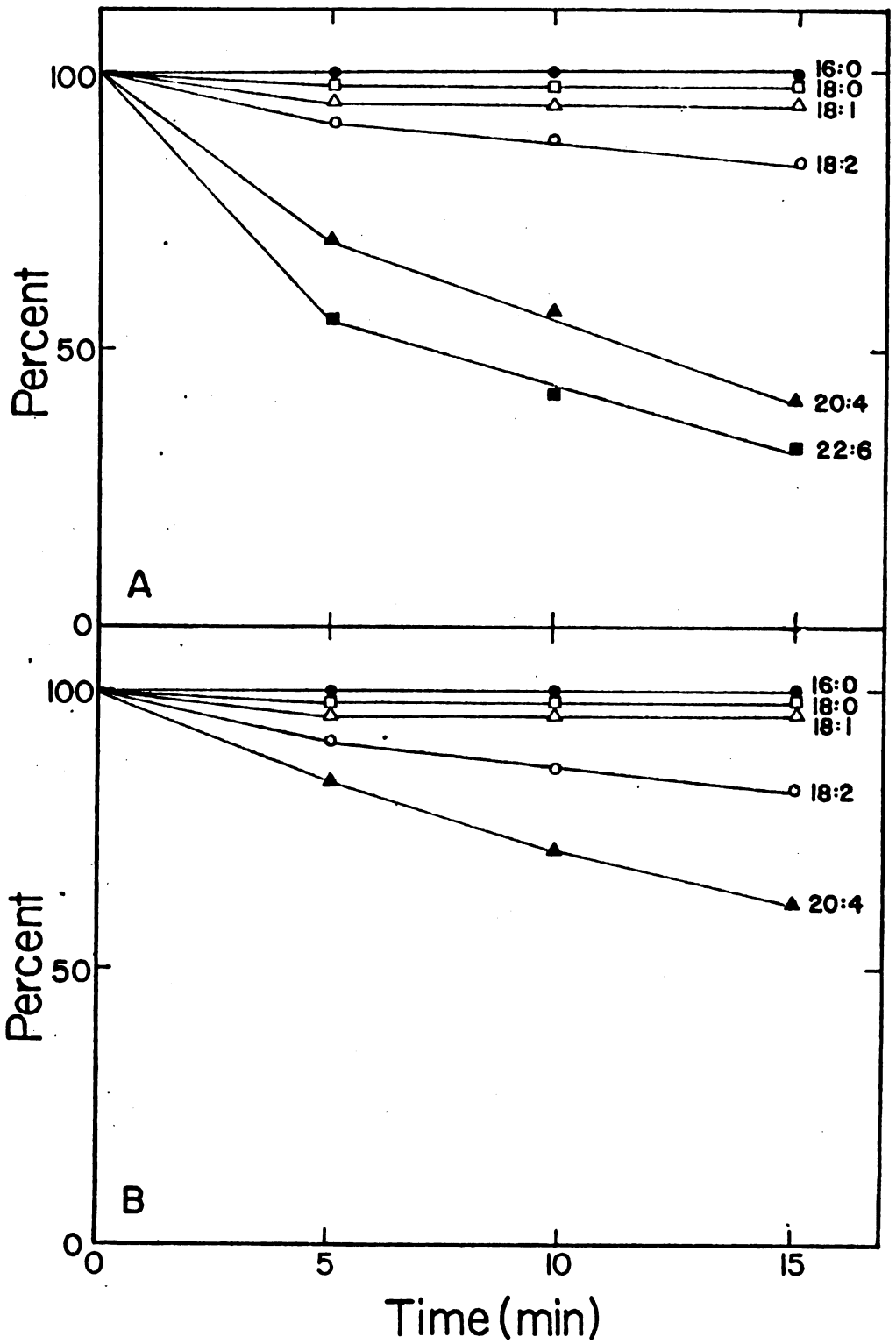

100
50
0
Percent
16:0
18:0
18:1
18:2
20:4
22:6
A
100
50
0
Percent
16:0
18:0
18:1
18:2
20:4
B
0
5
10
15
Time (min)

Table 6. Enhancement of Rabbit Liver Microsomal Lipid Peroxidation by Phospholipids.

Additions	MDA	LOOH
	(nmol/min/mg)	
NADPH	0.15	0.6
None	1.14	6.2
Liposomes (0.2 μmol/ml)	1.64	9.1
Liposomes (0.5 μmol/ml)	1.97	11.4

Microsomal reaction mixtures contained 0.5 mg microsomal protein/ml, ADP-Fe^{3+} (3 mM ADP, 0.15 mM $FeCl_3$) and 0.1 mM NADPH in 50 mM Tris-Cl, pH 7.5 at 37°C. Liposomes were made from extracted rabbit liver microsomal lipid and added at the indicated lipid phosphate concentrations. Incubation and assay conditions are described in Materials and Methods.

Enhancement of Microsomal Lipid Peroxidation

To further examine whether polyunsaturated fatty acids are the limiting component in rabbit liver microsomal LP, increasing concentrations of microsomal lipid was added to the microsomal reaction mixture. Table 6 shows that the addition of microsomal lipid (0.2 μmol lipid phosphate/ml) to the microsomal reaction mixture resulted in a 44% and 46% increase in the rate of MDA and LOOH formation, respectively. The addition of microsomal lipid, yielding final concentration of 0.5 μmol lipid phosphate/ml, caused a 74% increase in the rate of MDA formation and an 83% increase in the rate of LOOH formation.

DISCUSSION

Previous studies have shown that rabbit liver microsomes do not readily catalyze LP (Levin et al., 1973; Gram and Fouts, 1966; Mishin et al., 1976b). This present study confirms the previous reports and shows that the lower rates of LP, when compared to rat liver microsomes, are not attributable to differences in incubation conditions. Some investigators have attributed the differences in rates to the presence of endogenous antioxidants or inhibitors in rabbit liver microsomes rather than to a lower specific activity of NADPH-cytochrome P450 reductase (Mishin et al., 1976b). Our results also showed that the lower rate of LP observed for rabbit liver microsomes is not due to the reductase. It is also unlikely that the lower rates of peroxidation are due to antioxidants or other inhibitors since the addition of rabbit microsomal lipid did not inhibit the rate of LP of rat microsomal lipid catalyzed by rat microsomal reductase. Low concentrations of rat microsomal lipid (1 μmol lipid phosphate/ml) were required to produce maximal rates of LP in a system reconstituted with rat microsomal reductase. Increasing rat liver microsomal lipid concentration beyond 1 μmol lipid phosphate/ml did not result in a significant increase in the rate of LP. However, increasing rabbit liver microsomal lipid concentration from 0 to 6 μmol lipid phosphate/ml resulted in a linear increase in the rate of LP. This suggests that rabbit microsomal lipid is not as susceptible to peroxidation as rat microsomal lipid.

Gas-liquid chromatographic analysis of the fatty acid composition of microsomal phospholipids revealed that rat liver microsomes are enriched in the polyunsaturated fatty acids arachidonic and docosahexaenoic. Rabbit liver microsomes were found devoid of docosahexaenoic acid, contained a lower percentage (w/w) of arachidonic acid, and were enriched in linoleic acid. These differences in the fatty acid composition may have two possible effects on LP: 1) The rate of initiation may be lower in rabbit liver microsomes due to lower availability of polyunsaturated fatty acids. 2) The rate of chain propagation reactions are enhanced when the unsaturated centers of fatty acids are in close proximity (Porter et al., 1972). This configuration maximizes the possibility of chain reactions. Due to the greater polyunsaturated fatty acid content, such might be the case with rat liver microsomes to a greater degree than rabbit liver microsomes. The work of May and McCay (1968a) with microsomal LP demonstrated that the increased unsaturation of a fatty acid rendered it more susceptable to peroxidation. Our results with both rat and rabbit liver microsomal LP are in accord with this observation. Within 15 minutes after initiation, 70% of the docosahexaenoic acid was lost from the rat liver microsomal reaction mixture, as well as over 50% of the arachidonic acid. In the rabbit liver microsomal reaction mixture, over 30% of the arachidonic acid and 15% of the linoleic acid was lost during this time span. The saturated fatty acids were not significantly altered in either system.

Increasing the concentration of polyunsaturated lipid in the rabbit liver microsomal reaction mixture by the addition of extracted microsomal lipid increased the rate of LP. These results suggest that the availability of polyunsaturated fatty acids is the rate limiting step in rabbit

liver microsomal LP. Furthermore, they indicate that the deleterious effects of LP to membrane bound proteins and other cellular components may be controlled by the degree of membrane phospholipid unsaturation, in addition to the presence of cellular antioxidants (Csallany, 1971), superoxide dismutase (McCord and Fridovich, 1968a), catalase (Sies et al., 1972), and other protective enzymes (Christophersen, 1968).

CHAPTER II

ON THE QUESTION OF HYDROXYL RADICAL INVOLVEMENT IN NADPH-DEPENDENT LIPID PEROXIDATION

ABSTRACT

The mechanism of NADPH-cytochrome P450 reductase catalyzed lipid peroxidation (LP) was studied in two different model systems. The reductase, NADPH, and iron chelates were incubated with liposomes made from extracted microsomal phospholipid or with sodium linoleate dispersed by the addition of detergent (1% Lubrol). The rate of LP was measured by malondialdehyde (MDA) formation in liposomes and by diene conjugation in linoleate. The mechanism of initiation of LP is dependent upon the iron chelator in both systems. Reductase catalyzed NADPH oxidation, dioxygen consumption, and LP required added EDTA-chelated Fe^{3+} for activity. None of these activities occurred in the absence of added iron or if the iron was chelated by ADP. In the liposomal system, LP did not occur with the addition of EDTA-Fe^{3+} or ADP-Fe^{3+} alone but required both iron chelates for activity (1.76 nmol MDA/min/ml). Hydroxyl radical (•OH) traps (mannitol, benzoate, and Tris-Cl) at a concentration (10 mM) which caused complete inhibition of •OH-dependent LP (initiated by the Fenton's reagent) did not inhibit reductase catalyzed peroxidation of liposomes. Catalase (1 unit/ml) was also without effect. In the peroxidation of linoleate, the incubation of the reductase and NADPH with EDTA-Fe^{3+} did initiate LP (ΔA_{234}=0.078/min/ml). This peroxidation was inhibited by •OH traps (100 mM mannitol, 42% inhibition; 100 mM ethanol, 42% inhibition) and by catalase (1 unit/ml, 37% inhibition). The rate of linoleate diene conjugation was enhanced by the addition of

EDTA-Fe^{3+} with ADP-Fe^{3+} (ΔA_{234}=0.306/min/ml). Reductase catalyzed peroxidation of detergent-dispersed linoleate with both iron complexes was less sensitive to •OH traps (100 mM mannitol, 22% inhibition; 100 mM ethanol, 13% inhibition) and to catalase (1 unit/ml, 8% inhibition). EPR spectroscopy demonstrated the formation of the •OH in incubations of reductase and NADPH with EDTA-Fe^{3+}. However, incubations with both EDTA-Fe^{3+} and ADP-Fe^{3+}, the system which catalyzed maximal rates of LP, showed no evidence for •OH formation. It is proposed that reductase catalyzed LP with EDTA-Fe^{3+} occurs via a •OH mechanism but in the presence of ADP-Fe^{3+}, the mechanism involves an ADP-Fe^{3+}-oxygen complex.

INTRODUCTION

In 1963 Hochstein and Ernster reported an enhancement in NADPH-dependent lipid peroxidation upon the addition of ADP to microsomes. Subsequent investigations revealed that the enhancement in activity was due to iron contamination of commercial preparations of ADP (Hochstein et al., 1964). Thus microsomal lipid peroxidation was attributed to the ability of microsomes to reduce iron (Beloff-Chain et al., 1965). By reconstituting lipid peroxidation with purified microsomal proteins, Pederson et al. (1973) demonstrated that NADPH-dependent microsomal lipid peroxidation was catalyzed by NADPH-cytochrome P450 reductase. These workers proposed that the first step in initiation of microsomal lipid peroxidation was the direct enzymatic reduction of ADP chelated iron (III) by the reductase. The subsequent reaction of ADP-Fe^{2+} with dioxygen would form the ADP-perferryl ion ($ADP\text{-}Fe^{2+}\text{-}O_2 \longleftrightarrow ADP\text{-}Fe^{3+}\text{-}O_2^{\cdot-}$). Initiation of lipid peroxidation was proposed to occur through the ADP-perferryl ion catalyzed abstraction of methylene hydrogen from polyunsaturated fatty acids. A second iron chelate, EDTA-Fe^{3+}, was found to greatly enhance the rate of reconstituted NADPH-dependent lipid peroxidation (Pederson et al., 1973). The catalytic role of EDTA-Fe^{3+} was proposed to be distinct from that of ADP-Fe^{3+} since enhancement of lipid peroxidation by EDTA-Fe^{3+} occurred only in the presence of ADP-Fe^{3+}. Since EDTA-Fe^{3+} was not required for NADPH-dependent microsomal lipid

peroxidation, the authors proposed that EDTA-Fe^{3+} replaced an endogenous microsomal component that participated in lipid peroxidation.

The discovery that erythrocuprein (renamed superoxide dismutase) catalyzed the dismutation of superoxide ($O_2^{\cdot-}$) expanded the horizon of research in biological free radicals and in oxygen mediated toxicity (McCord and Fridovich, 1968). With the knowledge that excessive $O_2^{\cdot-}$ production can result in lipid peroxidation (Bus et al., 1974, 1975), the mechanism of microsomal lipid peroxidation was re-examined. It was found that purified NADPH-cytochrome P450 reductase, like numerous other flavoproteins (Massey et al., 1969), produced low levels of $O_2^{\cdot-}$ during its redox cycle (Aust et al., 1972). The ability of microsomes to catalyze a NADPH-dependent lipid peroxidation is now attributed to $O_2^{\cdot-}$ formation via the oxidation of NADPH by the reductase. The role of iron in microsomal lipid peroxidation is now widely proposed to be catalysis of secondary reactions involving $O_2^{\cdot-}$ to generate radicals rather than a direct interaction with microsomal protein (Fong et al., 1973; Kameda et al., 1979).

Numerous workers have reported that the role of chelated iron in microsomal lipid peroxidation is to catalyze the Haber-Weiss reaction (Fong et al., 1973; McCay et al., 1972). Two chelates of iron frequently used are ADP-Fe^{3+} and EDTA-Fe^{3+}.

$$2O_2 + NADPH \xrightarrow{\text{reductase}} NADPH^+ + 2O_2^{\cdot-} + H^+$$

$$O_2^{\cdot-} + \text{chelate-}Fe^{3+} \longrightarrow O_2 + \text{chelate-}Fe^{2+}$$

$$2H^+ + 2O_2^{\cdot-} \longrightarrow H_2O_2$$

$$\text{chelate-}Fe^{2+} + H_2O_2 \longrightarrow {\cdot}OH + {}^-OH + \text{chelate-}Fe^{3+}$$

This sequence of reactions generates the highly reactive hydroxyl radical (•OH) which can readily initiate lipid peroxidation. Evidence for the formation of the •OH in microsomes via the iron-catalyzed Haber-Weiss reaction comes from inhibition of lipid peroxidation by catalase, superoxide dismutase and by •OH trapping agents in addition to observing •OH formation with EPR spin trapping techniques (Lai and Piette, 1977, 1978).

Several objections have been raised by other investigators concerning the iron-catalyzed Haber-Weiss reaction occurring in microsomes. First, the proposal that reductase generated $O_2^{\cdot-}$ participates in NADPH-dependent lipid peroxidation has been questioned since other investigators have not shown significant generation of O_2 by the reductase (Svingen et al., 1978). Second, microsomal preparations are usually contaminated with catalase, yet they readily catalyze lipid peroxidation. Unless precautions are taken to inhibit catalase, hydrogen peroxide production in microsomes during NADPH oxidation cannot be demonstrated (Hildebrant and Roots, 1975). Third, the participation of the •OH in NADPH-dependent lipid peroxidation has not been confirmed by other investigators. Svingen et al. (1980) failed to inhibit NADPH-dependent lipid peroxidation in a reconstituted system with catalase or with •OH trapping agents. These workers proposed separate roles for ADP-Fe^{3+} and EDTA-Fe^{3+} in NADPH-dependent lipid peroxidation. In accord with the work of Pederson et al. (1973), initiation was proposed to be catalyzed by the ADP-perferryl ion. The enhancement of lipid peroxidation by EDTA-Fe^{3+} was attributed to its ability (upon reduction by the reductase) to catalyze the decomposition of lipid hydroperoxides (LOOH) formed by ADP-perferryl-dependent reactions.

$$LOOH + EDTA\text{-}Fe^{2+} \longrightarrow LO\cdot + {}^{-}OH + EDTA\text{-}Fe^{3+}$$

These proposals were based on the observation that: 1) The addition of ADP-Fe^{3+} to a reconstituted NADPH-dependent lipid peroxidation reaction mixture resulted in the formation of low levels of LOOH. 2) The addition of EDTA-Fe^{3+} alone did not result in lipid peroxidation. 3) EDTA-Fe^{3+} enhanced the rate of lipid peroxidation only in the presence of ADP-Fe^{3+}.

Using detergent-dispersed linoleate and liposomes made from extracted microsomal lipid as substrates, this study further examines the role of ADP-Fe^{3+} and EDTA-Fe^{3+} in NADPH-dependent lipid peroxidation. The effect of catalase and $\cdot OH$ trapping agents on lipid peroxidation was investigated to assess the involvement of the iron-catalyzed Haber-Weiss reaction. The mechanism of $\cdot OH$ formation from reductase-dependent reactions was also investigated by EPR spin trapping techniques.

MATERIALS AND METHODS

Materials. Cytochrome c (Type VI), thiobarbituric acid, NADPH, ADP, butylated hydroxytoluene (BHT), mannitol, Lubrol, and benzoic acid were purchased from Sigma Chemical Company. Linoleic acid was obtained from Nu Chek Prep, Elysian, MN. The spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) was purchased from Aldrich Chemical Co. All buffers and reagents were passed through Chelex 100 (Bio-Rad Laboratories) ion exchange resin to free them of contaminants. DMPO was vacuum distilled prior to use.

Enzymes. Superoxide dismutase (SOD) and catalase were obtained from Sigma. Gel filtration chromatography on Sephadex G-25 was used to remove the antioxidant thymol from catalase. NADPH-cytochrome P450 reductase was purified by the method of Pederson et al. (1973) except that EDTA was omitted from the storage buffer. SOD activity was measured by the method of McCord and Fridovich (1968). Catalase activity was measured by the method of Pederson et al. (1973).

Microsomal Lipid. Male Sprague-Dawley rats (250-274 g) were obtained from Spartan Research Animals (Haslett, MI). Liver microsomes were isolated by the method of Pederson (1973). Microsomal lipid was extracted from freshly isolated microsomes by the method of Folch et al. (1957). All solvents used for extractions were purged with argon and all steps were performed at 4°C to minimized autoxidation of unsaturated lipids. Extracted lipid was stored in argon saturated $CHCl_3$:CH_3OH

(2:1) at -20°C. Lipid phosphate determinations were performed by the method of Bartlett (1959).

Reaction Mixtures. Liposomes were prepared by sonication of the extracted microsomal lipid in argon-saturated distilled deionized water at 4°C (Pederson et al., 1973). Liposomal peroxidation reactions initiated by the Fenton's reagent were accomplished by the addition of $FeCl_2$ (0.2 mM) to incubations containing H_2O_2 (0.1 mM) and liposomes (1 μmol lipid phosphate/ml) in 30 mM NaCl, pH 7.5. Other additions or deletions are as specified in the legends to the figures and tables. NADPH-cytochrome P450 reductase-dependent peroxidation of liposomes was performed by incubating liposomes (1 μmol lipid phosphate/ml) with the purified reductase (0.1 unit/ml), EDTA-Fe^{3+} (0.11 mM EDTA, 0.1 mM $FeCl_3$), ADP-Fe^{3+} (1.7 mM ADP, 0.1 mM $FeCl_3$), and 0.1 mM NADPH in 30 mM NaCl, pH 7.5. Reactions were initiated by the addition of NADPH. Other additions or deletions are as specified in the table and figure legends. Incubations were done in a metabolic shaking water bath at 37°C, under an air atmosphere. Although the reaction mixtures were unbuffered, the pH did not change during the course of the reactions. Liposomal lipid peroxidation was assessed by MDA (Buege and Aust, 1978). To prevent further peroxidation of lipid during the assay procedure for MDA, 0.03 volumes of 2% BHT in ethanol was added to the thiobarbituric acid reagent (Svingen and Aust, 1980).

Linoleate stock solution were made by suspending linoleic acid (100 mg) into argon-purged Chelexed water by adding 5-10 drops of 6 N NaOH to yield a clear solution. The pH of this solution was then slowly lowered to 7.5 by the addition of 6 N HCl. The resultant micelle solution was taken up to a final volume of 50 ml and used immediately. Lipid

peroxidation of detergent-dispersed linoleate was accomplished by incubating sodium linoleate (5.7 mM) with NADPH (0.1 mM), NADPH-cytochrome P450 reductase (0.1 unit/ml), Lubrol (1%), EDTA-Fe^{3+} (0.11 mM EDTA, 0.1 mM $FeCl_3$), and ADP-Fe^{3+} (0.5 mM ADP, 0.1 mM $FeCl_3$) in 30 mM NaCl, pH 7.5, at 37°C under air. These incubations were carried out in a cuvette of a Cary 219 spectrophotometer. Diene conjugation during lipid peroxidation was continuously monitored by the absorbance change at 234 nm (Adams and Wardman, 1977).

Other Methods. ADP and EDTA chelated iron solutions were prepared by the addition of $FeCl_3$ to chelate solutions adjusted to pH 7.5. Due to the pH change from the addition of $FeCl_3$, the pH of these chelate solutions were readjusted to 7.5. Water used in the preparation of the ferrous solutions were argon-purged to minimize Fe^{2+} autoxidation. Since the reaction mixtures were unbuffered, the pH of all reagents were carefully adjusted to 7.5 prior to use. NADPH-cytochrome P450 reductase-dependent consumption of NADPH and dioxygen were measured decrease in 340 nm absorbance and the Clark electrode, respectively. EPR spectra were recorded with a Varian Century E112 spectrometer under the following conditions: 3370 G magnetic field, 9.41208 GHz, 10 mW microwave power, 100 KHz modulation frequency, 2.5 modulation amplitude, 0.25 seconds time constant and 4 minute scan time. Spectra recording began within 8 minutes of initiation of reactions.

RESULTS

Lipid Peroxidation Measured by Diene Conjugation and Malondialdehyde Formation

Table 7 shows the components required for reconstitution of NADPH-cytochrome P450 reductase-dependent lipid peroxidation. Using liposomes made from extracted microsomal phospholipid as substrate, lipid peroxidation was assayed by the rate of MDA formation. It is clear that peroxidation is dependent on NADPH and the reductase. In the absence of added iron, the incubation of NADPH with the reductase did not result in lipid

Table 7. NADPH-Dependent Peroxidation of Liposomes

Additions	MDA (nmol/min/ml)
NADPH, reductase	0.01
NADPH, reductase, ADP-Fe^{3+}	0.36
NADPH, reductase, EDTA-Fe^{3+}	0.06
NADPH, reductase, ADP-Fe^{3+}, EDTA-Fe^{3+}	1.76
NADPH, EDTA-Fe^{3+}, ADP-Fe^{3+}	0.01
Reductase, EDTA-Fe^{3+}, ADP-Fe^{3+}	0.01

Reaction mixtures contained liposomes (1 μmol lipid phosphate/ml) in 30 mM NaCl, pH 7.5 with the following additions: NADPH-cytochrome P450 reductase (0.1 unit/ml), NADPH (0.1 mM), EDTA-Fe^{3+} (0.11 mM EDTA, 0.1 mM $FeCl_3$) and ADP-Fe^{3+} (0.5 mM ADP, 0.1 mM $FeCl_3$). Incubation and assay conditions are described under Materials and Methods.

peroxidation. The addition of 0.1 mM ADP-Fe^{3+} caused low levels of MDA formation while no lipid peroxidation was observed with the addition of 0.1 mM EDTA-Fe^{3+}. When both iron complexes were added, the highest rate of MDA formation was observed (1.76 nmol/min/ml). The increase in rates of lipid peroxidation when both iron complexes were added was not due to an increase in the total iron content. In incubations with only one iron chelate, doubling that iron chelate concentration did not result in increased rates of lipid peroxidation.

The measurement of MDA by the thiobarbituric acid test is not always an accurate assessment of peroxidation (Buege and Aust, 1978). For this reason, lipid peroxidation was also measured by diene conjugation. However, the incubation conditions had to be modified. Liposomes could not be used for this assay due to the light scattering observed at 234 nm. Linoleate micelles could not be utilized for the same reasons. The dispersion of the fatty acid micelles with 1% Lubrol gave clear solutions which permitted conjugated diene to be assayed. The components which are required to catalyze the NADPH-dependent peroxidation of detergent-dispersed linoleate are shown in Table 8. As in the peroxidation of phospholipid liposomes, no peroxidation was observed in the absence of added iron. Again, the addition of both ADP-Fe^{3+} and EDTA-Fe^{3+} resulted in the maximal rate of lipid peroxidation (ΔA_{234}=0.306/min/ml). When the only iron chelate added was EDTA-Fe^{3+}, the rate of lipid peroxidation observed was 25% of that observed when both iron complexes were added. This is a far greater percentage than that observed in the peroxidation of liposomes (3.4%) (Table 7). In contrast to the peroxidation of liposomes (Table 7), the

Table 8. NADPH-Dependent Peroxidation of Linoleate

Additions	ΔA_{234}/min/ml
NADPH, reductase	0.001
NADPH, reductase, ADP-Fe^{3+}	0.002
NADPH, reductase, EDTA-Fe^{3+}	0.078
NADPH, reductase, EDTA-Fe^{3+}, ADP-Fe^{3+}	0.306
NADPH, EDTA-Fe^{3+}, ADP-Fe^{3+}	0.002
Reductase, EDTA-Fe^{3+}, ADP-Fe^{3+}	0.002

Reaction mixtures contained sodium linoleate (5.7 mM) and Lubrol (1%) in 30 mM NaCl, pH 7.5. The additions are as specified in Table 7. Incubation and assay conditions are described under Materials and Methods.

addition of ADP-Fe^{3+} resulted in exceedingly low rates of linoleate peroxidation as assayed by conjugated diene formation (Table 8).

Reduction of Iron Chelates

The results obtained in the conjugated diene and MDA assay show an absolute requirement for iron in lipid peroxidation. It is well known that iron must be reduced to initiate lipid peroxidation. Aust et al. (1972) reported that $O_2^{\cdot-}$ is produced from the redox cycle of the purified reductase. If the reduction of iron is through $O_2^{\cdot-}$, then the action of superoxide dismutase should inhibit this reduction and consequently inhibit lipid peroxidation. Table 9 demonstrates that superoxide dismutase, at concentrations of 1, 5, and 10 units/ml, had little effect on the rate of diene conjugation. In fact, superoxide dismutase caused a modest increase in the rate of lipid peroxidation in incubations containing either EDTA-Fe^{3+} or EDTA-Fe^{3+} with ADP-Fe^{3+}. It may be argued that the flux of $O_2^{\cdot-}$ produced by the

Table 9. Effect of Superoxide Dismutase on Peroxidation of Linoleate

Additions	EDTA-Fe^{3+}	EDTA-Fe^{3+}, ADP-Fe^{3+}
	(ΔA_{234}/min/ml)	
None	0.096	0.341
SOD, 1 unit/ml	0.096	0.312
SOD, 5 unit/ml	0.102	0.332
SOD, 10 unit/ml	0.106	0.368

The effect of varying concentrations of SOD on NADPH-dependent lipid peroxidation was studied in the presence of EDTA-Fe^{3+} or EDTA-Fe^{3+} plus ADP-Fe^{3+}. Reaction mixtures contained NADPH (0.1 mM), NADPH-cytochrome P450 reductase (0.1 unit/ml), and the specified iron chelate: EDTDA-Fe^{3+} (0.11 mM EDTA, 0.1 mM $FeCl_3$), ADP-Fe^{3+} (0.5 mM ADP, 0.1 mM $FeCl_3$). Incubation and assay conditions are described under Materials and Methods.

reductase is sufficient for iron reduction and that superoxide dismutase serves to enhance the rate of hydrogen peroxide formation, thus facilitating the rate of ·OH formation (Fong et al., 1973). This would result in increased rates of lipid peroxidation.

In view of this interpretation, the path in which electrons flow from the reductase to iron was also examined by monitoring NADPH oxidation. Table 10 shows that in the absence of added iron, the rate of NADPH oxidation catalyzed by the reductase is not significantly different from that observed by NADPH autoxidation. The addition of ADP, $FeCl_3$, or ADP-Fe^{3+} was also without effect on the rate of NADPH oxidation. The only chelate of iron that did stimulate NADPH oxidation (7 μmol/min/ml) was EDTA-Fe^{3+} (0.1 mM). This activity was dependent on both iron and EDTA since no NADPH oxidation was observed with EDTA in the absence of iron.

Table 10. Reductase Catalyzed Consumption of NADPH and Dioxygen

Additions	NADPH (nmol/min/ml)	O_2
NADPH	<0.2	0.0
NADPH, reductase	<0.2	0.0
NADPH, reductase, $FeCl_3$	<0.2	0.0
NADPH, reductase, ADP	<0.2	0.0
NADPH, reductase, ADP-Fe^{3+}	<0.2	0.0
NADPH, reductase, EDTA	<0.2	0.0
NADPH, reductase, EDTA-Fe^{3+}	7.1	3.2

NADPH consumption was measured by decrease in 340 nm absorbance. Decrease in dioxygen partial pressure was measured with a YSI Model 53 Oxygen Monitor. Reaction mixtures contained the following additions: $FeCl_3$ (0.1 mM), ADP (0.5 mM) ADP-Fe^{3+} (0.5 mM ADP, 0.1 mM $FeCl_3$), EDTA (0.11 mM), EDTA-Fe^{3+} (0.11 mM EDTA, 0.1 mM $FeCl_3$), NADPH-cytochrome P450 reductase (0.1 unit/ml) and NADPH (0.1 mM) in 30 mM NaCl, pH 7.5.

In a separate experiment, the rate of oxygen consumption was monitored to assess reductase-dependent production of $O_2^{\cdot-}$ (Table 10). These experiments verified the results obtained by observing NADPH oxidation. No detectable changes in the oxygen partial pressure were observed from incubating NADPH (0.1 mM) with NADPH-cytochrome P450 reductase (0.1 units/ml). Again, Fe^{3+}, ADP, ADP-Fe^{3+} and EDTA had no effect while incubations containing 0.1 mM EDTA-Fe^{3+} consumed oxygen at a rate of 3.2 nmol/min/ml. This observaton is consistent with the report that NADPH-cytochrome P450 reductase can directly reduce EDTA-Fe^{3+} (Kamin and Master, 1968). Upon reduction to the ferrous state, EDTA-Fe^{2+} readily autoxidizes to produce $O_2^{\cdot-}$, which accounts for

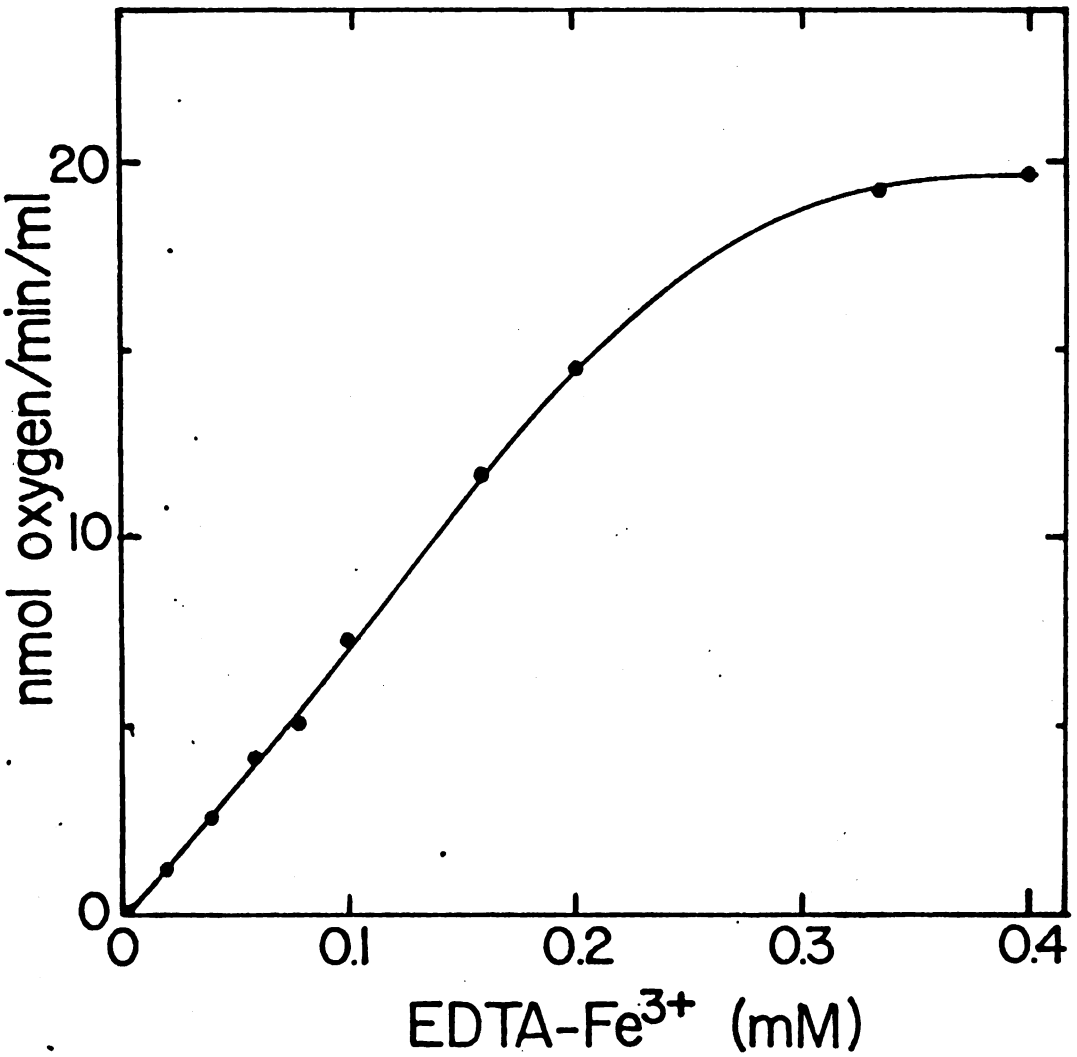
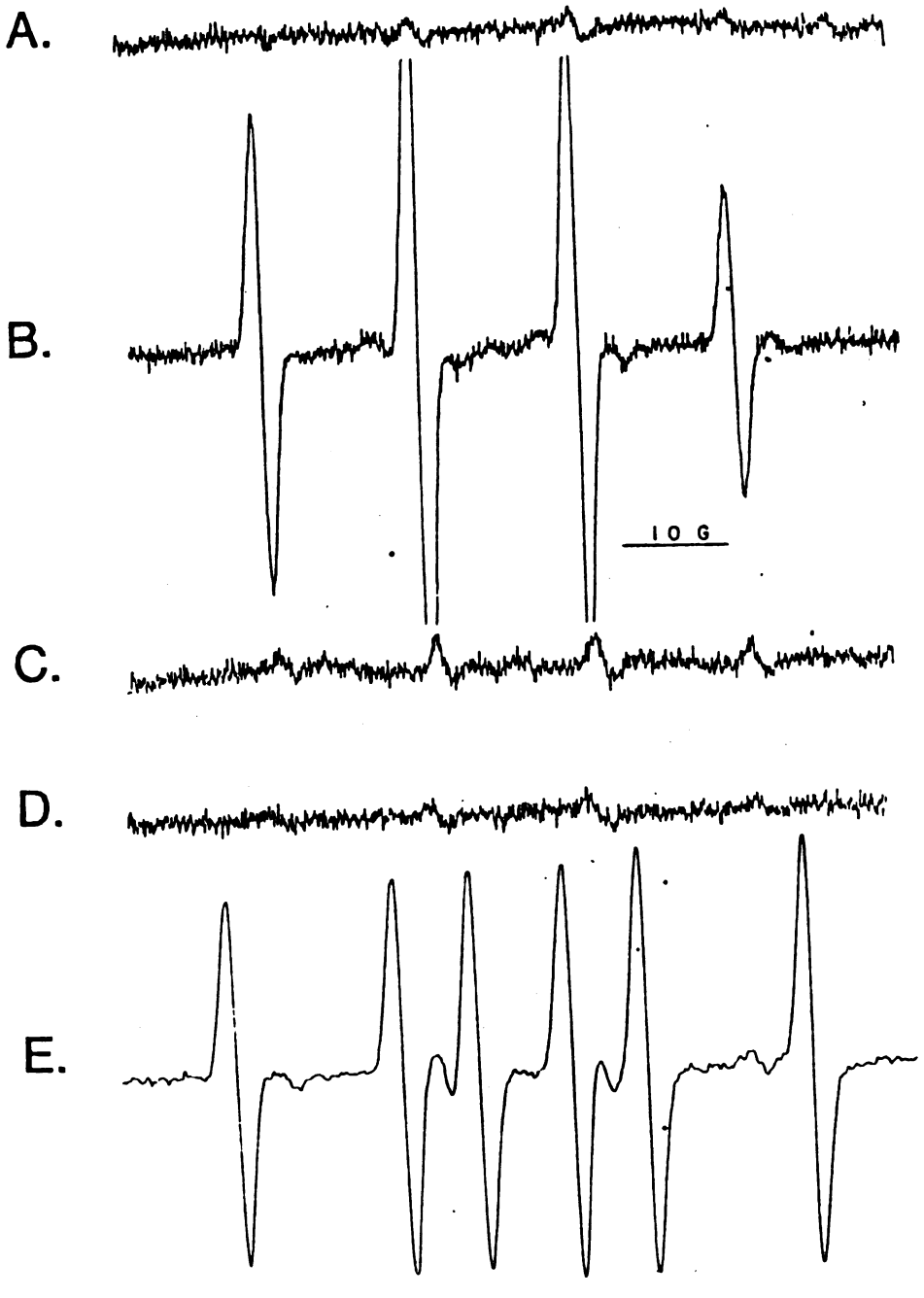


Figure 6. EDTA-Fe^{3+} Enhancement of Reductase Catalyzed Oxygen Consumption. The rate of oxygen consumption in reaction mixtures containing NADPH-cytchrome P450 reductase (0.1 unit/ml) NADPH (0.1 mM) and EDTA-Fe^{3+} in 30 mM NaCl, pH 7.5 is plotted as a function of EDTA-Fe^{3+} concentration (1.1 EDTA to $FeCl_3$ molar ratio). Oxygen consumption was measured on a YSI Model 53 Oxygen Monitor.

Figure 7. EPR Spectra of Reductase Catalyzed Radical Formation. Reaction mixtures contained NADPH-cytochrome P450 reductase (0.1 unit/ml) and 60 mM DMPO in 0.3 M NaCl, pH 7.5 at 25°C. The EPR spectra were obtained by scanning the reaction mixtures containing the following additions: A. NADPH (0.1 mM); B. NADPH (0.1 mM), EDTA-Fe^{3+} (0.11 mM EDTA, 0.1 mM $FeCl_3$); C. NADPH (0.1 mM), EDTA-Fe^{3+} (0.11 mM EDTA, 0.1 mM $FeCl_3$), ADP-Fe^{3+} (0.5 mM ADP, 0.1 mM $FeCl_3$); D. Same additions as C plus ethanol (100 mm). EPR instrument settings are described in Materials and Methods.


A.
B.
10 G
C.
D.
E.

the observed oxygen consumption. The EDTA-Fe^{3+}-dependent consumption of oxygen is concentration dependent. Figure 6 shows that reductase (0.1 units/ml) catalyzed reduction of EDTA-Fe^{3+} by NADPH (0.1 mM) follows Michaelis-Menten kinetics with a calculated Km of 0.15 mM for EDTA-Fe^{3+}.

NADPH-Cytochrome P450 Reductase-Dependent Production of Hydroxyl Radical

It has been reported that NADPH-cytochrome P450 reductase-dependent lipid peroxidation occurs via the formation of the •OH. Central to the evidence for such a mechanism is the observation of the •OH adduct signal in EPR spin trapping experiments. The •OH adduct of DMPO has a characteristic spectrum with hyperfine splitting (hps) constants of A_N=14.9 G and A_H=14.9 G (Harbour and Bolton, 1978). Figure 7 shows the EPR spectra of various reaction mixtures containing the reductase. These reaction mixtures contained 60 mM DMPO in the absence of liposomes or linoleate. No radical signals were observed in incubations containing 0.1 mM NADPH and 0.1 units/ml NADPH-cytochrome P450 reductase (Figure 7A). An intense •OH-DMPO adduct signal was observed upon the addition of 0.1 mM EDTA-Fe^{3+} (Figure 7B). The •OH signal was observed with the addition of ADP-Fe^{3+} (Figure 7C), however, the intensity of this signal was much less than that observed with EDTA-Fe^{3+}. The addition of both ADP-Fe^{3+} and EDTA-Fe^{3+} resulted in a diminished signal intensity when compared to incubations containing just EDTA-Fe^{3+}. It should be noted that the addition of both iron complexes resulted in maximal rates of lipid peroxidation as assayed by diene conjugation (Table 8) and MDA formation (Table 7).

The detection of the •OH-DMPO adduct signal does not necessarily mean that the •OH has been trapped. One method for verifying that

•OH trapping has occurred is to utilize the ability of spin trapping to distinguish between radical species. The •OH oxidizes ethanol to form the α-hydroxy ethyl radical (Adams and Wardman, 1977). The α-hydroxy ethyl radical can then react with DMPO to yield an EPR spectrum with hps constants of A_N=15.8 G and A_H=22.8 G. The observation of the α-hydroxy ethyl DMPO radical adduct has been utilized as verification of •OH formation. Figure 7D shows that the α-hydroxy ethyl DMPO EPR spectrum was detected upon the addition of ethanol (100 mM) to an incubation of reductase, NADPH, EDTA-Fe^{3+}, and ADP-Fe^{3+}.

The effect of various •OH trapping agents on the intensity of the EPR signal is shown in Figure 8. Mannitol (100 mM), benzoate (100 mM), and Tris-Cl (100 mM) all caused a dramatic decrease in the EPR signal intensity of the •OH-DMPO adduct. This decrease is a result of the trapping agents competing with DMPO for the •OH. Like ethanol, these substrates are oxidized by the •OH, but with the exception of mannitol, the resultant radical is not trapped by DMPO. In the case of mannitol, the decrease in the •OH-DMPO signal correlated with the formation of an EPR signal with hps constants of A_N=15.8 G and A_H=22.8 G.

Effect of Catalase and Hydroxyl Radical Trapping Agents on Lipid Peroxidation

The often repeated observation that catalase and •OH trapping agents inhibit NADPH-dependent lipid peroxidation was the subject of further examination. To assess whether these trapping agents or catalase were effective inhibitors of •OH-dependent reactions, they were tested on a lipid peroxidation system dependent upon the •OH. The •OH was generated by a Fentons's reagent of 0.2 mM $FeCl_2$ and 0.1 mM H_2O_2. Table 11 shows that generation of the •OH by this reagent readily

Figure 8. Effect of Catalase and Hydroxyl Radical Trapping Agents on the EPR Signal Intensity of the Hydroxyl Radical-DMPO Adduct. Reaction mixtures contained NADPH-cytochrome P450 reductase (0.1 unit/ml), NADPH (0.1 mM), EDTA-Fe^{3+} (0.11 mM EDTA, 0.1 mM $FeCl_3$) and 60 mM DMPO in 0.3 M NaCl, pH 7.5 at 25°C. The EPR specta were obtained by scanning the reaction mixtures containing the following additions: A. none; B. catalase (5 unit/ml); C. mannitol (100 mM); D. benzoate (100 mM); E. Tris-Cl (100mM). EPR instrument settings are described in Materials and Methods.

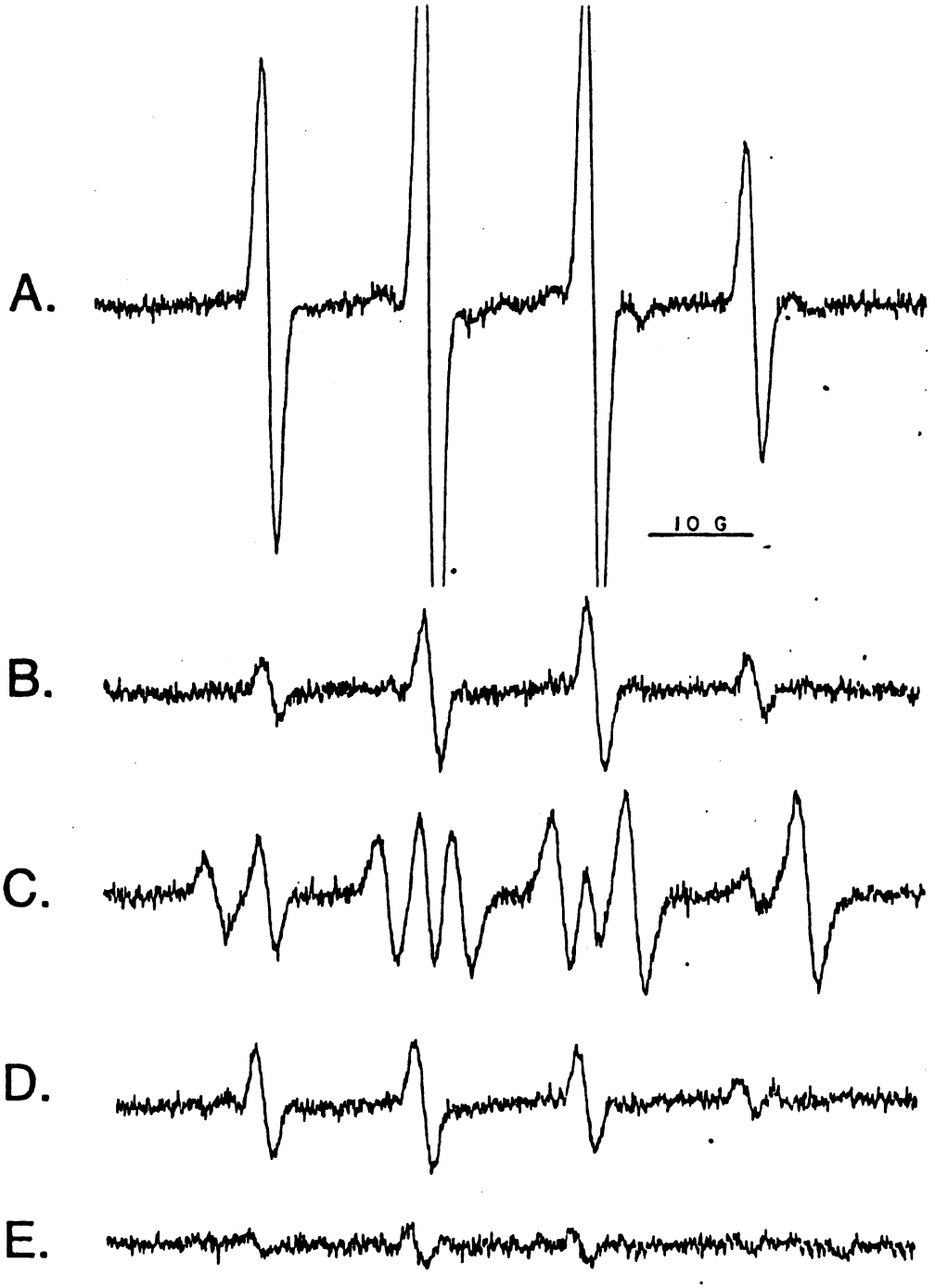

A.
10 G
B.
C.
D.
E.

Table 11. Effect of Trapping Agents on Hydroxyl Radical-Dependent Peroxidation of Liposomes

Conditions	% Inhibition
Complete	---
Complete, catalase	93%
Complete, mannitol	94%
Complete, benzoate	91%
Complete, Tris-Cl	92%

Complete reaction mixtures contained $FeCl_2$ (0.2 mM), H_2O_2 (0.1 mM), and liposome (1 μmol lipid phosphate/ml) in 30 mM NaCl, pH 7.5. The following additions were made as indicated: catalase (1 unit/ml), mannitol (10 mM), benzoate (10 mM) and Tris-Cl (10 mM). Rates are expressed as percentage inhibition of rates obtained from the complete reaction mixture (3.45 nmol MDA/min/ml). Incubation and assay conditions are described under Materials and Methods.

initiated the peroxidation of liposomes (3.45 nmol/min/ml). Catalase (1 unit/ml) caused 93% inhibition of activity while known ·OH trapping agents, mannitol (10 mM), benzoate (10 mM), and Tris-Cl (10 mM) cause 94%, 91%, and 92% inhibition respectively.

The concentrations of catalase and ·OH trapping agents which completely inhibited ·OH-dependent lipid peroxidation via the Fenton's reagent were without effect in NADPH-cytochrome P450 reductase-dependent peroxidation of liposomes (Table 12). The results clearly show that mannitol, benzoate, and Tris-Cl had no effect on the rates of MDA formation. Increasing the concentration of these trapping agents to 40 mM did not result in inhibition (data not shown) while catalase (1 unit/ml) was also without effect.

Table 12. Effect of Hydroxyl Radical Traps on NADPH-Dependent Peroxidation of Liposomes

Conditions	% Inhibition
Complete	---
Complete, mannitol	0%
Complete, benzoate	4%
Complete, Tris-Cl	8%
Complete, catalase	4%

Complete reaction mixtures contained liposomes (1 μmol lipid phosphate/ml), NADPH (0.1 mM), NADPH-cytochrome P450 reductase (0.1 unit/ml), EDTA-Fe^{3+} (0.11 mM EDTA, 0.1 mM $FeCl_3$) and ADP-Fe^{3+} (0.5 mM ADP, 0.1 mM $FeCl_3$) in 30 mM NaCl, pH 7.5. Additions are specified in Table 11. Rates are expressed as percentage inhibition of the complete reaction mixture (1.83 nmol MDA/min/ml). Incubation and assay conditions are described under Materials and Methods.

The effect of catalase and •OH trapping agents on NADPH-dependent peroxidation of detergent-dispersed linoleate was also investigated. The EPR spin trapping experiments indicated that the incubation of NADPH, reductase, and EDTA-Fe^{3+} generate the •OH, yet this system did not initiate peroxidation of liposomes. It did, however, initiate the peroxidation of detergent-dispersed linoleate (ΔA_{234}=0.096/min/ml) (Table 13). Catalase (1 unit/ml) caused 43% inhibition of diene conjugation while 5 unit/ml did not increase the percent inhibition. At concentrations of 10 mM and 100 mM, mannitol caused 27% and 42% inhibition respectively, while ethanol caused 15% and 42% inhibition. Benzoate could not be utilized as a •OH trapping agent in these experiments due to its intense ultraviolet absorbance.

Table 13. Effect of Hydroxyl Radical Traps and Catalase on NADPH-Dependent Peroxidation of Linoleate

Additions	$EDTA\text{-}Fe^{3+}$	$EDTA\text{-}Fe^{3+}$, $ADP\text{-}Fe^{3+}$
	(% Inhibition)	
None	---(0.096)	---(0.341)
Mannitol, 10 mM	27%	21%
Mannitol, 100 mM	42%	22%
Ethanol, 10 mM	15%	2%
Ethanol, 100 mM	42%	13%
Catalase, 1 unit/ml	43%	8%
Catalase, 5 unit/ml	38%	0%

The effect of hydroxyl radical traps on NADPH-dependent peroxidation of linoleate was studied in the presence of $EDTA\text{-}Fe^{3+}$ (0.11 mM EDTA, 0.1 mM $FeCl_3$) or $EDTA\text{-}Fe^{3+}$ plus $ADP\text{-}Fe^{3+}$ (0.5 mM ADP, 0.1 mM $FeCl_3$). Reaction mixtures contained linoleate (5.7 mM), Lubrol (1%), NADPH (0.1 mM), NADPH-cytochrome P450 reductase (0.1 unit/ml), and the specified iron chelate. Addiitons are as specified. Rates are expressed as percentage inhibition. Values in parenthesis represent the rates of 234 nm absorbance increase (ΔA_{234}/min/ml) in the absence of $\cdot OH$ traps or catalase. Incubation and assay conditions are described under Materials and Methods.

In accord with the results obtained in liposomal peroxidation, the peroxidation of linoleate in the presence of $ADP\text{-}Fe^{3+}$ and $EDTA\text{-}Fe^{3+}$ was not affected by catalase (1 unit/ml and 5 unit/ml) (Table 9). Mannitol and ethanol caused a slight inhibition of activity. At concentrations of 10 mM and 100 mM, mannitol caused 21% and 22% inhibition of activity, respectively, while ethanol caused 2% and 13% inhibition.

DISCUSSION

The results of this study show little evidence for NADPH-cytochrome P450 reductase catalyzed formation of $O_2^{\cdot-}$. No detectable changes in NADPH concentration or in oxygen partial pressure were observed in incubation of the reductase with NADPH. EPR spin trapping, which is a sensitive technique for $O_2^{\cdot-}$ detection (Finkelstein et al., 1980), also failed to show any evidence for $O_2^{\cdot-}$ formation by the reductase. It is therefore doubtful that the rate of $O_2^{\cdot-}$ generation reported by others (Aust et al., 1972; Mishin et al., 1976a, Gnosspelius et al., 1969-1970) can account for the observed rates of lipid peroxidation catalyzed by the reductase.

Kamin and Master (1968) reported that iron, when chelated by EDTA, can be directly reduced by NADPH-cytochrome P450 reductase. This was demonstrated by monitoring NADPH oxidation under anaerobic conditions, thus eliminating dioxygen or $O_2^{\cdot-}$ as an obligatory intermediate in EDTA-Fe^{3+} reduction. Their results are in accord with the results of this paper which indicates that the reductase catalyzed passage of electrons from NADPH to dioxygen and the resultant radicals formed by this process, requires the catalysis of EDTA-Fe^{3+}. The calculated Km for EDTA-Fe^{3+} is 150 μM. Neither ADP-Fe^{3+} nor free Fe^{3+} were capable of catalyzing this electron transfer. These results clearly indicate that the EDTA-Fe^{3+}-dependent reductase catalyzed transfer of

electrons from NADPH to dioxygen can account for the observed rates of lipid peroxidation.

The radicals formed after EDTA-Fe^{3+} reduction were identified as •OH by EPR spin trapping techniques with DMPO. Hydrogen peroxide appears to be an intermediate in •OH formation since catalase decreased the EPR signal intensity of the •OH-DMPO adduct. The intensity of this signal could also be affected by known •OH trapping agents mannitol, benzoate, and Tris-Cl. The incubation of ADP-Fe^{3+} alone with the reductase and NADPH did not result in radical formation as determined by EPR spin trapping techniques.

In a heterogenous system containing liposomes, the apparent generation of the •OH by the incubation of EDTA-Fe^{3+} with NADPH and the reductase did not result in lipid peroxidation as assayed by MDA formation. However, in a homogenous system of detergent-dispersed linoleate, the generation of •OH by this system did result in lipid peroxidation as assayed by diene conjugation. The inhibition by catalase and •OH trapping agents confirmed that hydrogen peroxide and •OH are involved in initiation. The addition of ADP-Fe^{3+} caused an enhancement in lipid peroxidation in both systems. In the liposomal system, NADPH-dependent lipid peroxidation shows an absolute requirement for ADP-Fe^{3+} for activity, while in the detergent dispersed-linoleate system, ADP-Fe^{3+} caused a four fold increase in the rate of diene conjugation.

In addition to augmenting NADPH-dependent lipid peroxidation of liposomes and detergent-dispersed linoleate, ADP-Fe^{3+} also caused an apparent shift to a non-hydroxyl radical dependent mechanism of lipid peroxidation. In the detergent-dispersed linoleate system, catalase,

mannitol and ethanol were much less effective inhibitors of lipid peroxidation in the presence of ADP-Fe^{3+}. In the liposomal system, 1 unit/ml catalase and 10 mM mannitol, benzoate, and Tris-Cl did not inhibit NADPH-dependent lipid peroxidation in the presence of ADP-Fe^{3+} while catalase and these trapping agents at the same concentration caused greater than 90% inhibition of •OH-dependent lipid peroxidation via the Fenton's reagent.

One possible explanation for these results is that ADP-Fe^{3+} localizes the generation of the •OH in closer proximity to the lipid than EDTA-Fe^{3+} so that water soluble •OH trapping agents and catalase can no longer trap •OH or eliminate hydrogen peroxide. This interpretation is brought about by the differences observed in NADPH-dependent lipid peroxidation of liposomes versus detergent-dispersed linoleate. In the case of liposomes, •OH generation by EDTA-Fe^{3+} catalyzed reactions would probably occur in the aqueous phase. It is unlikely that the highly reactive •OH would diffuse away from the site of formation to liposomes to initiate lipid peroxidation (Walling, 1975). Dispersion of the lipid with detergent to yield a homogenous phase would bring the lipid in closer proximity to the site of •OH formation. Thus in detergent-dispersed linoleate, EDTA-Fe^{3+} catalyzed •OH formation does initiate lipid peroxidation while in liposomes, lipid peroxidation does not occur. Lipid peroxidation would be less susceptible to •OH trapping agents with the lipid in closer proximity. Since these trapping agents inhibits lipid peroxidation by competing with the lipid for the •OH, having the lipid in closer proximity to the site of formation would render the agents less effective. Furthermore, in the liposomal system, peroxidation initiated by the •OH generated by the Fenton's

reagent was totally inhibited by •OH trapping agents at a concentration of 10 mM. However, these trapping agents at a concentration of 100 mM only caused 42% inhibition of EDTA-Fe^{3+} catalyzed •OH-dependent peroxidation of detergent-dispersed linoleate. The interpretation of ADP-Fe^{3+} localizing the generation of •OH in closer proximity would suggest an affinity of ADP for the lipid phase. However, preliminary experiments where liposomes and ADP-Fe^{3+} were incubated prior to filtration to separate the liposomes from the aqueous phase showed no evidence for loss of ADP in the aqueous phase or enrichment of iron in the lipid phase.

Another possible explanation is that lipid peroxidation in the presence of ADP-Fe^{3+} proceeds by a mechanism not dependent upon the free •OH. EPR spin trapping experiments demonstrated that the addition of ADP-Fe^{3+} to the incubation of NADPH, EDTA-Fe^{3+} and the reductase caused a dramatic decrease in the EPR signal intensity of the •OH-DMPO adduct. Paradoxically, this system with both iron complexes catalyzed the highest rates of NADPH-dependent lipid peroxidation. Whether these results can be construed as a mechanism of initiation not dependent on the •OH is unclear. The observation of the α-hydroxyl ethyl-DMPO adduct with EPR spectroscopy in the incubation of ADP-Fe^{3+}, EDTA-Fe^{3+}, reductase, and NADPH with ethanol would suggest that the •OH is being generated in the presence of ADP-Fe^{3+}. However, this criteria for •OH involvement lacks specificity since the formation of the α-hydroxyl ethyl radical is dependent on the redox potential of the oxidant. The possibility of another oxidant aside from •OH oxidizing ethanol remains open.

Svingen et al. (1980) proposed that initiation of NADPH-dependent lipid peroxidation occurred via the ADP-perferryl ion ($ADP\text{-}Fe^{2+}\text{-}O_2$). These workers also proposed the enhancement of lipid peroxidation by EDTA-Fe^{3+} was due to its ability to catalyze LOOH decomposition. In their system, the ADP-perferryl ion is generated by the direct reduction of ADP-Fe^{3+} by the reductase followed by subsequent complexation with dioxygen. Due to the lack of precedence for ADP-perferryl as a strong oxidant, Tien et al. (1980) proposed that initiation of lipid peroxidation occurred through further reduction of perferryl ion to yield the ferryl ion (FeO^{2+}).

$$ADP\text{-}Fe^{2+}\text{-}O_2 \xrightarrow{e^-} ADP\text{-}Fe^{2+}\text{-}O_2^-$$

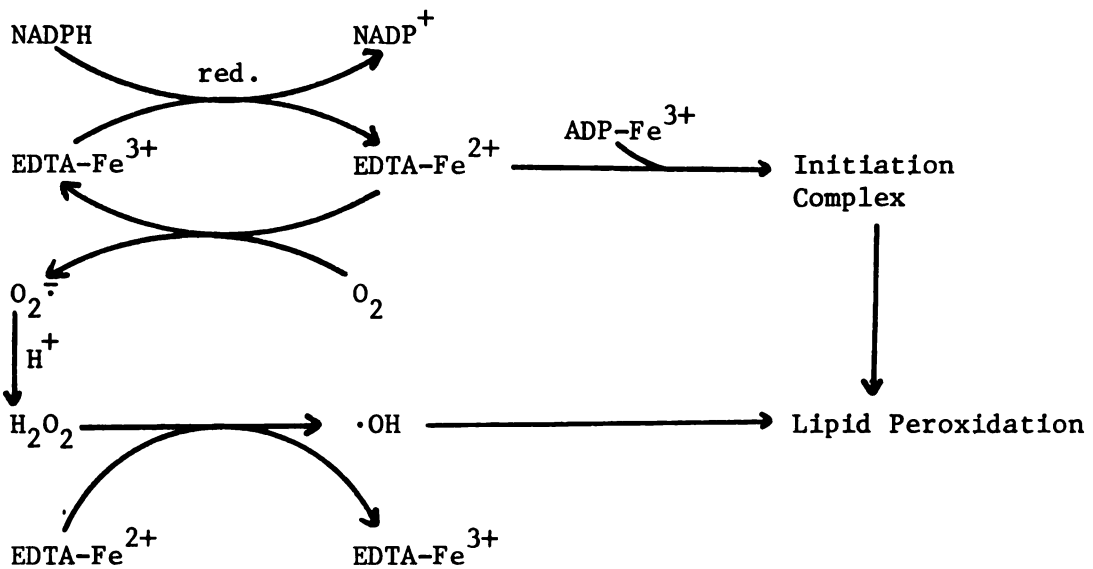
$$ADP\text{-}Fe^{2+}\text{-}O_2^- \xrightarrow{e^-} (ADP\text{-}Fe^{2+}\text{-}O_2^{=}) \longrightarrow ADP\text{-}FeO^{2+} + H_2O$$

The reactivity of the ferryl ion has been equated to that of the •OH (Walling, 1975). This mechanism would account for the lack of inhibition by catalase since free peroxide is not involved. It would also be required that the ferryl ion is not scavenged by •OH trapping agents.

The basic drawback of these proposed alternate mechanisms is the lack of positive evidence. Although the exact nature of this oxidant remains unknown, it is apparent that the generation of the free •OH is not required for its lipid peroxidation initiating activity. However, our results clearly indicate that such complexes of ADP-Fe^{3+} cannot be generated by direct enzymatic reduction of ADP-Fe^{3+}. The reduction of ADP-Fe^{3+} to form the ADP-perferryl ion would require catalysis by EDTA-Fe^{3+}. The ability of EDTA-Fe^{3+} to catalyze the

transfer of electrons from reduced NADPH-cytochrome P450 reductase to either dioxygen or ADP-Fe^{3+} may offer an alternate explanation for EDTA-Fe^{3+} enhancement of NADPH-dependent lipid peroxidation observed by Svingen et al. (1980). It is also important to point out that most procedures for purification of NADPH-cytochrome P450 reductase utilize EDTA in their storage buffer (Pederson et al., 1973; Lu et al., 1972; Dignam and Strobel, 1975; van der Hoeven and Coon, 1974; Yasukochi and Masters, 1976). Numerous studies which have reported that EDTA-Fe^{3+} is not required for reconstitution of NADPH-dependent lipid peroxidation are in fact using enzyme preparations that are contaminated with EDTA (Fong et al., 1973; Svingen, et al., 1980).

In conclusion, the results of this paper suggest that two mechanisms for initiation of NADPH-dependent lipid peroxidation are possible: a ·OH-dependent mechanism in the presence of EDTA-Fe^{3+} and a non-·OH-dependent mechanism in the presence of ADP-Fe^{3+} as shown in the following scheme.



The direct demonstration of lipid peroxidation initiated by the ADP-perferryl ion or the ADP-ferryl ion must await for further experimental development. In the presence of EDTA-Fe^{3+}, lipid peroxidation occurs via a •OH-dependent mechanism. This mechanism is a slight modification of the iron-catalyzed Haber-Weiss reaction proposed by numerous other investigators for NADPH-dependent lipid peroxidation (Lai and Piette, 1977, 1978; McCay et al., 1972; Fong et al., 1973). In this case, reduced iron is the source of $O_2^{\cdot-}$ and H_2O_2 rather than $O_2^{\cdot-}$ being the source of reduced iron and H_2O_2. Although both mechanisms generate the Fenton's reagent for •OH formation, the mechanism proposed above has profound implications for defense against lipid peroxidation. In cases where iron can be directly reduced by a mechanism not dependent upon $O_2^{\cdot-}$, superoxide dismutase may not provide defense against lipid peroxidation. As demonstrated by this study, superoxide dismutase may, in fact, augment lipid peroxidation.

CHAPTER III

ON THE QUESTION OF HYDROXYL RADICAL INVOLVEMENT IN SUPEROXIDE-DEPENDENT LIPID PEROXIDATION

ABSTRACT

A model lipid peroxidation system dependent upon the hydroxyl radical, generated by Fenton's reagent, was compared to another model system dependent upon the enzymatic generation of superoxide by xanthine oxidase. Hydroxyl radical generation by Fenton's reagent ($FeCl_2$ + H_2O_2) in the presence of phospholipid liposomes resulted in lipid peroxidation as evidenced by malondialdehyde and lipid hydroperoxide formation. Catalase, mannitol and Tris-Cl were capable of completely inhibiting activity. The addition of EDTA resulted in complete inhibition of activity when the concentration of EDTA exceeded the concentration of Fe^{2+}. The addition of ADP resulted in slight inhibition of activity, however, the activity was less sensitive to inhibition by mannitol. At an ADP to Fe^{2+} molar ratio of 10 to 1, 10 mM mannitol caused 24% inhibition of activity.

Lipid peroxidation dependent on the enzymatic generation of superoxide by xanthine oxidase was studied in liposomes and in detergent-dispersed linoleate. This system was also sensitive to iron chelation. No activity was observed in the absence of added iron. The addition of EDTA-chelated iron to detergent-dispersed linoleate resulted in lipid peroxidation as evidenced by diene conjugation. This activity was inhibited by catalase and hydroxyl radical trapping agents. In contrast, no activity was observed in liposomes with the addition of EDTA-chelated iron. The peroxidation of liposomes required both EDTA and ADP-chelated

iron for maximal activity. Tris-Cl, mannitol or catalase had no effect on this activity. The peroxidation of detergent-dispersed linoleate was also enhanced by ADP-chelated iron. Again, this peroxidation in the presence of ADP-chelated iron was not sensitive to catalase or hydroxyl radical trapping agents. It is proposed that initiation of superoxide-dependent lipid peroxidation in the presence of EDTA-chelated iron occurs via the hydroxyl radical. However, in the presence of ADP-chelated iron, the participation of the free hydroxyl radical is minimal.

INTRODUCTION

The traditional view on the basis of oxygen toxicity was expanded by the discovery that certain enzymes liberate superoxide radical ($O_2^{\cdot-}$) (McCord and Fridovich, 1968) and that other enzymes catalytically scavenge this radical (McCord and Fridovich, 1969). Superoxide was soon seen to be a commonplace product of dioxygen reduction and an important agent of oxygen toxicity. One of the most significant findings in the field of oxygen toxicity is that $O_2^{\cdot-}$ can initiate lipid peroxidation (Bus et al., 1974, 1975).

Using the aerobic action of xanthine oxidase on acetaldehyde or xanthine as a source of $O_2^{\cdot-}$ and of hydrogen peroxide, numerous workers have investigated the mechanism by which $O_2^{\cdot-}$ initiates lipid peroxidation. Kellogg and Fridovich (1975, 1977) studied $O_2^{\cdot-}$-dependent lipid peroxidation in the absence of added iron and proposed that initiation occurred by the $O_2^{\cdot-}$-dependent formation of singlet oxygen and hydroxyl radical (·OH). The authors proposed that singlet oxygen and ·OH were formed by the Haber-Weiss reaction (Haber and Weiss, 1934).

$$H_2O_2 + O_2^{\cdot-} \longrightarrow {\cdot}OH + {}^-OH + {}^1O_2$$

The proposed mechanism was based on the findings that lipid peroxidation was inhibited by superoxide dismutase, catalase, singlet oxygen trapping agents and ·OH trapping agents.

Fong and coworkers (1973) also studied $O_2^{\cdot-}$-dependent lipid peroxidation promoted by xanthine oxidase and found evidence for •OH involvement. The rate of lipid peroxidation was enhanced by the addition of ADP-Fe^{3+} while catalase and •OH trapping agents such as ethanol, mannitol, and benzoate (Neta and Dorfman, 1968) afforded protection from lipid peroxidation. Paradoxically, superoxide dismutase enhanced the rate of lipid peroxidation. These workers postulated that the reaction of $O_2^{\cdot-}$ with hydrogen peroxide, both produced by xanthine oxidase, produced low levels of •OH via a Haber-Weiss mechanism. The addition of chelated iron to the system would enhance the rate of •OH formation by catalyzing the Haber-Weiss reaction.

$$O_2^{\cdot-} + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {\cdot}OH + {}^-OH$$

If the flux of $O_2^{\cdot-}$ is sufficient for iron reduction, superoxide dismutase may serve to enhance the rate of hydrogen peroxide formation and thus facilitate the rate of •OH formation.

Although numerous reports have proposed that $O_2^{\cdot-}$-dependent lipid peroxidation is mediated by the iron-catalyzed Haber-Weiss reaction (Fridovich and Porter, 1981; Lai and Piette, 1978; Halliwell, 1978), the reactive form of oxygen involved in $O_2^{\cdot-}$-dependent lipid peroxidation is yet to be clearly established. Numerous other workers have failed to inhibit $O_2^{\cdot-}$-dependent lipid peroxidation with catalase (Pederson and Aust, 1973; Tyler, 1975; Svingen and Aust, 1980) or •OH trapping agents (Tyler, 1975; Svingen and Aust, 1980).

To further examine the involvement of the •OH in $O_2^{\bar{\cdot}}$-dependent lipid peroxidation, this study utilized Fe^{2+} and hydrogen peroxide, termed the Fenton's reagent, to generate the •OH for the purpose of initiating lipid peroxidation. The response of this •OH-dependent lipid peroxidation system to the presence of iron chelation agents and to known inhibitors of •OH-dependent reactions is characterized and compared to the results of similar studies utilizing a lipid peroxidation system dependent upon $O_2^{\bar{\cdot}}$.

MATERIALS AND METHODS

Materials. Cytochrome c (Type VI), 2-thiobarbituric acid, NADPH, ADP, butylated hydroxytoluene (BHT), mannitol, Lubrol, and sodium benzoate were purchased from Sigma Chemical Company. Linoleic acid was obtained from Nu Chek Prep, Elysian, MN. All other chemicals used were of analytical grade. All buffers and reagents were passed through Chelex 100 (Bio-Rad Laboratories) ion exchange resin to free them of contaminants.

Enzymes. Superoxide dismutase (SOD), beef liver catalase, and xanthine oxidase were obtained from Sigma. Gel filtration chromatography on Sephadex G-25 was used to remove the antioxidant, thymol, from catalase and the ammonium sulfate from xanthine oxidase. Xanthine oxidase activity was measured by aerobic reduction of cytochrome c or by uric acid formation (McCord and Fridovich, 1969). SOD activity was measured by the method of McCord and Fridovich (1969). Catalase activity was measured by the procedure of Holmes and Masters (1970).

Microsomal Lipid. Male Sprague-Dawley rats (250-274 g) were obtained from Spartan Research Animals (Haslett, MI). Liver microsomes were isolated by the method of Pederson et al. (1973). Microsomal lipid was extracted from freshly isolated microsomes by the method of Folch et al. (1957). All solvents used for extractions were purged with argon and all steps were performed at 4°C to minimize autoxidation of unsaturated lipids. Extracted lipid was stored in argon-saturated $CHCl_3$:CH_3OH

(2:1) at -20°C. Lipid phosphate determinations were performed by the method of Bartlett (1959).

Reaction Mixtures. Liposomes were prepared by sonication of the extracted microsomal lipid in argon-saturated distilled deionized water at 4°C (Pederson et al., 1973). Liposomal peroxidation reactions initiated by the Fenton's reagent were accomplished by the addition of $FeCl_2$ to incubations containing H_2O_2 and liposomes (1 μmol lipid phosphate/ml) in 30 mM NaCl, pH 7.5. The concentrations of $FeCl_2$ and H_2O_2 are as specified in the figures and tables. Other additions or deletions are as specified in the legends to the figures and tables. Xanthine oxidase-dependent peroxidation of liposomes was performed by incubating liposomes (1 μmol lipid phosphate/ml) with xanthine oxidase (0.1 unit/ml), EDTA-Fe^{3+} (0.11 mM EDTA, 0.1 mM $FeCl_3$), ADP-Fe^{3+} (1.7 mM ADP, 0.1 mM $FeCl_3$) and 0.33 mM xanthine in 30 mM NaCl, pH 7.5. Reactions were initiated by the addition of xanthine oxidase. Other additions or deletions are as specified in the table and figure legends. Incubations were done in a metabolic shaking water bath at 37°C under an air atmosphere. Although the reaction mixtures were unbuffered, the pH did not change during the course of reactions. Liposomal lipid peroxidation was assessed by malondialdehyde (MDA) or lipid hydroperoxide (LOOH) formation (Buege and Aust, 1978). To prevent further peroxidation of lipid during the assay procedure for MDA, 0.03 volumes of 2% BHT in ethanol was added to the thiobarbituric acid reagent (Buege and Aust, 1978).

Linoleate stock solutions were prepared as described in Chapter II. Lipid peroxidation of detergent-dispersed linoleate was accomplished by incubating sodium linoleate (5.7 mM) with xanthine oxidase

(6.6×10^{-3} unit/ml), Lubrol (1%), 35 mM acetaldehyde, EDTA-Fe^{3+} (0.11 mM EDTA, 0.1 mM $FeCl_3$), and ADP-Fe^{3+} (0.5 mM ADP, 0.1 mM $FeCl_3$) in 30 mM NaCl, pH 7.5, at 37°C under air. These incubations were carried out in a cuvette of a Cary 219 spectrophotometer. Diene conjugation during lipid peroxidation was continuously monitored by the absorbance change at 234 nm (Adams and Wardman, 1977).

Other Methods. ADP and EDTA chelated iron solutions were prepared by the addition of either $FeCl_2$ or $FeCl_3$ to chelate solutions adjusted to pH 7.5. Due to the pH change from the addition of $FeCl_3$, the pH of these chelate solutions were readjusted to 7.5. No changes in pH were observed from the addition of $FeCl_2$ to the chelate solutions. Water used in the preparation of the ferrous solutions were argon-purged to minimize Fe^{2+} autoxidation. Since most buffers are iron chelators or reactive with hydroxyl radical, the reaction mixtures were unbuffered. Consequently, pH of all reagents were carefully adjusted to 7.5 prior to use.

RESULTS

Hydroxyl Radical-Dependent Lipid Peroxidation

The reaction of H_2O_2 with Fe^{2+} (Fenton's reagent) has been demonstrated to produce the •OH (Ilan and Zapski, 1977). The formation of this radical has been demonstrated with EPR spin trapping techniques utilizing DMPO (Finkelstein et al., 1980). The EPR spectrum has a 1:2:2:1 signal intensity pattern, a g value of 2.006 and hyperfine splitting constants of A_N=14.95 G and A_H=14.95 G. To confirm the formation of the •OH in our system, this spectrum was reproduced by the reaction of 0.15 mM Fe^{2+} and 0.1 mM H_2O_2 in the presence of 60 mM DMPO (not shown). The generation of the •OH in the presence of liposomes resulted in lipid peroxidation as evidenced by a rapid rate of LOOH and MDA formation (Figure 9). The rate of MDA formation was constant at 6.24 nmol/min/ml up to 2 minutes after initiation of the reaction. The LOOH content increased at a much greater rate (approximately 150 nmol/min/ml) but, the rate of LOOH formation decreased after the first minute of the reaction. No detectable MDA or LOOH formation resulted from the separate addition of $FeCl_2$ or H_2O_2 to liposomes.

The effect of H_2O_2 concentration of the rate of lipid peroxidation is illustrated in Figure 10. At 0.2 mM $FeCl_2$, the rate of MDA formation reached a maximum when H_2O_2 concentration was 0.1 mM. Higher concentrations of H_2O_2 had an inhibitory effect on the rate of lipid peroxidation. At a constant H_2O_2 concentration of 0.1 mM,

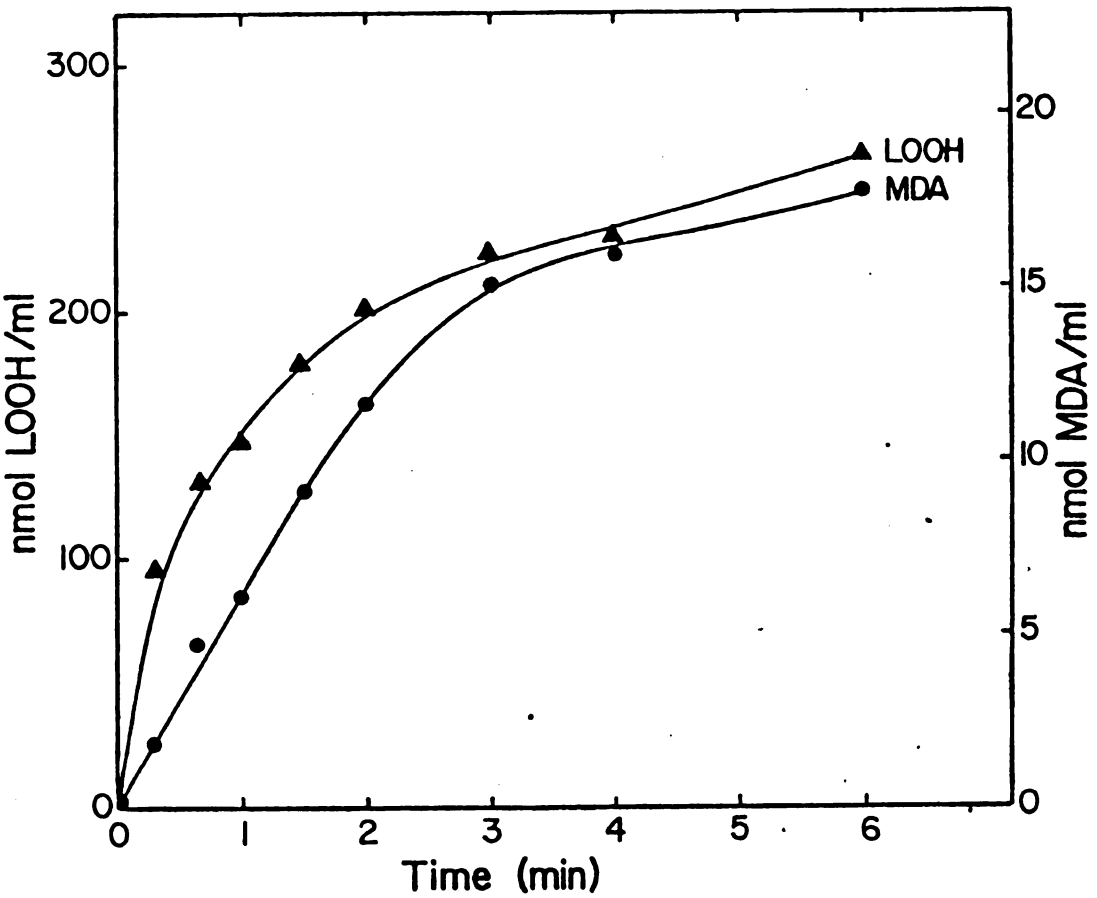


Figure 9. Time Course of Hydroxyl Radical-Dependent Lipid Peroxidation. Reaction mixtures contained liposomes (1umol lipid phosphate/ml), 0.1 mM H_2O_2 and 0.2 mM $FeCl_2$ in 30 mM NaCl, pH 7.5 at 37^0C. Incubation and assay conditions are described under "Materials and Methods".

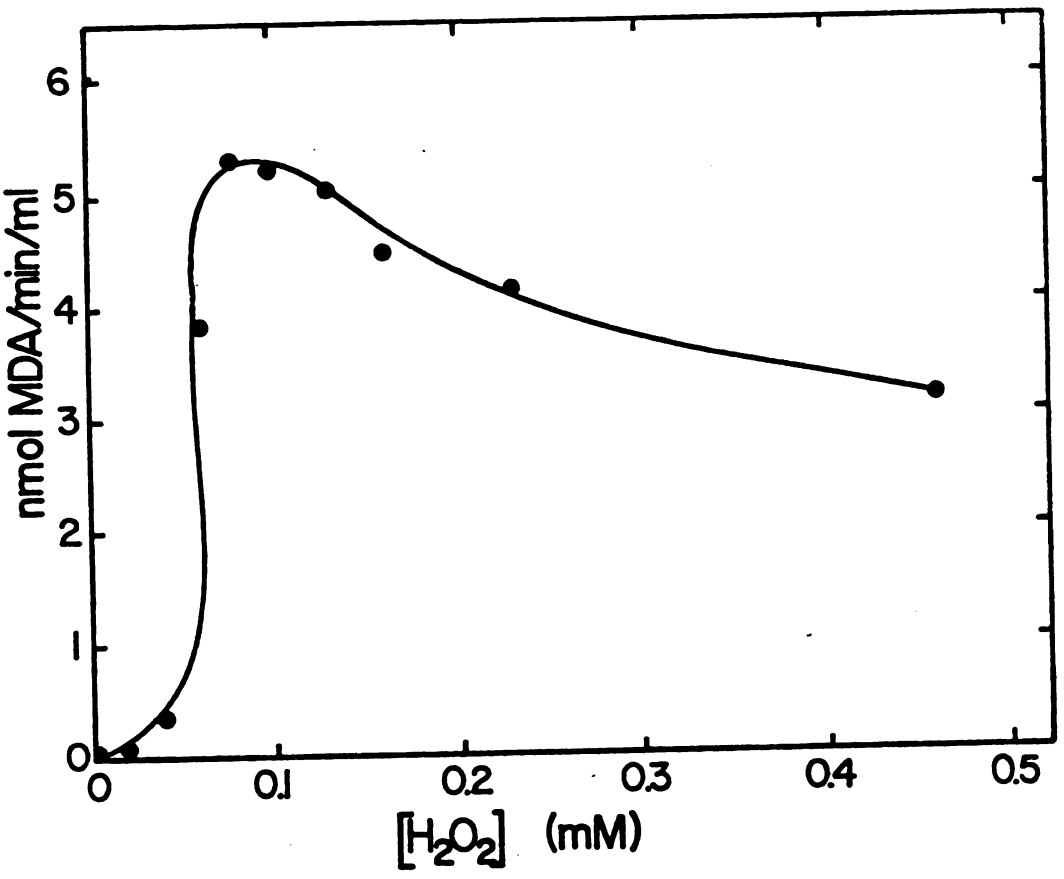


Figure 10. Effect of Hydrogen Peroxide Concentration on Hydroxyl Radical-Dependent Lipid Peroxidation. Reaction mixtures contained liposomes (1 μmol lipid phosphate/ml), 0.2 mM $FeCl_2$ and the specified amount of H_2O_2 in 30 mM NaCl, pH 7.5 at 37°C. Incubation and assay conditions are described under Materials and Methods.

Table 14. Hydroxyl Radical-Dependent Lipid Peroxidation.

Additions	MDA nmol/min/ml
None	0.01
Fe^{2+}	0.15
H_2O_2	0.10
Fe^{2+}, H_2O_2	3.45
Fe^{2+}, H_2O_2, benzoate	0.41
Fe^{2+}, H_2O_2, mannitol	0.22
Fe^{2+}, H_2O_2, Tris-Cl	0.29
Fe^{2+}, H_2O_2, catalase	0.25

Liposomes (1 μmol lipid phosphate/ml) were incubated in 30 mM NaCl, pH 7.5 at 37°C. The following additions were made as indicated: 0.2 mM $FeCl_2$, 0.1 mM H_2O_2, 1 unit catalase/ml, and 10 mM benzoate, mannitol, or Tris-Cl.

increasing $FeCl_2$ concentrations to 0.2 mM resulted in a linear increase in the rate of MDA formation (Figure 11). At $FeCl_2$ concentrations above 0.2 mM, the rate of MDA formation was constant.

Inhibition by mannitol or benzoate has been used as a criterion to assess •OH involvement in reaction mechanisms (Fong et al., 1973; Kellogg and Fridovich, 1975, 1977; Fridovich and Porter, 1981). As shown in Table 14, peroxidation of liposomes by Fenton's reagent could be effectively inhibited by these •OH traps. At 10 mM, mannitol completely inhibited activity while 10 mM benzoate caused 88% inhibitions. Tris-Cl is also an effective •OH trap (Tien et al., 1980) and 10 mM Tris caused 85% inhibition of activity. Addition of 1 unit/ml catalase completely abolished activity (Table 14) while boiled catalase had no effect.

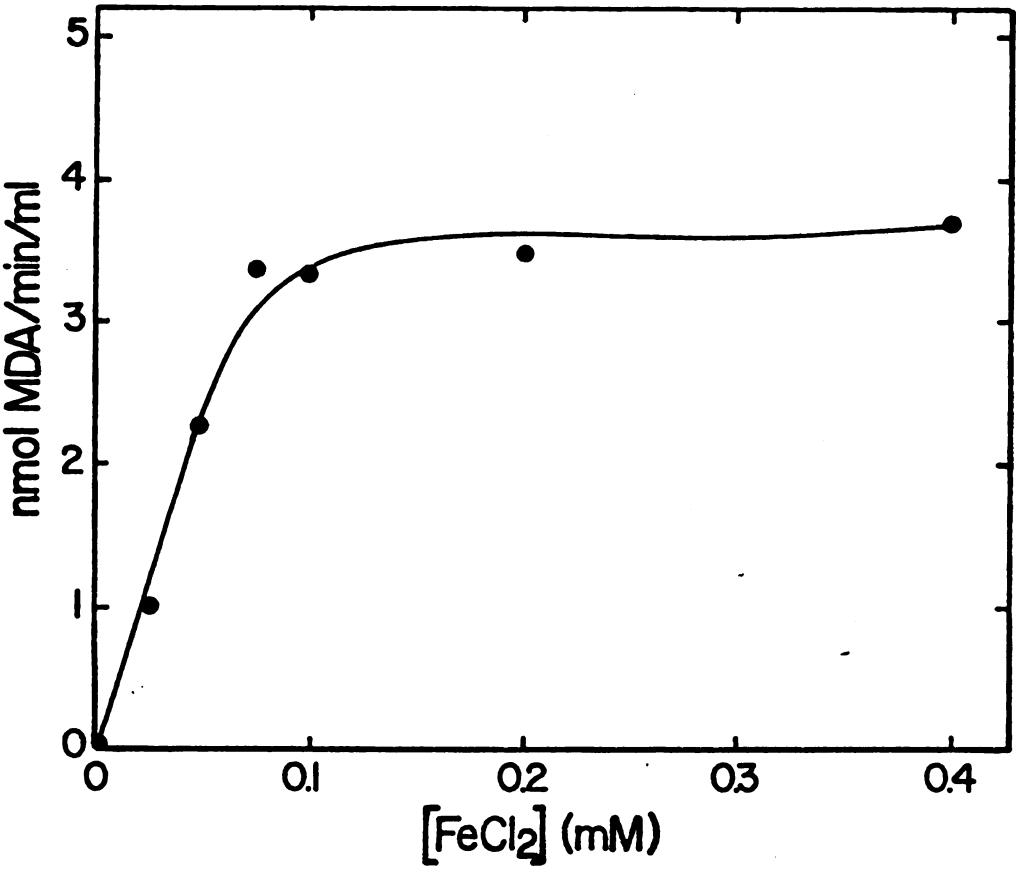


Figure 11. Effect of $FeCl_2$ Concentration on Hydroxyl Radical-Dependent Lipid Peroxidation. Reaction mixtures contained liposomes (1 μmol lipid phosphate/ml), 0.1 mM H_2O_2 and the specified amount of $FeCl_2$ in 30 mM NaCl, pH 7.5 at 37°C. Incubation and assay conditions are described under Materials and Methods.

Most model lipid peroxidation systems employ iron chelates. In addition, biological systems are abundant in substances which can chelate iron. For these reasons, the effect of iron chelation on •OH dependent lipid peroxidation was examined. ADP and EDTA were examined because both have been implicated in Fenton-type reactions to yield the •OH (Walling, 1975; Lai and Piette, 1978). The chelation of Fe^{2+} by EDTA in the Fenton's reagent resulted in inhibition of MDA formation (Figure 12). Complete inhibition occurred when the ratio of EDTA to Fe^{2+} was equimolar. The chelation of Fe^{2+} by ADP resulted in slight inhibition of activity (Figure 13). Activity in the absence of ADP could be completely inhibited by mannitol but as the concentration of ADP increased, the amount of activity inhibited by mannitol decreased, reaching 24% inhibition at an ADP to Fe^{2+} molar ratio of 10 to 1.

Xanthine Oxidase-Dependent Peroxidation of Liposomes

The requirements for xanthine oxidase-dependent peroxidation of liposomes are shown in Table 15. In the absence of iron, the generation of O_2 and H_2O_2 by the action of xanthine oxidase on xanthine did not result in lipid peroxidation as assayed by LOOH and MDA formation. The addition of chelated iron to this reaction mixture stimulated the activity, however, this activity was exceedingly sensitive to the iron chelators. Very low rates of lipid peroxidation were obtained by the addition of Fe^{3+} chelated by EDTA (Table 15). The inclusion of Fe^{3+} chelated by ADP resulted in low levels of MDA and LOOH formation. Much greater rates of lipid peroxidation (2.02 nmol MDA/min/ml and 15.3 nmol LOOH/min/ml) occurred when both ADP-Fe^{3+} and EDTA-Fe^{3+} were included in the reaction mixture. The effect of unchelated Fe^{3+}

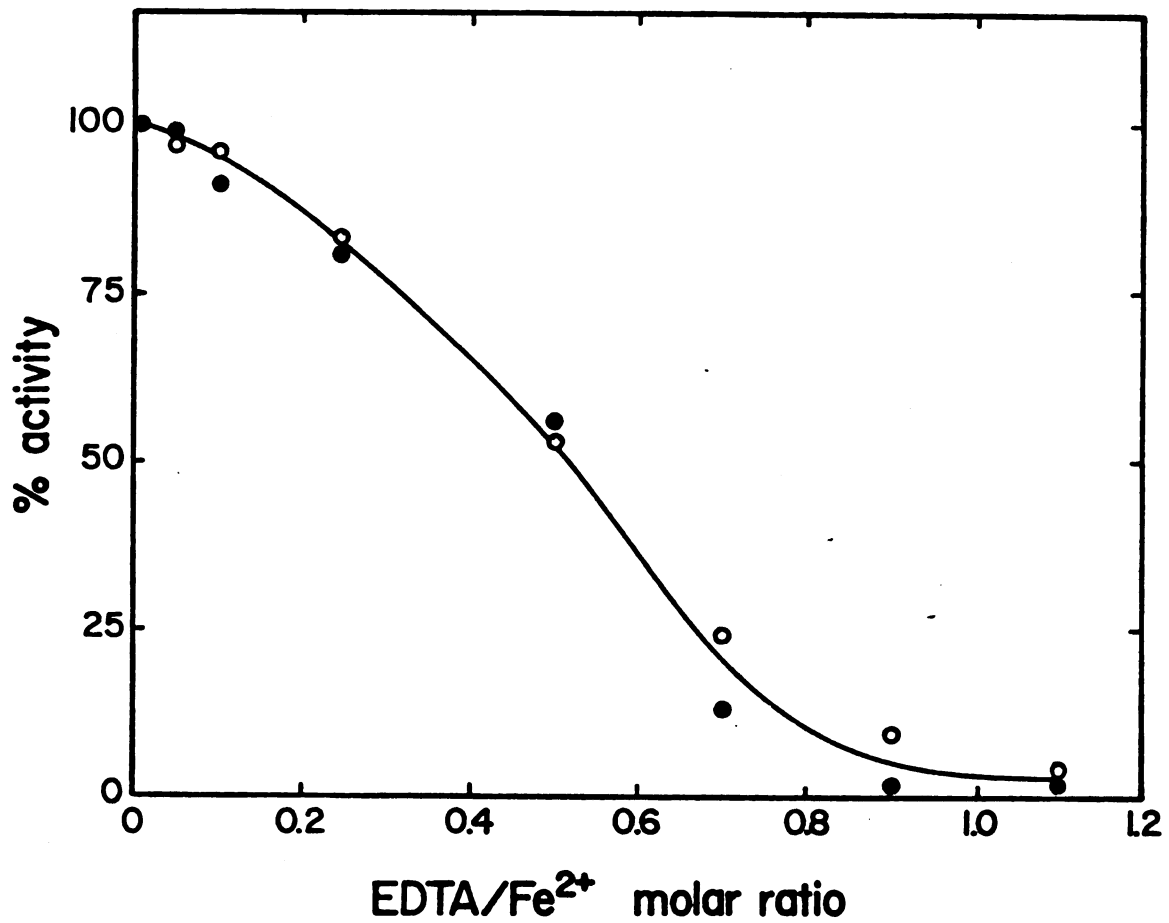


Figure 12. Inhibition of Hydroxyl Radical-Dependent Lipid Peroxidation by EDTA. Reaction mixtures contained liposomes (1 μmol lipid phosphate/ml), and 0.1 mM H_2O_2 in 30 mM NaCl, pH 7.5 at 37°C. EDTA concentration is expressed as molar ratios to $FeCl_2$. The $FeCl_2$ concentrations were either 0.05 mM (open circles) or 0.1 mM (closed circles). Incubation and assay conditions are described under Materials and Methods.

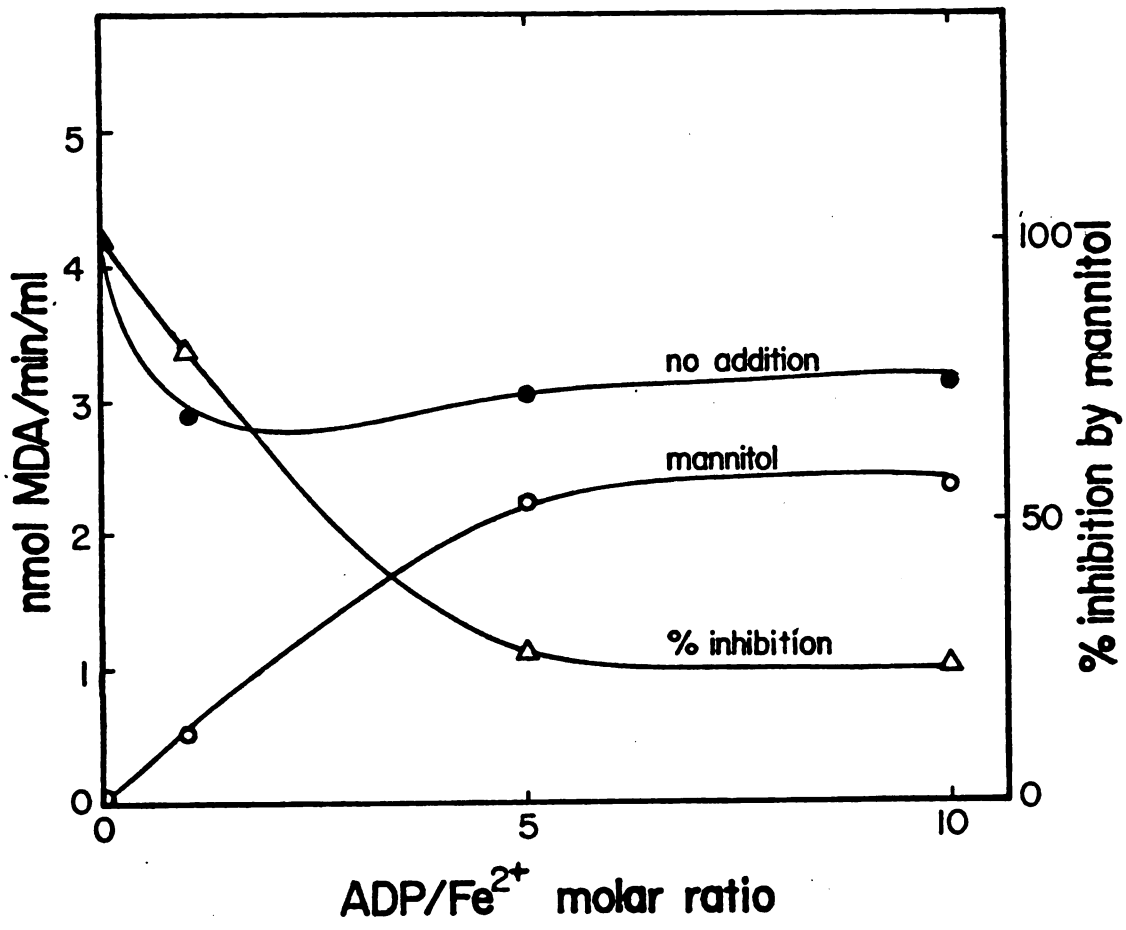


Figure 13. Effect of ADP on Hydroxyl Radical-Dependent Lipid Peroxidation. Reaction mixtures contained liposomes (1 μmol lipid phosphate/ml), 0.1 mM H_2O_2, 0.2 mM $FeCl_2$ and the specific amount of ADP in 30 mM NaCl, pH 7.5 at 37°C. ADP concentration is expressed as molar ratios of $FeCl_2$. Incubations were performed in the presence (closed circles) and absence (open circles) of 10 mM mannitol. The percent inhibition by mannitol as a function of ADP is plotted as open triangles. Incubation and assay conditions are described under Materials and Methods.

Table 15. Effect of Iron Chelates on Xanthine Oxidase-Dependent Peroxidation of Liposomes.

Additions	MDA	LOOH
	(nmol/min/ml)	
None	0.02	0.1
ADP-Fe^{3+}, EDTA-Fe^{3+}	0.02	0.4
Xanthine oxidase	0.02	0.6
Xanthine oxidase, ADP-Fe^{3+}	0.29	2.2
Xanthine oxidase, EDTA-Fe^{3+}	0.18	1.0
Xanthine oxidase, ADP-Fe^{3+}, EDTA-Fe^{3+}	2.02	15.3

Reaction mixtures contained liposomes (1 μmol lipid phosphate/ml) and 0.33 mM xanthine in 30 mM NaCl, pH 7.5 at 37°C. The following additions were made as indicated: ADP-Fe^{3+}, (1.7 mM ADP, 0.1 mM $FeCl_3$), EDTA-Fe^{3+}, (0.11 mM EDTA, 0.1 mM $FeCl_3$) and 0.1 unit xanthine oxidase/ml.

on xanthine oxidase dependent peroxidation of liposomes was also examined, however, no activity was observed.

The involvement of the •OH in xanthine oxidase dependent peroxidation of liposomes was investigated by the criteria utilized in investigating peroxidation of liposomes initiated by the Fenton's reagent. Mannitol (10 mM) and Tris-Cl (50 mM), both very effective inhibitors of lipid peroxidation initiated by the Fenton's reagent, were without effect in lipid peroxidation promoted by xanthine oxidase (Table 16). SOD (1 unit/ml) caused complete inhibition of activity, presumably by blocking the reduction of iron by $O_2^{\cdot-}$. However, catalase (1 unit/ml) did not inhibit the rate of lipid peroxidation, suggesting that the involvement of H_2O_2 in xanthine oxidase-dependent lipid peroxidation is minimal. In fact, catalase caused a slight enhancement of activity.

Table 16. Xanthine Oxidase-Dependent Lipid Peroxidation of Liposomes.

Additions	MDA	LOOH
	(nmol/min/ml)	
None	2.02	15.3
Catalase	2.23	17.3
SOD	0.03	5.0
Mannitol	2.32	14.5
Tris-Cl	2.25	17.1

The complete system contained liposomes (1 μmol lipid phosphate/ml), ADP-Fe^{3+} (1.7 mM ADP, 0.1 mM $FeCl_3$), EDTA-Fe^{3+} (0.11 mM EDTA, 0.1 mM $FeCl_3$), 0.33 mM xanthine and 0.1 unit xanthine oxidase/ml in 30 mM NaCl, pH 7.5 at 37°C. The following additions were made as indicated: 1 unit catalase/ml, 1 unit SOD/ml 10 mM mannitol, and 50 mM Tris-Cl.

The direct addition of H_2O_2 caused a sharp decrease in the rates of MDA and LOOH formation (Figure 14). The addition of 0.25 mM H_2O_2 resulted in complete inhibition of MDA and LOOH formation. Xanthine oxidase activity, as measured by uric acid formation, was also decreased by H_2O_2, however, this decrease was minimal and did not correlate with the decrease in the rate of lipid peroxidation. Hydrogen peroxide had no effect on the assays for MDA or LOOH.

Xanthine Oxidase-Dependent Peroxidation of Detergent-Dispersed Linoleate

In a recent report, Fridovich and Porter (1981) demonstrated that EDTA-Fe^{3+} facilated the xanthine oxidase-dependent peroxidation of detergent-dispersed archidonate. Our results with liposomes indicate that EDTA-Fe^{3+} was not effective in catalyzing xanthine oxidase-dependent lipid peroxidation. For these reasons, xanthine oxidase-dependent lipid peroxidation was also studied using detergent-dispersed

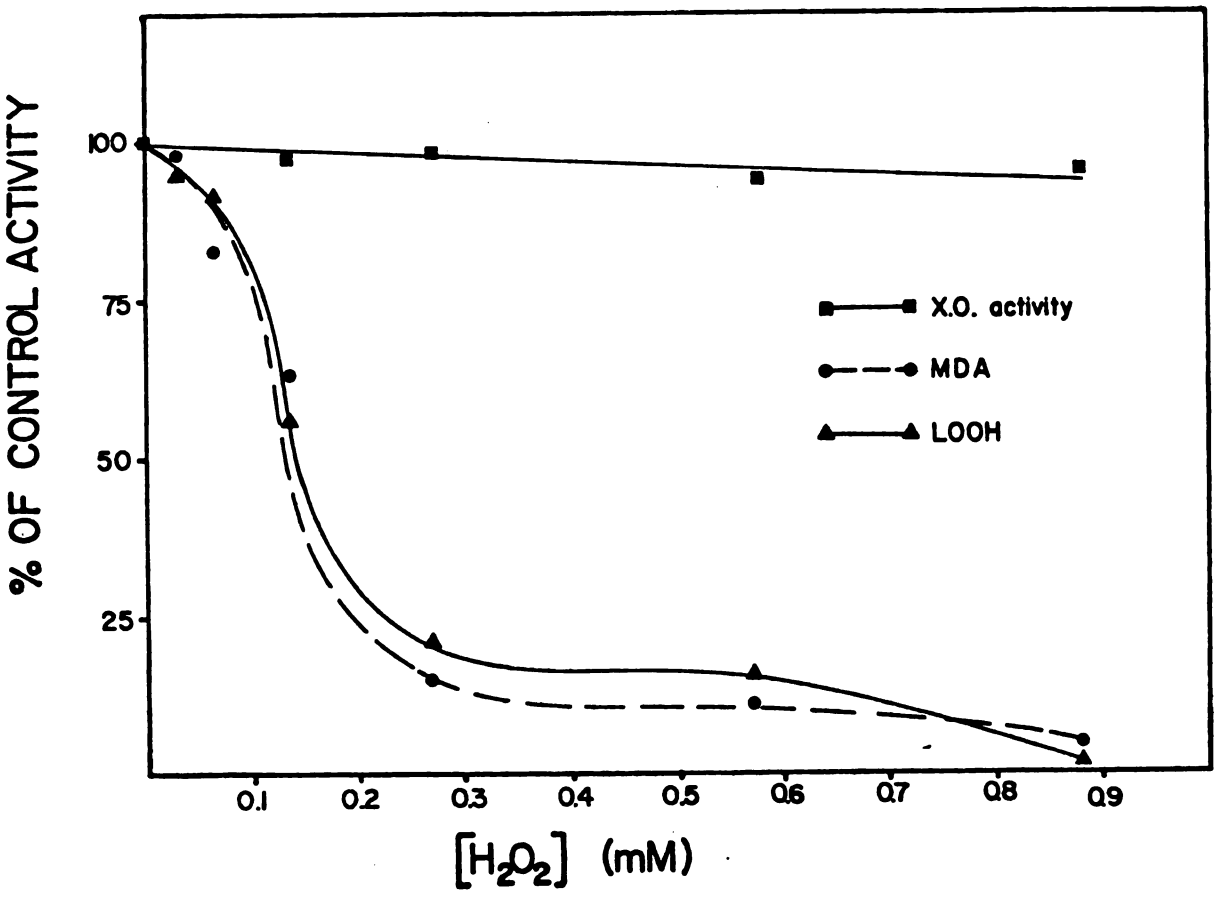


Figure 14. Inhibition of Xanthine Oxidase-Dependent Lipid Peroxidation by H_2O_2. Reaction mixtures contained liposomes (1 µmol lipid phosphate/ml), ADP-Fe^{3+} (1.7 mM ADP, 0.1 mM $FeCl_3$), EDTA-Fe^{3+} (0.11 mM EDTA, 0.1 mM $FeCl_3$), 0.33 mM xanthine, 0.1 unit xanthine oxidase/ml and the specific amount of H_2O_2 in 30 mM NaCl, pH 7.5 at 37°C. Incubation and assay conditions are described under Materials and Methods.

Table 17. Effect of Iron Chelates on Xanthine Oxidase-Dependent Peroxidation of Linoleate.

Additions	ΔA_{234}/min/ml
None	<0.001
ADP-Fe^{3+}	<0.001
EDTA-Fe^{3+}	<0.001
ADP-Fe^{3+}, EDTA-Fe^{3+}	<0.001
Xanthine oxidase	<0.001
Xanthine oxidase, ADP-Fe^{3+}	0.085
Xanthine oxidase, EDTA-Fe^{3+}	0.070
Xanthine oxidase, ADP-Fe^{3+}, EDTA-Fe^{3+}	0.092

Reaction mixtures contained 5.7 mM sodium linoleate, 1% Lubrol, and 35 mM acetaldehyde in 30 mM NaCl, pH 7.5. The following additions were made as indicated: ADP-Fe^{3+} (0.5 mM ADP, 0.1 mM $FeCl_3$), EDTA-Fe^{3+} (0.11 mM EDTA, 0.1 mM $FeCl_3$), and xanthine oxidase (6.6×10^{-3} unit/ml). Diene conjugation was assessed by continuous monitor of increase in 234 nm absorbance. Incubations and assay conditions are described under Materials and Methods.

linoleate as the substrate. The extent of lipid peroxidation was monitored by the increase in absorbance at 234 nm due to conjugated diene formation. The formation of conjugated diene hydroperoxides, and the corresponding increase in absorbance at 234 nm was verified by HPLC analysis of the products. The extracted products of xanthine oxidase-dependent peroxidation of linoleate were shown to have similiar retention times as linoleate hydroproxides generated by soybean lipoxidase (not shown). Due to the intense unltraviolet absorbance of xanthine and ADP, modifications in the experimental conditions were necessary to monitor diene conjugation. Acetaldehyde was utilized as the substrate for

xanthine oxidase and the ADP concentration of ADP-Fe^{3+} solutions was lowered from 1.7 mM to 0.5 mM. Further studies with xanthine oxidase-dependent peroxidation of liposomes demonstrated that this change in ADP concentration did not affect the rate of peroxidation (not shown).

Table 17 shows the effect of different iron chelates on the peroxidation of detergent-dispersed linoleate. Again, no activity was observed in the absence of added iron. In contrast to the results with liposomes, the addition of EDTA-Fe^{3+} resulted in peroxidation of detergent-dispersed linoleate (ΔA_{234}=0.070/min/ml). The rate of diene conjugation was slightly higher in the presence of ADP-Fe^{3+} (ΔA_{234}= 0.085/min/ml) while the addition of both iron complexes resulted in maximal rates of lipid peroxidation (ΔA_{234}=0.092/min/ml). The effects of SOD, catalase, and various •OH trapping agents on xanthine oxidase dependent lipid peroxidation were studied in the presence of EDTA-Fe^{3+}, ADP-Fe^{3+}, and EDTA-Fe^{3+} plus ADP-Fe^{3+}. The results are summarized in Table 18. It is clear that SOD (10 units/ml) was a potent inhibitor of lipid peroxidation in all three systems. Essentially no inhibition was observed with catalase (1, 5, and 10 unit/ml), mannitol (10 and 50 mM), or ethanol (10 and 50 mM) on lipid peroxidation in the presence of ADP-Fe^{3+} while significant inhibition occurred in the presence of EDTA-Fe^{3+}. These agents caused only modest inhibition of lipid peroxidation in the presence of both iron complexes.

Table 18. Xanthine Oxidase-Dependent Peroxidation of Linoleate.

Additions	EDTA-Fe^{3+}	ADP-Fe^{3+}	EDTA-Fe^{3+}, ADP-Fe^{3+}
		% Inhibition	
None	---(0.070)	---(0.085)	---(0.092)
10 unit/ml SOD	91%	90%	83%
1 unit/ml catalase	20%	3%	0%
5 unit/ml catalase	37%	3%	3%
10 unit/ml catalase	40%	0%	4%
10 mM mannitol	19%	0%	8%
50 mM mannitol	31%	0%	9%
10 mm ethanol	26%	1%	4%
50 mM ethanol	37%	2%	9%

The effect of SOD catalase and various •OH trapping agents were studied on lipid peroxidation in the presence of EDTA-Fe^{3+}, ADP-Fe^{3+}, or EDTA-Fe^{3+} plus ADP-Fe^{3+}. The iron chelate concentrations were: EDTA-Fe^{3+} (0.11 mM EDTA, 0.1 mM $FeCl_2$), and ADP-Fe^{3+} (0.5 mM ADP, 0.1 mM $FeCl_3$). All reaction mixtures contained 5.7 mM sodium linoleate, 1% Lubrol, 35 mM acetaldehyde, and 6.6×10^{-3} unit/ml xanthine oxidase in 30 mM NaCl, pH 7.5. Additions were made as indicated. Rates are expressed as the percentage inhibition. The value in parenthesis represent the initial velocities of the reaction expressed in ΔA_{234}/min/ml. Diene conjugation was assessed by continuous monitor of increase in 234 nm absorbance. Incubation and assay conditions are described under Materials and Methods.

DISCUSSION

The results of these experiments demonstrate that peroxidation of liposomes can be initiated by •OH generated by Fenton's reagent. EPR spin trapping techniques with DMPO confirmed the formation of •OH by Fe^{2+} and H_2O_2. The addition of mannitol or benzoate effectively inhibited lipid peroxidation of liposomes initiated by Fenton's reagent. The involvement of H_2O_2 was also illustrated by inhibition of lipid peroxidation by catalase. The rate of lipid peroxidation was highly sensitive to H_2O_2 concentration. This may be due to the dual role of H_2O_2 as a substrate for •OH formation and also as a •OH trap. The rate constant for •OH reacting with H_2O_2 is 4.5×10^7 $M^{-1}s^{-1}$ (Anbar and Netta, 1967).

$$\bullet OH + H_2O_2 \longrightarrow H_2O + O_2^{\bullet -} + H^+$$

Thus at high concentrations, H_2O_2 may be competing with polyunsaturated lipid for the •OH resulting in decreased rates of lipid peroxidation. Ferrous iron has also been demonstrated to be highly reactive with •OH, resulting in a termination reaction (Anbar and Netta, 1967).

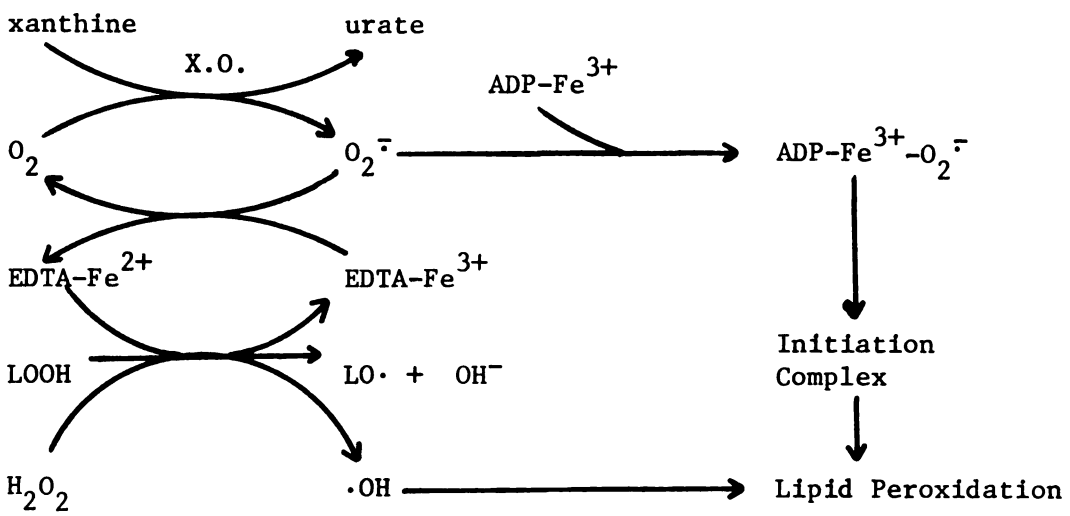
$$Fe^{2+} + \bullet OH \longrightarrow Fe^{3+} + -OH$$

Although this reaction proceeds with a second order rate constant of 3×10^8 $M^{-1}s^{-1}$ (Anbar and Netta, 1967), high Fe^{2+} concentrations did not inhibit lipid peroxidation under the experimental conditions employed. The participation of Fe^{2+} in secondary initiation reactions with LOOH, thus augmenting the rates of lipid peroxidation (Sosnovsky and Rawlinson, 1971), may account for the lack of inhibition observed at high $FeCl_2$ concentrations.

The effect of iron chelation by EDTA or ADP on the Fenton's reagent was examined since both these chelates have been used to study xanthine oxidase-dependent lipid peroxidation (Tyler, 1975; Fong, et al., 1973; Fridovich and Porter, 1981). EDTA chelation of Fe^{3+} has been reported to facilitate catalysis of the Haber-Weiss reaction (Ilan and Czapski, 1977; Halliwell, 1978). Catalysis of the Haber-Weiss reaction involves initial reduction of Fe^{3+} by $O_2^{\cdot-}$ followed by reduction of H_2O_2 by the reduced iron. Our results show that the latter reaction, the Fenton-type reaction, is inhibited by EDTA chelation of Fe^{2+}. Further studies indicated that EDTA chelation resulted in a rapid autoxidation of Fe^{2+}. Thus the inhibition of $\cdot OH$-dependent lipid peroxidation observed by EDTA chelation of Fe^{2+} is most likely the result of a decreased concentration of Fe^{2+} due to autoxidation. These results suggest that the observed enhancement of the iron catalyzed Haber-Weiss reaction by EDTA is due to EDTA facilation of Fe^{3+} reduction by $O_2^{\cdot-}$. The inclusion of ADP in Fenton's reagent caused a slight decrease in the rate of lipid peroxidation. Oxygen consumption experiments indicated that ADP chelation also enhanced the rate of Fe^{2+} autoxidation, however, these rates were much slower than those

observed with EDTA (not shown). Although purine nucleotides have been shown to react with the •OH (Scholes et al., 1960), 10 mM ADP only caused a 10% decrease in activity. In addition, lipid peroxidation initiated by $FeCl_2$ and H_2O_2 in the presence of ADP was less sensitive to mannitol inhibition. These results suggest that ADP chelation of Fe^{2+} results in lipid peroxidation that is not dependent upon the •OH. However, it is possible that ADP reacts with the •OH to yield a radical product that does not react with mannitol but is capable of initiating lipid peroxidation. These possibilities are currently under investigation.

Our results on xanthine oxidase-dependent lipid peroxidation are summarized on the following scheme:



In accord with the report of Lai and Piette (1978), our results indicate that in the presence of EDTA-Fe^{3+}, initiation of xanthine oxidase-dependent lipid peroxidation occurs via the •OH. In a homogenous system of detergent-dispersed linoleate, the EDTA-Fe^{3+}-dependent generation of the •OH resulted in lipid peroxidation as evidenced by diene conjugation. The inhibition of activity by SOD, catalase, mannitol, and ethanol suggest that initiation occurs via the iron catalyzed Haber-Weiss reaction. However, in a heterogenous system of liposomes, the generation of $O_2^{\cdot-}$ and H_2O_2 by the action of xanthine oxidase on xanthine in the presence of EDTA-Fe^{3+} did not result in lipid peroxidation. This difference in activity would suggest a correlation between proximity of •OH generation and its lipid peroxidation initiating activity. In the case of liposomes, •OH generation would probably occur in the aqueous phase. It is unlikely that that the highly reactive •OH would diffuse away from the site of formation to liposomes to initiate lipid peroxidation (Walling, 1975). Dispersion of the lipid with detergent to yield a homogenous phase would bring the lipid in closer proximity to the site of •OH formation, thus resulting in lipid peroxidation.

In contrast to the results with EDTA-Fe^{3+}, •OH participation in xanthine oxidase-dependent lipid peroxidation appears minimal in the presence of ADP-Fe^{3+}. Catalase and •OH trapping agents, at concentrations which caused complete inhibition of Fenton's reagent-dependent peroxidation of liposomes were without effect on xanthine oxidase-dependent peroxidation of liposomes in the presence of ADP-Fe^{3+}. The addition of H_2O_2 to xanthine oxidase-dependent peroxidation of liposomes

actually resulted in decreased rates of peroxidation. In the detergent-dispersed linoleate system, the concentrations of catalase and •OH trapping agents which caused significant inhibition of EDTA-Fe^{3+}-dependent peroxidation were not effective in the presence of ADP-Fe^{3+}. In liposomes and detergent-dispersed linoleate, the addition of both EDTA-Fe^{3+} and ADP-Fe^{3+} resulted in the maximal rate of lipid peroxidation. Again, catalase and •OH trapping agents were without effect. It could be argued that ADP-Fe^{3+} localizes the generation of the •OH closer to the lipid such that water-soluble trapping agents cannot effectively scavenge the •OH. This would suggest an affinity of ADP for the lipid phase. However, preliminary experiments show no evidence for such an affinity (Chapter II). Moreover, detergent dispersion of the lipid phase into a homogenous system did not render ADP-Fe^{3+}-dependent peroxidation more susceptable to catalase or •OH trapping agents.

Our results on xanthine oxidase-dependent lipid peroxidation in the presence of ADP-Fe^{3+} are in agreement with the results of Svingen *et al.* (1978) and Tyler (1975). Svingen and coworkers proposed that initiation of lipid peroxidation occurred via an ADP-iron-oxygen complex. Although we have no direct evidence for such a complex, our results support this possibility. In addition, these workers proposed that enhancement of lipid peroxidation by EDTA-Fe^{3+} is a result of EDTA-Fe^{3+} (upon reduction to EDTA-Fe^{2+} by $O_2^{\cdot-}$) catalyzed decomposition of LOOH resulting in accelerated peroxidation through lipid hydroperoxide- dependent initiation reactions. Although our results and the results of others (Fridovich and Porter, 1981; Lai and Piette, 1978) suggest that the role of EDTA-Fe^{3+} in xanthine oxidase-dependent

lipid peroxidation is catalysis of the Haber-Weiss reaction, it is probable that once LOOH are formed, EDTA-Fe^{2+} acts to reductively cleave these lipid hydroperoxides as it can H_2O_2. This may account for the high rates of lipid peroxidation observed in the presence of both EDTA-Fe^{3+} and ADP-Fe^{3+}.

Fong and coworkers (1973) also studied xanthine oxidase-dependent lipid peroxidation in the presence of ADP-Fe^{3+}. These workers reported that SOD stimulated, while catalase and •OH trapping agent inhibited $O_2^{\cdot-}$-induced lysis of lysosomes. Aside from different methods used for assessing lipid peroxidation, we have no apparent explanation for the discrepancy in results. Our results with a model system of ADP-Fe^{3+} and liposomes, which we would anticipate to have more biological significance than EDTA-Fe^{3+} with detergent-dispersed linoleate indicate $O_2^{\cdot-}$-dependent lipid peroxidation may proceed through the interaction of $O_2^{\cdot-}$ with an iron chelate resulting in the production of an initiating complex. While the exact nature of this complex remains unknown, it is apparent that the generation of free •OH is not required for its lipid peroxidation initiating activity.

CHAPTER IV

INITIATION OF LIPID PEROXIDATION BY IRON CHELATES

ABSTRACT

Initiation of enzyme catalyzed lipid peroxidation has shown an absolute requirement for iron. That iron must be first reduced to initiate lipid peroxidation has been proposed by numerous investigators. The chelation of iron can alter the mechanism of initiation and its lipid peroxidation initiating activity. Two forms of iron frequently used for catalysis of enzymatic lipid peroxidation are EDTA-Fe^{3+} and ADP-Fe^{3+}. In order to investigate the mechanism by which these two iron chelates catalyze initiation reactions, lipid peroxidation was initiated by the direct addition of ADP-chelated and EDTA-chelated iron (ferrous and ferric forms) to liposomes made from extracted microsomal lipid. The addition of EDTA-Fe^{2+} (0.11 mM EDTA, 0.1 mM $FeCl_2$) to peroxide-free liposomes did not result in lipid peroxidation. The addition of ADP-Fe^{2+} (1.7 mM ADP, 0.1 mM $FeCl_2$) did result in lipid peroxidation, but only after a lag period. The lag period was not affected by catalase (1 unit/ml), mannitol (10 mM), or by superoxide dismutase (1 unit/ml), but was shortened in a concentration-dependent manner by H_2O_2. This would suggest involvement of the hydroxyl radical, however, peroxidation in the presence of added H_2O_2 was not inhibited by hydroxyl radical trapping agents. The addition of ADP-Fe^{3+} also shortened the lag period. Catalase, mannitol or superoxide dismutase were not effective inhibitors in the presence of added ADP-Fe^{3+}. Peroxidation of peroxide-free liposomes could also be initiated by the

addition of EDTA-Fe^{2+} with ADP-Fe^{3+}. Since the addition of either EDTA-Fe^{2+} or ADP-Fe^{3+} alone did not initiate lipid peroxidation, an interaction between these two iron chelates in initiating lipid peroxidation is proposed. Evidence is presented for superoxide involvement in this reaction. In contrast to peroxide-free liposomes, the addition of EDTA-Fe^{2+} readily initiated lipid peroxidation in partially-peroxidized liposomes. The addition of ADP-Fe^{2+} caused low rates of peroxidation while the addition of unchelated Fe^{2+} resulted in the highest rate of peroxidation. These results suggest that initiation in the presence of ADP-chelated iron is not dependent on the free hydroxyl radical but on an ADP-iron-oxygen complex. In addition, EDTA-Fe^{2+} augmentation of lipid peroxidation is proposed to occur by two mechanisms: interaction with ADP-Fe^{3+} to form an initiation complex in peroxide-free liposomes and by reacting with lipid hydroperoxides to yield free radical products. The significance of these results to enzyme catalyzed lipid peroxidation is discussed.

INTRODUCTION

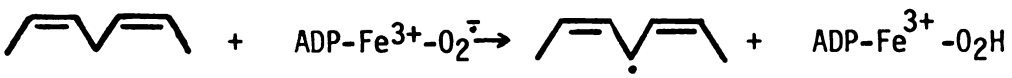
Initiation of lipid peroxidation by enzymatic catalysis has been observed with xanthine oxidase (King et al., 1975; Pederson and Aust, 1973) and NADPH-cytochrome P450 reductase (Pederson et al., 1973). The ability to catalyze the passage of electrons from a two-electron donor to a one-electron acceptor imparts these enzymes with their lipid peroxidation initiating activity. A large number of studies have shown an absolute requirement for iron to initiate xanthine oxidase catalyzed lipid peroxidation (Fong et al., 1973; Svingen and Aust, 1980; Lai and Piette, 1978) and NADPH-cytochrome P450 reductase catalyzed lipid peroxidation (Beloff-Chain et al., 1963; Hochstein et al., 1964; Wills, 1969a; Svingen et al., 1979). That iron must be first reduced to participate in lipid peroxidation is implicit in essentially all currently proposed mechanisms of xanthine oxidase catalyzed lipid peroxidation (Fong et al., 1973; Fridovich and Porter, 1981; Lai and Piette, 1978; Svingen and Aust, 1980) and NADPH-cytochrome P450 reductase catalyzed lipid peroxidation (Beloff-Chain et al., 1965; Svingen et al., 1979; Kameda et al., 1979). In the case of xanthine oxidase, dioxygen is reduced to superoxide ($O_2^{\cdot-}$) which in turn can reduce chelated iron (Ilan and Czapski, 1977) while NADPH-cytochrome P450 reductase can directly reduce EDTA-chelated iron (Kamin and Masters, 1968).

The mechanism of initiation is highly sensitive to the chelation of the iron. Lipid peroxidation in the presence of EDTA-Fe^{3+} appears to

proceed by an hydroxyl radical (•OH) mechanism (Chapter II and III). In this case, reduced iron is required for reductive cleavage of hydrogen peroxide to generate the •OH (Hardwick, 1957). However, the participation of the •OH appears to be minimal in lipid peroxidation systems with ADP-Fe^{3+} (Chapters II and III). An alternate mechanism for initiation of lipid peroxidation involving an iron-oxygen complex was first proposed by Hochstein and Ernster (1963) for microsomal NADPH-dependent lipid peroxidation. These workers proposed that reduction of ADP-Fe^{3+} followed by complexation with dioxygen would generate the ADP-perferryl ion. The ADP-perferryl ion can also be generated by $O_2^{\bullet-}$ complexing with ADP-Fe^{3+} as proposed by Svingen and Aust (1980) for xanthine oxidase catalyzed lipid peroxidation.

$$\text{ADP-Fe}^{2+} + O_2 \longrightarrow [\text{ADP-Fe}^{2+}\text{-}O_2] \longleftrightarrow [\text{ADP-Fe}^{3+}\text{-}O_2^{\bullet-}] \longleftarrow \text{ADP-Fe}^{3+} + O_2^{\bullet-}$$

Hochstein and Ernster (1963) proposed that the ADP-perferryl ion initiated lipid peroxidation by abstracting methylene hydrogens of polyunsaturated fatty acids analogous to the mechanism proposed for plant lipoxidase (Bergstron and Holman, 1948).



The carbon-hydrogen bond dissociation energy for the methylene hydrogen is 85 kcal/mole (Mead, 1976). The energy gained from the formation of the oxygen-hydrogen bond would be 47 kcal/mole (George, 1965). Assuming that the effect of Fe^{3+} on the oxygen-hydrogen bond energy is

minimal, this reaction would be extremely endothermic. Other reactions by which the perferryl ion can initiate lipid peroxidation have been proposed (Ingold, 1962).

$$Fe^{3+}\text{-}O_2^{\cdot-} + LH \longrightarrow Fe^{2+} + {\cdot}OH + LO\text{-}$$

$$Fe^{3+}\text{-}O_2^{\cdot-} + LH \longrightarrow Fe^{3+} + \text{-}OH + LO{\cdot}$$

However, evidence for the existence of these complexes and their reactivities would be useful. The biological analogies of the perferryl ion such as oxy-hemoglobin or oxy-myoglobin are not known to be strong oxidants. Yet the addition of ADP-Fe^{2+} to phospholipid liposomes resulted in low levels of lipid hydroperoxide and malondialdehyde (MDA) formation (Svingen et al., 1978). The addition of EDTA-Fe^{2+} to liposomes did not result in lipid peroxidation, presumably because it could not form the perferryl ion.

The objective of this study is to further examine the mechanism of lipid peroxidation initiated by chelates of ferrous ion. The effects of iron (ferrous and ferric) chelation by EDTA and ADP on lipid peroxidation of peroxide-free and hydroperoxide-containing lipid are characterized. The effect of superoxide dismutase, catalase and ·OH trapping agents on these lipid peroxidation systems is investigated to assess whether $O_2^{\cdot-}$, hydrogen peroxide or ·OH are involved in the mechanism.

MATERIALS AND METHODS

Materials. Cytochrome c (Type VI), thiobarbituric acid, ADP, butylated hydroxytoluene, sodium deoxycholate, and mannitol were obtained from Sigma Chemical Company. All buffers were passed through Chelex 100 (Bio-Rad Laboratories) ion exchange resin to free them of contaminants. All other reagents were analytical grade and used without further purification.

Enzymes and Microsomal Lipid. Superoxide dismutase (SOD), soybean lipoxygenase (Type I), and catalase were a product of Sigma. Further purification of catalase and activity determinations of SOD and catalase were performed as described in Chapters II and III. Microsomal lipid was extracted and liposomes were made as described in Chapters II and III. Lipid phosphate determinations were performed by the method of Barlett (1959).

Preparation of Lipid Hydroperoxides in Liposomes. Liposomal lipid hydroperoxides were generated by the action of soybean lipoxygenase. The reaction mixtures contained 4 μmol lipid phosphate/ml, 0.1 mg lipoxygenase/ml, 0.1 mM EDTA, and 0.3% sodium deoxycholate (w/v) in 20 ml of oxygen purged 0.05 M borate buffer, pH 9.0. Reactions were carried out in a 250 ml erlenmyer flask with the head space purged with oxygen. At the end of a 20 minute incubation period, total lipid was extracted (Folch et al., 1957) and stored in argon-purged $CHCl_3/CH_3OH$ (2:1) at -20°C. The lipid hydroperoxide content was determined by KI titration

(Buege and Aust, 1978). These lipid hydroperoxides were mixed with unperoxidized phospholipids to make liposomes for the study of lipid hydroperoxide-dependent lipid peroxidation.

Reaction Mixtures. Lipid hydroperoxide-dependent lipid peroxidation reaction mixtures contained 0.2 μmol lipid hydroperoxides/ml and 1 μmol lipid phosphate/ml in 30 mM NaCl, pH 7.5. Other additions are as specified in the table legend. Reactions were initiated by the addition of iron as specified in the table legend. Lipid peroxidation of peroxide-free lipid was accomplished by the addition of the ferrous chelates to liposomes (1 μmol lipid phosphate/ml) in 30 mM NaCl, pH 7.5. The concentration of the iron salts ($FeCl_2$ and $FeCl_3$) and iron chelators (EDTA and ADP) are as specified in the figure legends. Incubation conditions and assay conditions are described in Chapters II and III.

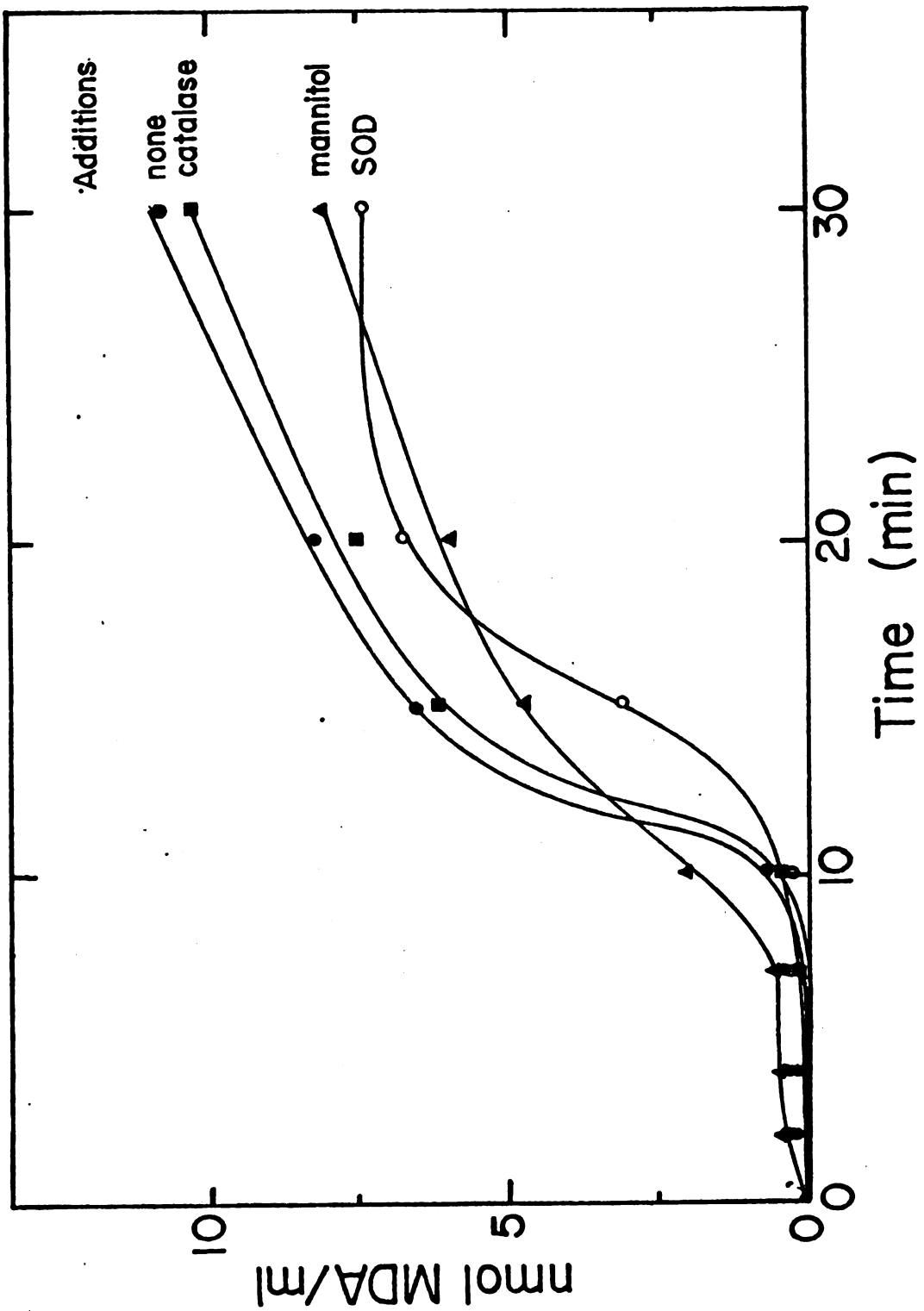
Other Methods. The iron chelate solutions were prepared as described in Chapter III. Oxygen consumption was measured with a YSI Model 53 Oxygen Monitor (Yellow Spring Instruments, Yellow Springs, Ohio).

RESULTS

ADP-Fe^{2+}-Dependent Initiation of Lipid Peroxidation

Peroxidation of liposomes initiated by xanthine oxidase or NADPH-cytochrome P450 reductase both require ADP-Fe^{3+} for activity (Chapters II and III). Based on the premise that iron must be first reduced to participate in lipid peroxidation, we postulated that the initial complex formed in these enzymatic systems is ADP-Fe^{3+}-$O_2^{\cdot-}$ and that the direct addition of ADP-Fe^{2+} to aerated solutions should form the valence isomers of this complex (ADP-Fe^{2+}-O_2). The mechanism by which this complex initiates lipid peroxidation was further investigated with a non-enzymatic model system by which lipid peroxidation was initiated by the direct addition of ADP-Fe^{2+} to liposomes (Figure 15). Contrary to previously published reports (Svingen et al., 1979), initial velocity studies indicated that the addition of ADP-Fe^{2+} to liposomes did not initiate even low levels of lipid peroxidation. However, when the reactions were monitored longer than 10 minutes, the addition of ADP-Fe^{2+} did result in lipid peroxidation. The lag period observed before the onset of peroxidation suggests the formation of a reactive intermediate essential for initiating lipid peroxidation. Catalase (1 unit/ml) did not extend this lag period nor the rate of peroxidation suggesting that H_2O_2 is not involved. Mannitol at a concentration which completely inhibited $\cdot OH$- dependent lipid peroxidation (10 mm) (Chapter III), had no effect on the

Figure 15. Initiation of Lipid Peroxidation by ADP-Fe^{2+}. Reaction mixtures contained ADP-Fe^{2+} (1.7 mM ADP, 0.1 mM $FeCl_2$) and liposomes (1 µmol lipid phosphate/ml) in 30 mM NaCl, pH 7.5 at 37°C. The following additions were made as indicated: catalase (1 unit/ml), mannitol (10 mM), and superoxide dismutase (SOD) (1 unit/ml). Reactions were initiated by the addition of ADP-Fe^{2+}. Incubation and assay conditions are described in Materials and Methods.

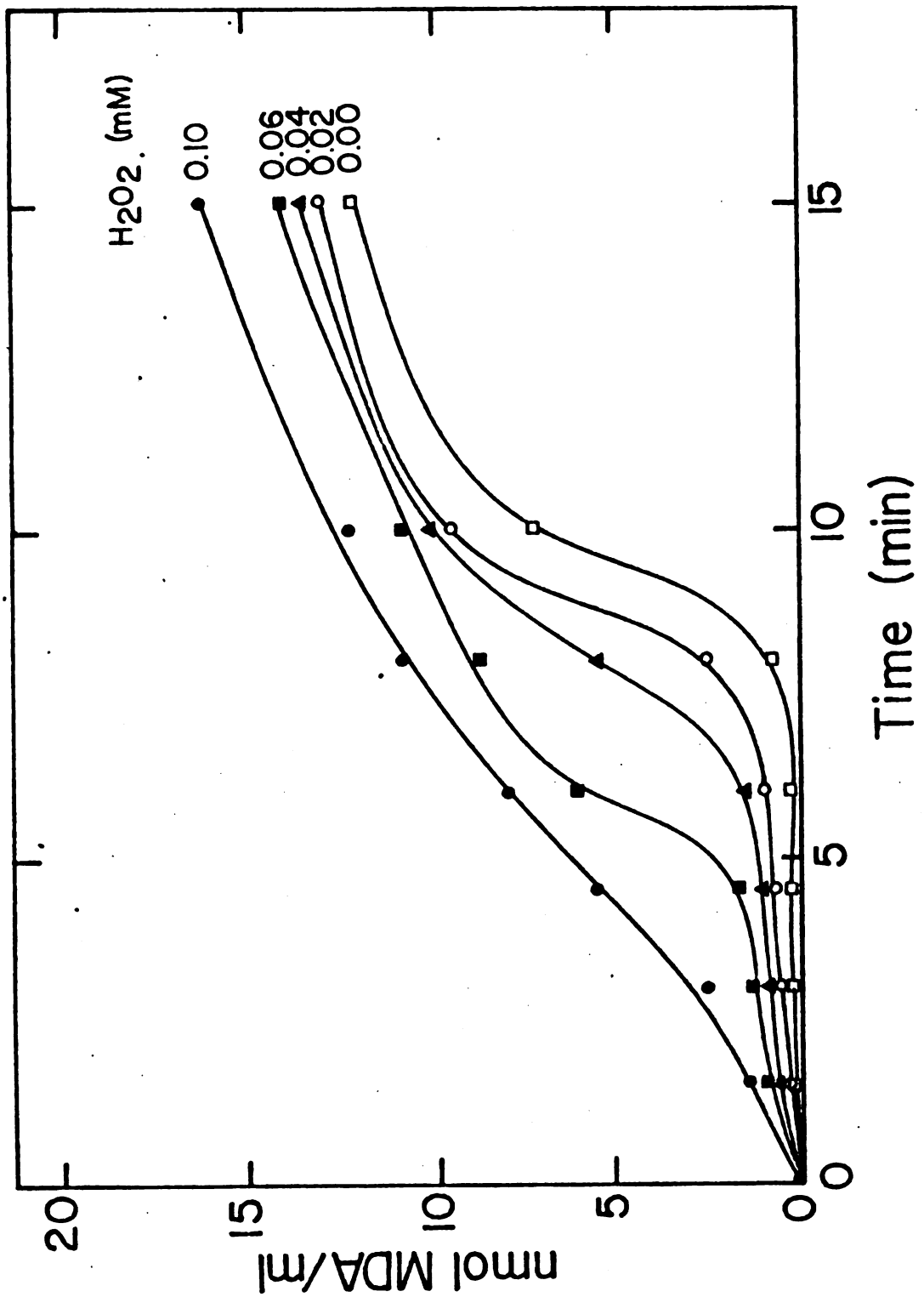

Additions
none
catalase
mannitol
SOD
nmol MDA/ml
0
5
10
0
10
20
30
Time (min)

lag period, however, the MDA content was slightly lower. Some inhibition was observed by the addition of SOD (1 unit/ml), suggesting that $O_2^{\cdot-}$ is involved in the mechanism, yet the lag period was not extended by SOD.

Due to the relative high Km of catalase for H_2O_2 (Sies et al., 1972), it is possible that catalase at the concentration utilized was not sufficient to scavenge all the H_2O_2 produced. Thus the involvement of H_2O_2 was further examined by the ability of added H_2O_2 to shorten the lag period (Figure 16). Increasing the H_2O_2 concentration effectively decreased the length of the lag period. In the absence of H_2O_2, the lag period was approximately 8 minutes. The lag period was shortened by approximately 2 minutes per increase in H_2O_2 concentration of 0.02 mM. At a concentration of 0.1 mM, a near linear increase in MDA formation was observed. This result would suggest that initiation occurred via ·OH formation from a Fenton's reagent of ADP-Fe^{2+} and H_2O_2. This would necessitate that the lag period is associated with H_2O_2 production (via ADP-Fe^{2+} autoxidation) at levels which are not effectively scavenged by catalase, yet capable of initiating lipid peroxidation. Figure 17 illustrates that even in the presence of 0.1 mM H_2O_2, ADP-Fe^{2+}-dependent initiation of lipid peroxidation was not inhibited by mannitol (10 mM).

Aside from H_2O_2, another product of ADP-Fe^{2+} autoxidation would be ADP-Fe^{3+}. As shown in Figure 18, the lag period of ADP-Fe^{2+}-dependent lipid peroxidation was also shortened by the inclusion of ADP-Fe^{3+} in the reaction mixture. The extent of this lag period had an inverse relation to the concentration of ADP-Fe^{3+}. In the presence of 0.1 mM ADP-Fe^{2+}, 0.3 mM ADP-Fe^{3+} totally eliminated the lag period. At an equimolar ratio of ADP-Fe^{2+}

Figure 16. Effect of H_2O_2 on Lipid Peroxidation Initiated by ADP-Fe^{2+}. Reaction mixture contained liposomes (1 μmol lipid phosphate/ml), ADP-Fe^{2+} (1.7 mM ADP, 0.1 mM $FeCl_2$) and H_2O_2 at the concentrations specified in 30 mM NaCl, pH 7.5 at 37°C. Reactions were initiated by the addition of ADP-Fe^{2+}. Incubation and assay conditions are described in Materials and Methods.


nmol MDA/ml
Time (min)
H_2O_2 (mM)
0.10
0.06
0.04
0.02
0.00
0
5
10
15
20

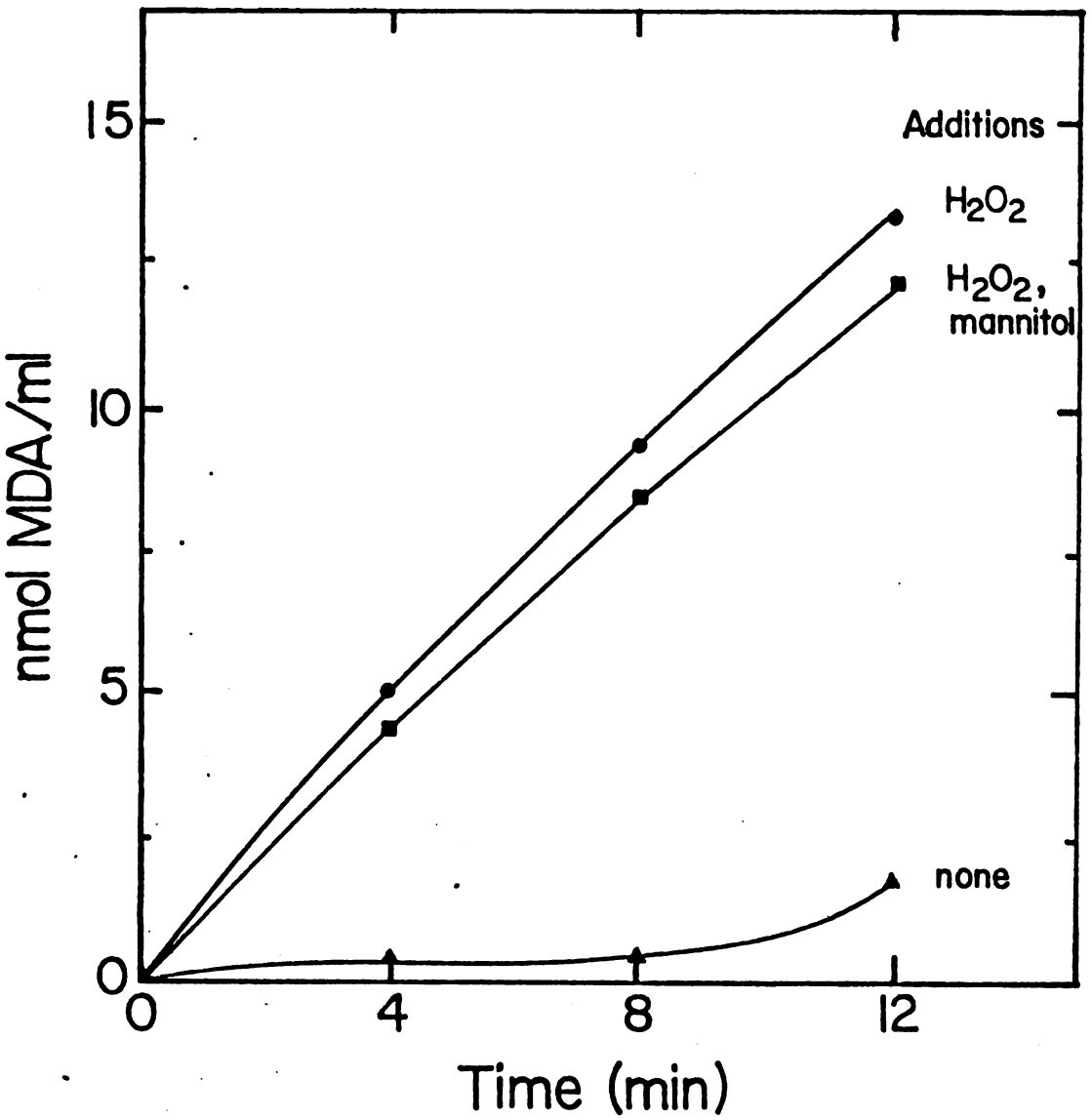
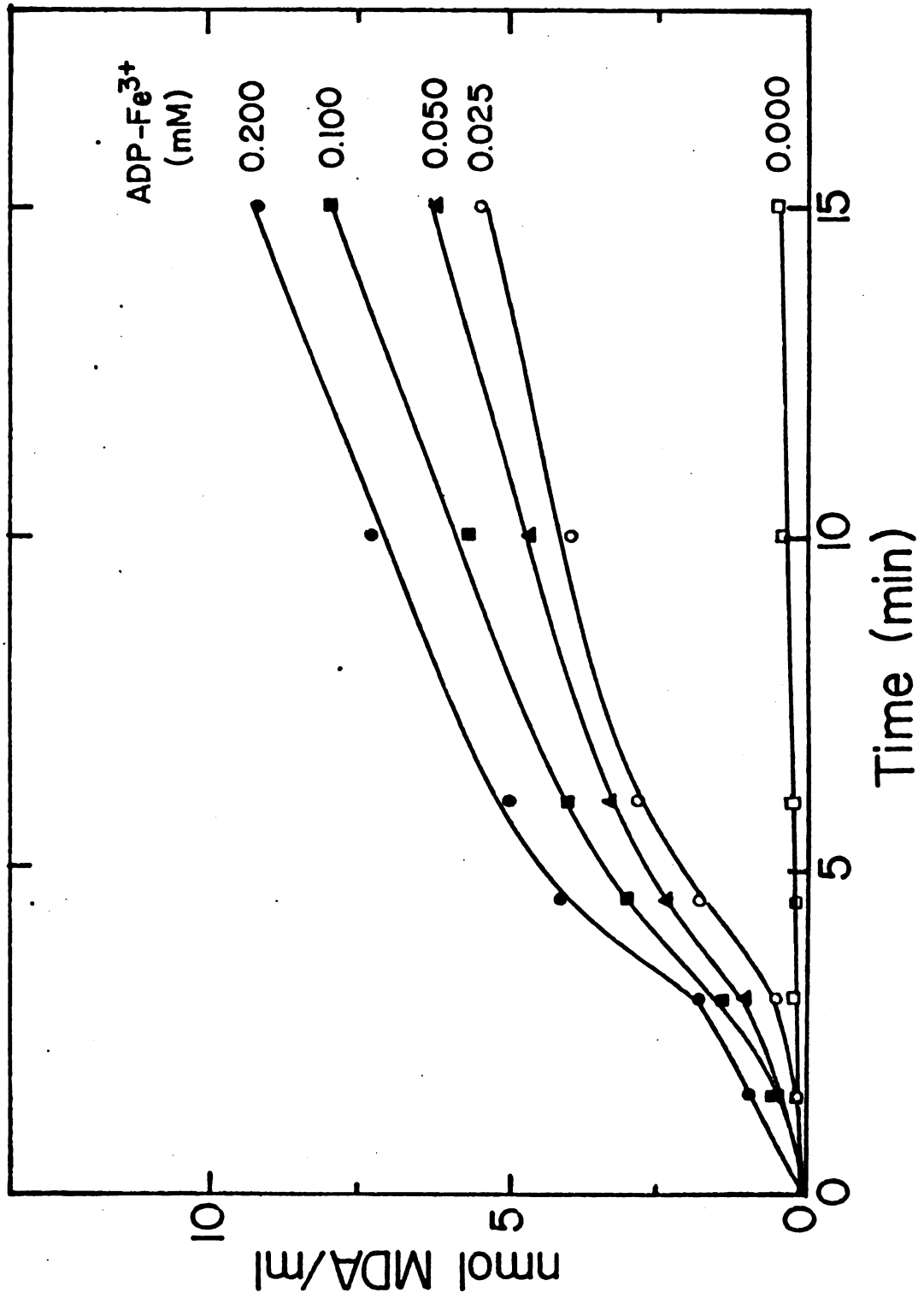


Figure 17. Effect of Mannitol on Lipid Peroxidation Initiated by ADP-Fe^{2+} and H_2O_2. Reaction mixtures contained liposomes (1 μmol lipid phosphate/ml), ADP-Fe^{2+} (1.7 mM ADP, 0.1 mM $FeCl_2$) and H_2O_2 (0.1 mM) and mannitol (10 mM) in 30 mM NaCl, pH 7.5 at 37°C. Reactions were initiated by the addition of ADP-Fe^{2+}. Incubation and assay conditions are described in Materials and Methods.

Figure 18. Effect of ADP-Fe^{3+} on Lipid Peroxidation Initiated by ADP-Fe^{2+}. Reaction mixtures contained liposomes (1 μmol lipid phosphate/ml), ADP-Fe^{2+} (1.7 mM ADP-Fe^{2+}) and the specified ADP-Fe^{3+} concentration in 30 mM NaCl, pH 7.5 at 37°C. ADP-Fe^{3+} concentration are specified as the concentration of $FeCl_3$ (17 ADP to 1 $FeCl_3$ molar ratio). Reactions were incubated by addition of ADP-Fe^{2+}. Incubation and assay conditions are described in Materials and Methods.


nmol MDA/ml
0
5
10
0
5
10
15
Time (min)
ADP-Fe3+
(mM)
0.200
0.100
0.050
0.025
0.000

(0.05 mM) and ADP-Fe^{3+} (0.05 mM), the lag period was approximately 4 minutes (Figure 19). Again, catalase (1 unit/ml) and mannitol (10 mM) had no effect on the lag period or the extent of peroxidation. The addition of SOD (1 unit/ml) was without effect on the lag period, yet the rate of peroxidation was slightly lower after the lag period. These results suggest that ADP-Fe^{2+}-dependent initiation of lipid peroxidation occurs via an additive effect of ADP-Fe^{2+} and ADP-Fe^{3+}.

EDTA-Fe^{2+}-Dependent Initiation of Lipid Peroxidation

The augmentation of lipid peroxidation by EDTA-Fe^{3+}, upon reduction to EDTA-Fe^{2+}, has been proposed to occur via a lipid hydroperoxide-dependent mechanism (Svingen et al., 1979). To further investigate the effect of different iron chelates on lipid hydroperoxide-dependent lipid peroxidation, lipid hydroperoxides were generated by preincubation of liposomes with soybean lipoxygenase. These lipid hydroperoxides were mixed with unperoxidized phospholipids to make liposomes with a known content of lipid hydroperoxides (0.2 μmol LOOH/μmol lipid phosphate) for the studies summarized in Table 19. In agreement with the results of Svingen and coworkers (1979), the addition of EDTA-Fe^{2+} to liposomes in the absence of lipid hydroperoxides did not result in lipid peroxidation (0.24 nmol MDA/min/ml). In the presence of lipid hydroperoxides, the addition of EDTA-Fe^{2+} caused a rapid rate of MDA formation (2.42 nmol/min/ml). ADP-Fe^{2+} (0.1 mM) was less effective in initiating lipid hydroperoxide-dependent lipid peroxidation (0.65 nmol MDA/min/ml). Since these incubations were sampled at 0, 2.5, and 5 minutes for MDA formation, no lipid peroxidation was observed in reaction mixtures containing ADP-Fe^{2+} and unperoxidized liposomes. The rate of lipid hydroperoxide-dependent lipid peroxidation promoted by

Figure 19. Effect of Catalase and Hydroxyl Radical Traps on Lipid Peroxidation Initiated by ADP-Fe^{2+} and ADP-Fe^{3+}. Reaction mixtures contained liposomes (1 μmol lipid phosphate/ml). ADP-Fe^{2+} (0.85 mM ADP, 0.05 mM $FeCl_2$), ADP-Fe^{3+} (0.85 mM ADP, 0.05 $FeCl_3$) in 30 mM NaCl, pH 7.5 at 37°C. The following additions were made as indicated: catalase (1 unit/ml), mannitol (10 mM), and superoxide dismutase (1 unit/ml). Reactions were initiated by addition of ADP-Fe^{2+}. Incubation and assay conditions are described in Materials and Methods.

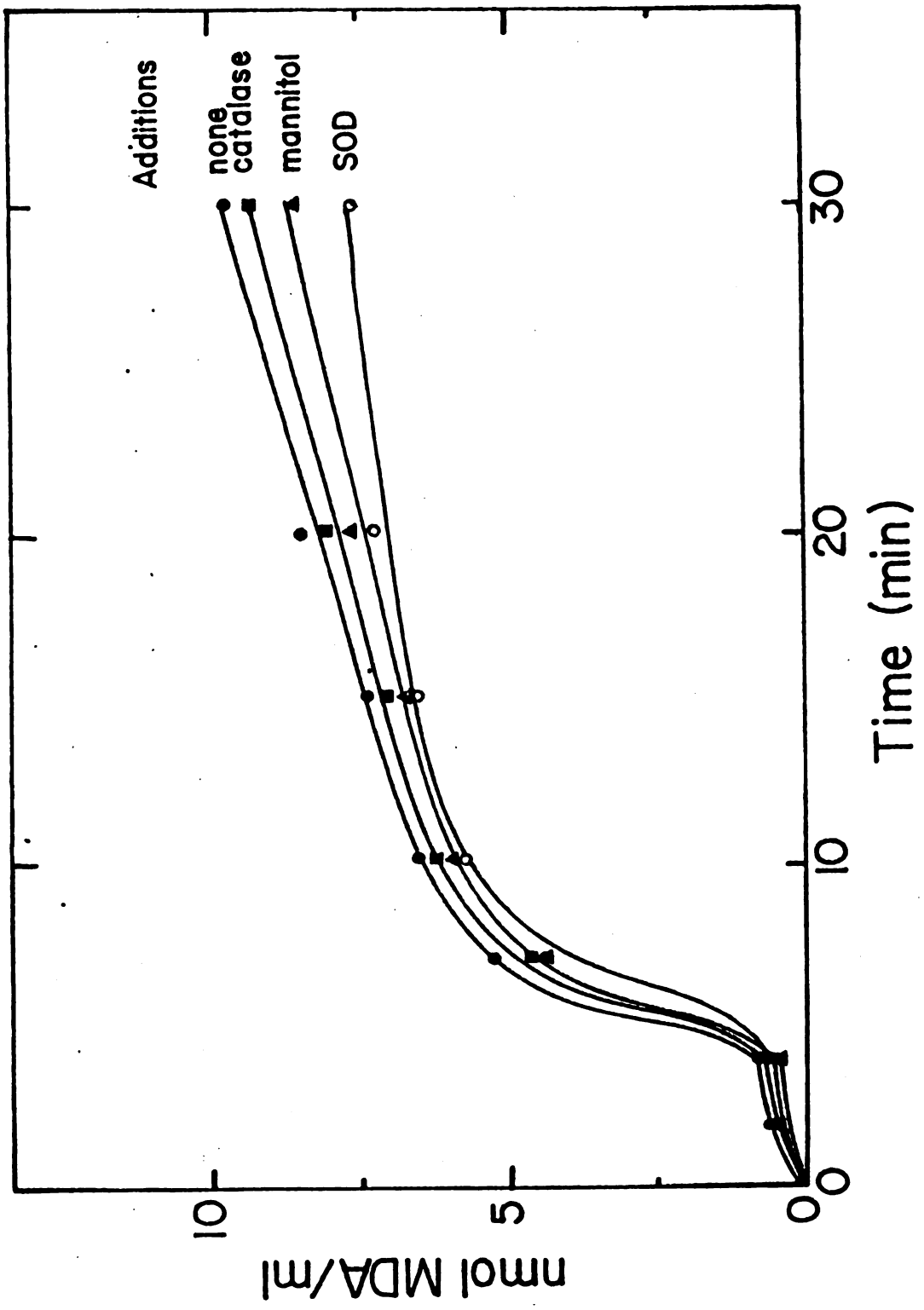

Additions
none
catalase
mannitol
SOD
nmol MDA/ml
0
5
10
0
10
20
30
Time (min)

Table 19. Initiation of Lipid Peroxidation by Iron Chelates

Additions	Partially-Peroxidized Liposomes	Unperoxidized Liposomes
	(nmol MDA/min/ml)	
None	0.13	0.01
ADP-Fe^{2+}	0.65	0.15
EDTA-Fe^{2+}	2.42	0.05
Fe^{2+}	4.98	0.32

Lipid peroxidation initiated by iron chelates was studied in partially-peroxidized liposomes, (1 μmole lipid hydroperoxide/ml, 1 μmole lipid phosphate/ml) and unperoxidized liposomes (1 μmole lipid phosphate/ml). Reaction mixture contained the liposome indicated in 30 mM NaCl, pH 7.5 at 37°C. The following additions were made as indicated: ADP-Fe^{2+} (1.7 mM ADP, 0.1 mM $FeCl_2$), EDTA-Fe^{2+} (0.11 mM EDTA, 0.1 mM $FeCl_2$) and Fe^{2+} (0.1 mM $FeCl_2$). Reactions were initiated by the addition of iron. Incubation and assay conditions are described in Materials and Methods.

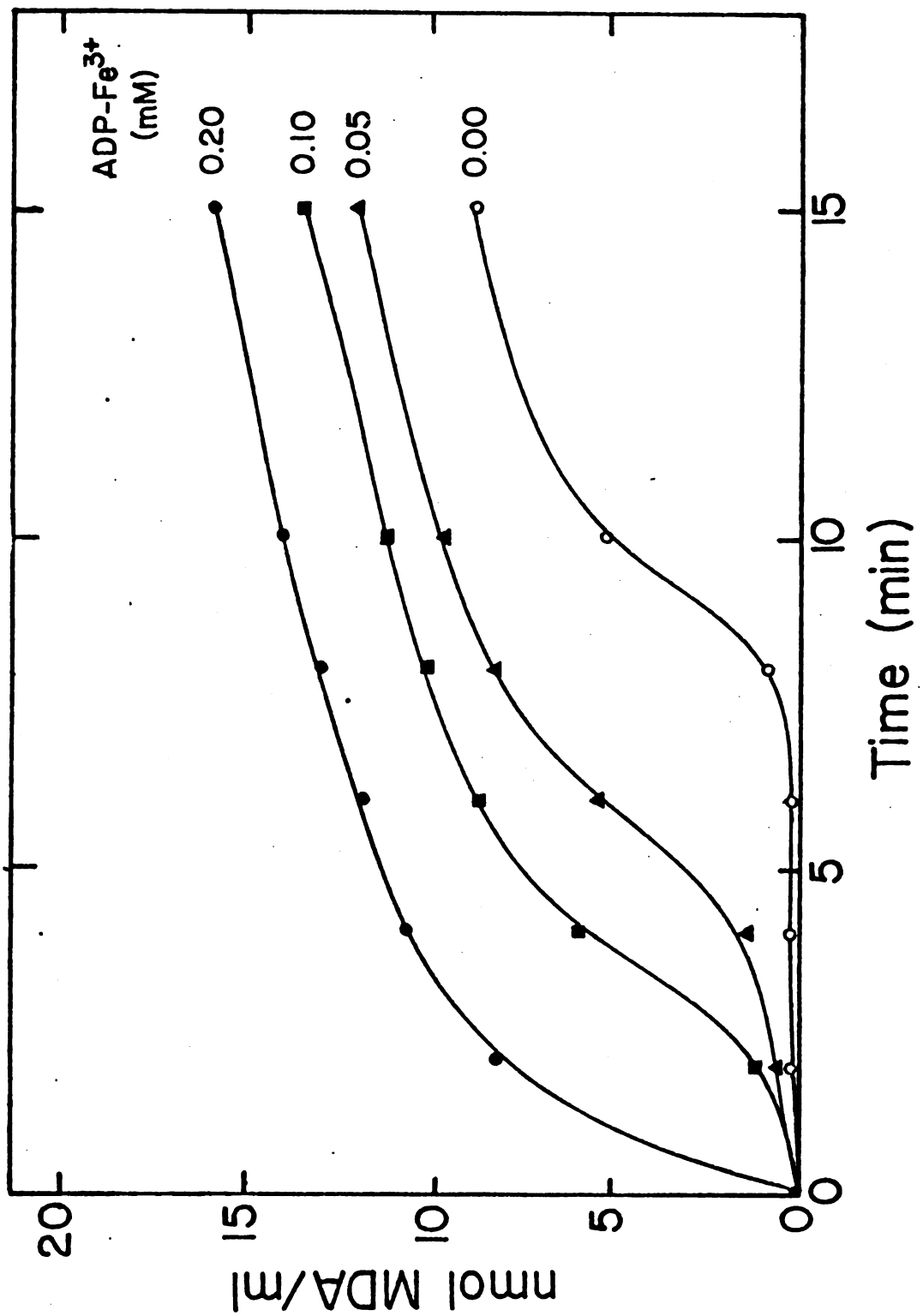
unchelated Fe^{2+} (4.98 nmol MDA/min/ml) was much greater than that observed with EDTA-Fe^{2+}. In unperoxidized liposomes, unchelated Fe^{2+} (0.1 mM) caused a modest increase in MDA content (0.32 nmol/min/ml). These results suggest that chelation of Fe^{2+} by EDTA does not impart any special characteristics to Fe^{2+} in promoting lipid hydroperoxide-dependent lipid peroxidation.

The results with NADPH-cytochrome P450 reductase-dependent lipid peroxidation indicate that chelation of Fe^{3+} by EDTA facilitates the reduction of Fe^{3+} (Chapter II). Reduction of Fe^{3+} was not facilitated by chelation with ADP. Since the initiation of lipid peroxidation in liposomes is dependent on ADP-Fe^{2+}, this would suggest a catalytic role for EDTA-Fe^{3+} in the passage of electrons from the reduced reductase to ADP-Fe^{3+}. This possibility was

examined by initiating lipid peroxidation with both ADP-Fe^{3+} and EDTA-Fe^{2+} (Figure 20). In the absence of ADP-Fe^{3+}, EDTA-Fe^{2+} did not initiate lipid peroxidation. However, increased concentrations of ADP-Fe^{3+} caused increased rates of lipid peroxidation. No activity was observed in reaction mixtures with ADP-Fe^{3+} in the absence of EDTA-Fe^{2+} (not shown).

As shown in Figure 21, EDTA chelation of Fe^{2+} resulted in rapid autoxidation of Fe^{2+}. This was demonstrated by the rapid uptake of oxygen upon the addition of EDTA to solutions of $FeCl_2$ (0.2 mM). Although ADP chelation of Fe^{2+} also facilitated oxygen uptake, the rates were much lower. Since Fe^{2+} is a one-electron reductant, it may be anticipated that $O_2^{\cdot-}$ is formed by EDTA-Fe^{2+} autoxidation. Complexation of $O_2^{\cdot-}$ with ADP-Fe^{3+} would result in the formation of an initiating complex or a precursor to an initiating complex. If $O_2^{\cdot-}$ is involved in lipid peroxidation initiated by EDTA-Fe^{2+} and ADP-Fe^{3+}, then SOD should cause inhibition of lipid peroxidation by scavenging $O_2^{\cdot-}$. Figure 22 shows that increased concentrations of SOD of 1, 5, and 10 units/ml caused a 31%, 35%, and 42% decrease, respectively, in the MDA content at 15 minutes. Boiled SOD caused modest inhibition of lipid peroxidation, but the inhibition was less than that of unboiled enzyme, indicating that some of the inhibition is non-specific.

Figure 20. Lipid Peroxidation Initiated by EDTA-Fe^{2+} and ADP-Fe^{3+}. Reaction mixtures contained liposomes (1 μmol lipid phosphate/ml), EDTA-Fe^{2+} (1.1 mM EDTA, 0.1 mM $FeCl_2$) and the specified ADP-Fe^{3+} concentration. ADP-Fe^{3+} concentration is specified as in Fig. 18. Reactions were initiated by addition of EDTA-Fe^{2+}. Incubation and assay conditions are described in Materials and Methods.


ADP-Fe3+
(mM)
0.20
0.10
0.05
0.00
nmol MDA/ml
Time (min)
0
5
10
15
20

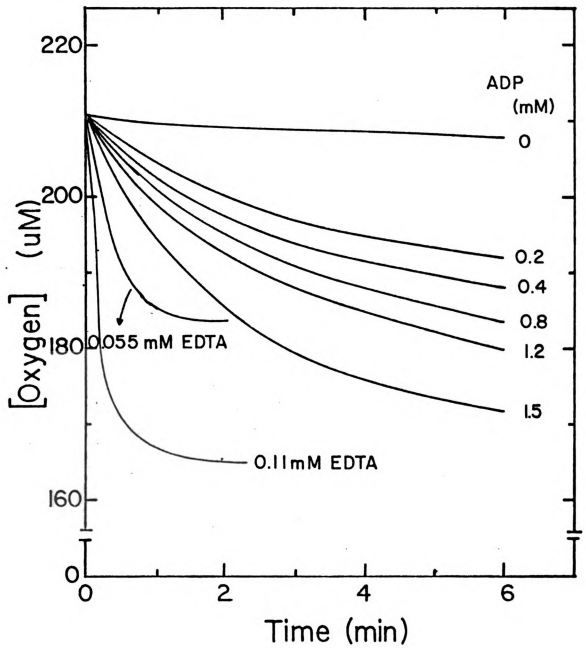


Figure 21. Effect of Chelation on Fe^{2+} Autoxidation. The autoxidation of Fe^{2+} was assessed by measuring the corresponding decrease in oxygen concentration. Decrease in oxygen concentration was measured with a YSI Model 531 Oxygen Monitor. Reaction mixtures contained 0.1 mM $FeCl_2$ in 30 mM NaCl, pH 7.5 at 37°C. The iron chelators (ADP or EDTA) were added at the concentrations indicated.

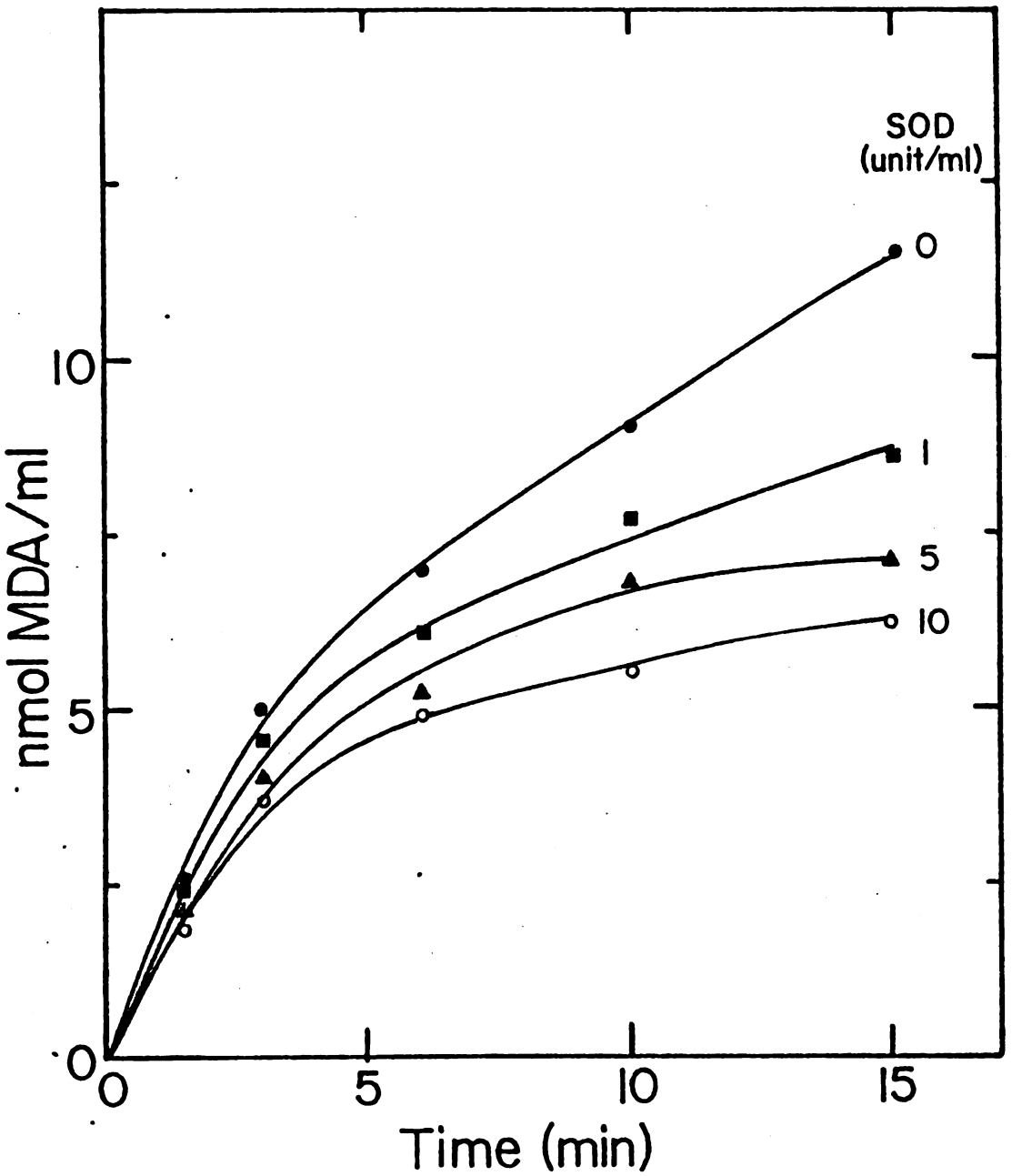


Figure 22. Effect of Superoxide Dismutase on Lipid Peroxidation Initiated by EDTA-Fe^{2+} and ADP-Fe^{3+}. Reaction mixtures contained liposomes, EDTA-Fe^{2+} (0.11 mM EDTA, 0.1 mM $FeCl_2$), and ADP-Fe^{3+} (1.7 mM ADP, 0.1 mM $FeCl_3$) in 30 mM NaCl, pH 7.5 at 37°C. Boiled (open circles) and unboiled (closed circles) superoxide dismutase (SOD) were added at the concentrations specified. Reactions were initiated by the addition of EDTA-Fe^{2+}. Incubation and assay conditions are described in Materials and Methods.

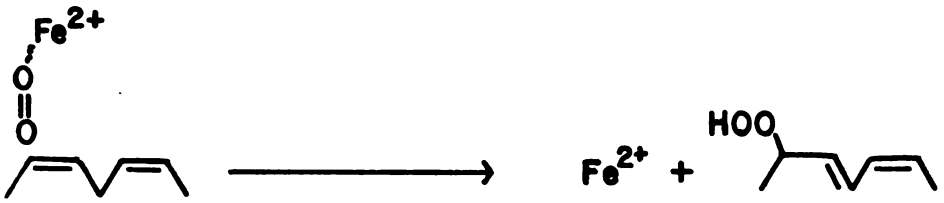
DISCUSSION

Recent studies have proposed that initiation of xanthine oxidase-dependent lipid peroxidation (Fridovich and Porter, 1980) and NADPH-cytochrome P450 reductase-dependent lipid peroxidation (Kameda et al., 1979) occur via •OH formation by an iron-catalyzed Haber-Weiss reaction. Yet the findings of numerous other studies are in discord with this proposal (Svingen and Aust, 1980; Tyler, 1975). Previous work concerning NADPH-cytochrome P450 reductase-dependent and xanthine oxidase-dependent lipid peroxidation has demonstrated that ADP-Fe^{3+} and EDTA-Fe^{3+} are required for maximal activity (Svingen and Aust, 1980). Svingen and coworkers (1978) added various iron chelates to unperoxidized liposomes and found that the addition of ADP-Fe^{2+} resulted in MDA and lipid hydroperoxide formation while EDTA-Fe^{2+} was without effect. However, in liposomes containing lipid hydroperoxides, EDTA-Fe^{2+} was much more effective than ADP-Fe^{2+} in initiating lipid peroxidation. Thus these workers proposed that initiation of lipid peroxidation occurred through the formation of the ADP-perferryl ion, formed by ADP-Fe^{2+} complexing with dioxygen. EDTA-Fe^{2+} was proposed to augment lipid peroxidation by catalyzing the decomposition of lipid hydroperoxides. We have reconfirmed the lipid peroxidation initiating activity of ADP-Fe^{2+} in unperoxidized liposomes. However our results show a lag period in ADP-Fe^{2+}-dependent initiation of lipid peroxidation suggesting that the ADP-perferryl ion is not the initiating species. The lag period

would suggest that the ADP-perferryl ion is a precursor to a more reactive species which initiates lipid peroxidation. The extent of peroxidation and the lag period were not affected by catalase or mannitol. SOD decreased the extent of peroxidation but had no effect on the lag period.

The ability of the ADP-perferryl ion to initiate lipid peroxidation has been attributed to the high oxidation state of the iron (Aust and Svingen, 1981). The formal charge of the iron is +6. However, the formal charge of an atom does not always accurately reflect the reactivity of that atom. Iron (II)-dioxygen complexes are not known to be strong oxidants. The charge of the iron is most likely a resonance between iron (II) and iron (III) ($Fe^{2+}O_2 \longleftrightarrow Fe^{3+}O_2^{\cdot-}$). If iron (VI) existed in such a complex, analogous complexes such as oxy-hemoglobin would reduce dioxygen to water resulting in the formation of a strong oxidant. It is doubtful that oxidation of Fe^{2+} by dioxygen would proceed past one-electron forming Fe^{3+} and $O_2^{\cdot-}$. The next oxidation would entail oxidation of Fe^{3+} by $O_2^{\cdot-}$. The redox couple of $O_2^{\cdot-}/H_2O_2$ is 0.94 v (Koppenol, 1978), much less than the expected redox couple of Fe^{3+}/Fe^{4+}. Another possible mechanism by which ADP-perferryl ion could initiate lipid peroxidation is analogous to the mechanism of dioxygenases. Dioxygenases activate dioxygen such that the spin restriction of singlet organic molecules reacting with triplet dioxygen is removed. The proposed mechanism by which dioxygenases remove this spin barrier is through overlapping the unpaired electrons in the p orbital of dioxygen with the unpaired electrons of the d orbital in Fe^{2+} (Hamilton, 1969). It is therefore spin-allowed for such a complex to react with singlet organic compounds by an ionic (nonradical) mechanism to give a

singlet oxidized product. The enzymes pyrocatechase and tryptophan pyrrolase have been proposed to proceed by this type of mechanism (Hamilton, 1974). This would suggest that for ADP-$Fe^{2+}O_2$ to initiate lipid peroxidation, it must proceed through a nonradical mechanism as shown below.



Reductive cleavage of the lipid hydroperoxide would result in free radical formation. This mechanism appears doubtful since the entropy of activation of such a mechanism would be high. In contrast to the active site of an enzyme where the specific binding of substrate and dioxygen would bring the three reactants in close proximity, initiation of lipid peroxidation would require formation of a collisional complex between free Fe^{2+}, dioxygen, and fatty acid. Moreover, ADP-Fe^{3+}-dependent initiation of lipid peroxidation is inhibited by antioxidants, suggesting a free radical mechanism (Svingen and Aust, 1980).

The lag period of ADP-Fe^{2+}-dependent lipid peroxidation was shortened and the extent of peroxidation was increased by the addition of either H_2O_2 or ADP-Fe^{3+} to the reaction mixture. The results obtained with H_2O_2 would suggest a •OH mechanism, yet 10 mM mannitol was without effect. One possibility is that the free •OH is not formed and that the ferryl ion (FeO^{2+}), which may be equated with the •OH (Walling, 1975) is formed.

$$ADP\text{-}Fe^{2+} + H_2O_2 \longrightarrow ADP\text{-}FeO^{2+} + H_2O$$

$$ADP\text{-}FeO^{2+} + H^+ \longrightarrow ADP\text{-}Fe^{3+} + \cdot OH$$

Perhaps chelation of iron with ADP favors the ferryl ion as opposed to dissociation to the free •OH. If the ferryl ion is not reactive with mannitol but capable of initiating lipid peroxidation, this would result in a mechanism appearing not to be dependent of the •OH. Another possible explanation is that the •OH is formed, but does not diffuse away from the site of its formation to initiate lipid peroxidation.

It follows that the oxidation of $ADP\text{-}Fe^{2+}$ by the added H_2O_2 would also bring about an increase in the $ADP\text{-}Fe^{3+}$ concentration. For this reason, $ADP\text{-}Fe^{3+}$ was added to the reaction mixture and a concentration-dependent decrease in the lag period was observed. The ability of $ADP\text{-}Fe^{3+}$ to shorten the lag period in a concentration-dependent manner similiar to H_2O_2 is additional evidence for a mechanism not dependent on the free •OH. It may be envisioned that the enhancement of $ADP\text{-}Fe^{2+}$-dependent lipid peroxidation by H_2O_2 is not due to •OH formation but rather due to $ADP\text{-}Fe^{3+}$ formation. This would suggest that lipid peroxidation is dependent on the ferrous-dioxygen-ferric complex.

$$ADP\text{-}Fe^{3+} + ADP\text{-}Fe^{2+}O_2 \longrightarrow ADP\text{-}Fe^{3+}O_2Fe^{2+}ADP$$

$$ADP\text{-}Fe^{3+}O_2Fe^{2+}ADP + 2\,H^+ \longrightarrow ADP\text{-}Fe^{3+} + H_2O + ADP\text{-}FeO^{3+}$$

Dissociation of this complex to yield $ADP\text{-}FeO^{3+}$ results in the formation of a strong oxidant analogous to the oxenoid complex proposed for cytochrome P450 (Hamilton, 1974). Catalase and mannitol were without

effect on lipid peroxidation initiated by the additive effect of ADP-Fe^{2+} and ADP-Fe^{3+}. Again, SOD decreased the extent of peroxidation but had no effect on the lag period. These results further suggest the involvement of higher oxidation states of iron (in iron-oxygen complexes) rather than free •OH in initiation of lipid peroxidation. We noticed that the length of the lag period of ADP-Fe^{2+}-dependent lipid peroxidation varied with different preparation of ADP-Fe^{2+}. These differences may reflect the difficulty in preparing ferrous solutions free of ferric iron. This may explain the discrepancies between the results of this study and that of Svingen et al. (1978).

The exact nature of the initiation complex(es) in lipid peroxidation systems containing ADP-Fe^{3+} remains unknown. Assignment of a mechanism must await further experimental evidence. As Mason (1957) has pointed out, even in the Fenton's reagent, the reactive intermediates may be •OH, $(FeO_2)^{2+}$, $Fe(OH)^{2+}$, $Fe(OH)^{3+}$, $Fe(H_2O)_5$, $(H_2O_2)^{3+}$, and FeO^{2+}. Chelation of these intermediates may effect their stability, reactivity toward •OH trapping agents, and their lipid peroxidation initiating activity.

Once lipid peroxidation is initiated by an ADP-Fe^{3+}-dependent process, secondary initiation reactions involving lipid hydroperoxides and ferrous iron can increase the rate of peroxidation. Confirming the results of Svingen et al. (1978), the addition of EDTA-Fe^{2+} to partially-peroxidized liposomes resulted in a rapid rate of MDA formation. ADP-Fe^{2+} was less effective while unchelated Fe^{2+} was most efficient in promoting lipid hydroperoxide-dependent lipid peroxidation. Our results with the oxygen electrode indicated that EDTA also facilates

Fe^{2+} autoxidation. This may explain why unchelated Fe^{2+} is more effective in promoting lipid hydroperoxide-dependent lipid peroxidation. These results suggest that chelation of Fe^{2+} by EDTA does not impart any special characteristics to Fe^{2+} in promoting lipid hydroperoxide-dependent lipid peroxidation. Since promotion of lipid hydroperoxide-dependent lipid peroxidation in the enzymatic system must involve initial reduction of Fe^{3+}, followed by reductive cleavage of the peroxide (Svingen and Aust, 1980), the ability of EDTA to promote this process may arise from facilitation of Fe^{3+} reduction by $O_2^{\cdot-}$. (Chapter III) and by NADPH-cytochrome P450 reductase (Chapter II).

In addition to catalyzing the decomposition of lipid hydroperoxides, we propose an additional role for EDTA-Fe^{3+} in lipid peroxidation. In NADPH-cytochrome P450 reductase-dependent lipid peroxidation, our results suggest that EDTA-Fe^{3+} catalyzes the electron transfer from reduced reductase to ADP-Fe^{3+} (Chapter II). Due to the rapid autoxidation of EDTA-Fe^{2+}, it may be anticipated that $O_2^{\cdot-}$ is also involved in this transfer as shown below.

$$\text{reduced reductase} + \text{EDTA-}Fe^{3+} \longrightarrow \text{reductase} + \text{EDTA-}Fe^{2+}$$

$$\text{EDTA-}Fe^{2+} + O_2 \longrightarrow \text{EDTA-}Fe^{3+} + O_2^{\cdot-}$$

$$\text{ADP-}Fe^{3+} + O_2^{\cdot-} \longrightarrow \text{ADP-}Fe^{3+}O_2^{\cdot-} \rightarrow \rightarrow \text{initiation complex}$$

The ability of EDTA-Fe^{2+} and ADP-Fe^{3+} to initiate peroxidation of liposomes supports this proposal. Neither EDTA-Fe^{2+} or ADP-Fe^{3+} alone were capable of initiating lipid peroxidation. It follows that SOD would inhibit lipid peroxidation initiated by this system, as it did. However, the extent of inhibition was low and a direct interaction

between EDTA-Fe^{2+} and ADP-Fe^{3+} in initiation of lipid peroxidation must be considered.

In microsomes, EDTA-Fe^{3+} is not required for initiation of NADPH-dependent lipid peroxidation as it is in the reconstituted system with the purified reductase (Pederson et al., 1973). The only iron chelate required for lipid peroxidation in microsomes is ADP-Fe^{3+}. Pederson et al. (1973) suggested that EDTA-Fe^{3+} replaced an endogenous microsomal component that participates in lipid peroxidation. Subsequent studies by Svingen and coworkers (1979) demonstrated that drug substrates such as SKF525-A or aminopyrene, which binds specifically to cytochrome P450, were effective inihibitors of microsomal NADPH-dependent lipid peroxidation. This inhibition could not be attributed to these compounds being antioxidants. The addition of EDTA-Fe^{3+} was found to reverse the inhibition of microsomal lipid peroxidation by drug substrates. Thus Svingen and coworkers (1979) proposed that cytochrome P450 was the endogenous microsomal component which EDTA-Fe^{3+} replaced. The ability of EDTA-Fe^{3+} to catalyze the NADPH-cytochrome P450 reductase-dependent decomposition of lipid hydroperoxides led these workers to propose that cytochrome P450 had similiar catalytic properties. Cytochrome P450 augmentation of lipid peroxidation by a lipid hydroperoxide-dependent mechanism has also been put forth by Maier and Tappel (1959).

The mechanism of microsomal NADPH-dependent lipid peroxidation proposed by Svingen and coworkers (1979) is predicated on the ability of the reductase to reduce both EDTA-Fe^{3+} and ADP-Fe^{3+}. However, the results of Chapter II clearly indicate that NADPH-cytochrome P450 reductase cannot catalyze the reduction of ADP-Fe^{3+}. This suggests

that another microsomal component in addition to the reductase is involved in ADP-Fe^{3+} reduction and therefore, initiation of lipid peroxidation. The capacity of EDTA-Fe^{3+} to be reduced by the reductase (Chapter II) and also to reverse the inhibition of microsomal NADPH-dependent lipid peroxidation by drug substrates would suggest that cytochrome P450 is the component in microsomes which catalyzes the electron transfer from the reductase to ADP-Fe^{3+}. It is evident that further studies are required to correlate the role of iron and iron chelation in reconstituted lipid peroxidation to that of <u>in vivo</u> lipid peroxidation.

CHAPTER V

IRON CATALYZED LIPID HYDROPEROXIDE-DEPENDENT LIPID PEROXIDATION AS A FUNCTION OF pH

ABSTRACT

Unchelated ferric ion catalyzes the decomposition of phospholipid hydroperoxides by a mechanism similiar to that proposed for ferric ion catalyzed decomposition of hydrogen peroxide. Both reactions show an acidic pH optimum. The products of lipid hydroperoxide decomposition were shown to be free radicals by EPR spin trapping techniques with α-(4-pyridyl-1-oxide)-N-tert-butylnitrone. EPR spin trapping experiments also demonstrated that the initial radical formed is the lipid peroxy radical. The resultant lipid peroxidation initiated by this reaction is dependent on lipid hydroperoxide concentration, ferric ion concentration, and pH. Using soybean lipoxidase to generate lipid hydroperoxides in liposomes, the rate of peroxidation was shown to be directly proportional to the lipid hydroperoxide content. At high lipid hydroperoxide to ferric ion ratios, the rate of peroxidation was found to be directly proportional to the ferric ion concentration.

INTRODUCTION

In the presence of oxygen and the proper catalyst, unsaturated lipid show a strong propensity to autoxidize by free radical mechanisms (Wills, 1965; Bateman et al., 1951; Bolland and Gee, 1946). The numerous products of autoxidation are usually breakdown products of lipid hydroperoxides (Gardner, 1975; Gardner et al., 1974) formed in the propagation step of lipid peroxidation (Pryor and Stanley, 1975; Pryor et al., 1977). Once formed, lipid hydroperoxides are relatively stable to uncatalyzed unimolecular decomposition (O'Brien, 1969). The calculated half-life for uncatalyzed unimolecular homolysis of tert-butyl hydroperoxide at 37°C is 10^9 years (Pryor, 1976). However, in the presence of transition metals, hydroperoxides are readily decomposed by a redox mechanism generating free radicals (Sosnovsky and Rawlinson, 1971; Uri, 1961). For example, iron readily catalyzes the decomposition of H_2O_2 (Barb et al., 1951).

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + \cdot OH + {}^-OH$$

$$Fe^{3+} + H_2O_2 \longrightarrow Fe^{2+} + HO_2 + H^+$$

The ferric ion reaction shows an inverse dependence on H^+ concentration, presumably because of the prior equilibria (Walling and Weil, 1974).

$$Fe^{3+} + H_2O_2 \rightarrow H^+ + FeOOH \rightarrow Fe^{2+} + HO_2^{\bullet}$$

Iron has also been shown to catalyze the decomposition of lipid hydroperoxides to free radical products by a redox mechanism (O'Brien, 1969). In the presence of unsaturated lipid, these free radicals can participate in the propagation of lipid peroxidation (Sosnovsky and Rawlinson, 1971; Walling and Weil, 1974). It is well known that in the presence of lipid hydroperoxides, lipid peroxidation can be catalyzed by numerous heme compounds (Maier and Tappel, 1959). Svingen and coworkers (1978, 1979) demonstrated that in the presence of lipid hydroperoxides, ferric cytochrome P450 and ferrous chelates such as EDTA-Fe^{2+} can readily initiate lipid peroxidation of extracted microsomal lipid. Using cumene hydroperoxide as a model for lipid hydroperoxides, these investigators demonstrated that decomposition could only occur with the ferrous chelates. Lipid peroxidation was proposed to be initiated by the free radical products of decomposition. At physiological pH, only ferrous chelates or heme compounds have been demonstrated to initiate lipid hydroperoxide-dependent lipid peroxidation. O'Brien (1969) has reported that unchelated iron can also catalyze the decomposition of lipid hydroperoxides. He reported that ferrous or ferric ion caused the disproportionation of linoleic acid 13-hydroperoxide (LAHPO) to a variety of products. Ferrous salts were much more effective than ferric salts in catalyzing LAHPO decomposition. Both systems exhibited a low pH optimum. A free radical mechanism was implicated.

While studying the effect of iron chelation on enzyme catalyzed lipid peroxidation, we observed that certain reaction mixtures containing unchelated Fe^{3+} and liposomes readily peroxidized if the pH of the

reaction mixture was not carefully adjusted. The extent of peroxidation varied with different preparations of liposomes. Liposomes made from old preparations of phospholipids peroxidized more readily than new preparations. It was subsequently shown that this peroxidation was dependent on lipid hydroperoxides, Fe^{3+}, and pH. The nature of these reactions and the resulting lipid peroxidation form the body of this study.

MATERIALS AND METHODS

Materials. Soybean lipoxidase (Type I), thiobarbituric acid, mannitol, butylated hydroxytoluene (BHT), and sodium deoxycholate were obtained from Sigma Chemical Co. (St. Louis, MO). Linoleic and arachidonic acid were purchased from Nu Chek Prep. (Elysian, MN). Cumene hydroperoxide was a product of K and K Laboratories, Inc. (Plainview, NY). The spin trap α-(4-pyridyl 1-oxide)-N-tert-butylnitrone (POBN) was purchased from Aldrich Chemical Co. (Milwaukee, WI). All other reagents were analytic grade and used without further preparation.

Microsomal Lipid. Microsomal lipid was extracted and liposomes were prepared as described in Chapters II and III. Lipid phosphate determinations were performed by the method of Bartlett (1959). Liposomal phospholipid hydroperoxides were generated by the action of soybean lipoxidase as described in Chapter IV.

Preparation of Linoleic 13-Hydroperoxide. Linoleic acid 13-hydroperoxide (13-hydroperoxy-9,11-octadecadieneoic acid) was prepared by soybean lipoxidase catalyzed oxidation of linoleic acid. Incubation mixtures contained sodium linoleate (2 mg/ml) in 2 ml of oxygen-purged 50 mM borate buffer, pH 9.0. Reactions were carried out at 25°C in 50 ml culture tubes with the head space purged with oxygen. At 0, 10, and 20 minutes, 0.2 ml of soybean lipoxidase (1 mg/ml in oxygen purged 50 mM borate, pH 9.0) was added. At 30 minutes, the pH of the reaction mixture was adjusted to 3.0 with 6 N HCl and immediately extracted with 10 ml

CH_3Cl:CH_3OH (2:1). The organic layer was washed 4 times with distilled water and then dried with anydrous $MgSO_4$. The decanted organic phase was evaporated to dryness under argon and taken up in absolute ethanol to a final concentration of approximately 50 mg/ml. Purification of LAHPO was carried out by HPLC on a LiChrosorb Si 60 column (24 cm, 0.46 cm, 10 μm) (EM Laboratories, Inc., Darmstadt, West Germany). The eluting solvent was 0.15% acetic acid and 1.5% anhydrous ethanol in hexane. The LAHPO peak was detected by absorption at 234 nm and was determined to be 99% pure. Lipid hydroperoxide content was measured by KI titration as described by Buege and Aust (1978).

Reaction Mixtures. Lipid hydroperoxide-dependent lipid peroxidation reaction mixtures contained 0.2 μmole lipid hydroperoxide/ml, 1 μmole lipid phosphate/ml, 0.1 mM $FeCl_3$ in 50 mM $HClO_4$, pH 2.2 unless specified otherwise. Other additions are as specified in the figure legends. Reactions were initiated by the addition of $FeCl_3$. Incubations were carried out in a metabolic shaking water bath at 37°C under an air atmosphere. Lipid peroxidation was assayed by the formation of malondialdehyde (MDA) and lipid hydroperoxides (LOOH) (Buege and Aust, 1977). Rates of MDA formation are reported as initial rates.

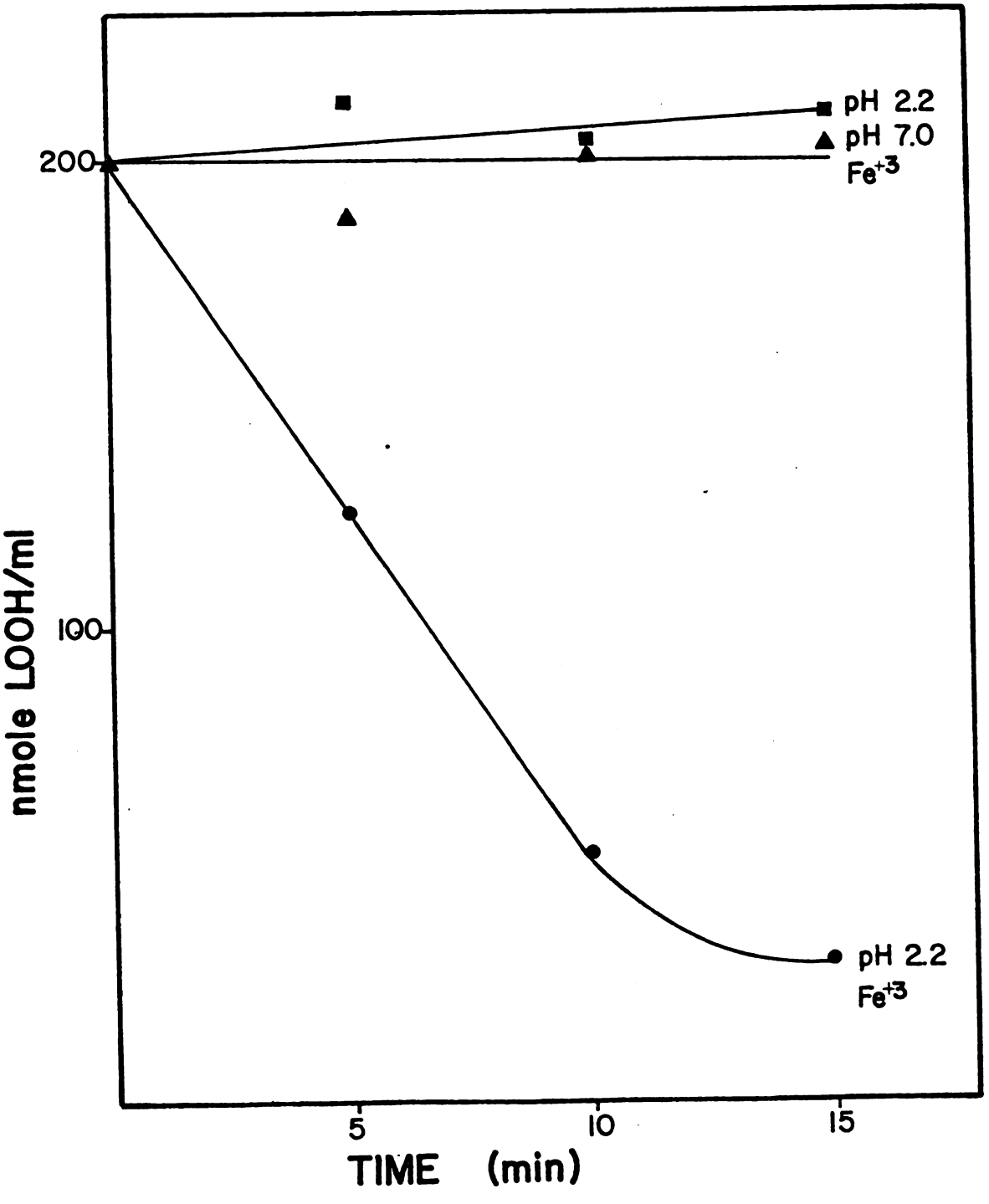
EPR Spectroscopy. EPR spectra were recorded with a Varian Century E112 spectrometer set at the following conditions: 3370 G magnetic field, 9.41208 GHz, 10 mW microwave power, 100 KHz modulation frequency, 2.5 modulation amplitude, 0.5 seconds time constant, 5×10^4 receiver gain and 4 minutes scan time. Reaction mixtures are as specified in the figure legend. Recording of all spectra began 2 minutes after initiation of the reaction.

RESULTS

Ferric Ion Catalyzed Decomposition of Lipid Hydroperoxides

Ferric ion has been demonstrated to catalyze the decomposition of hydrogen peroxide to a variety of products including $HO_2\cdot$ and $\cdot OH$ (Barb et al., 1951). An acidic pH optimum was observed for the rate of hydrogen peroxide decomposition. To investigate whether Fe^{3+} can also catalyze the decomposition of lipid hydroperoxides, phospholipid hydroperoxides were generated by the action of soybean lipoxidase. Soybean lipoxidase has been demonstrated to catalyze the incorporation of dioxygen into unsaturated fatty acids, fatty acid esters, and triglycerides (Christopher et al., 1969). The incorporation of dioxygen into unsaturated phospholipids to yield phospholipid hydroperoxides provided partially peroxidized liposomes for this study. The ability of Fe^{3+} to catalyze the decomposition of lipid hydroperoxides was studied at neutral and acidic pH. In order to assess lipid hydroperoxide decomposition by Fe^{3+}, the reactions were performed under anaerobic conditions to minimize possible further formation of lipid hydroperoxides in the unsaturated lipid. The results of this study are shown in Figure 23. At pH 7.0, Fe^{3+} (0.1 mM) had no effect on lipid hydroperoxides. However, upon lowering the pH to 2.2, 80% of the lipid hydroperoxides were decomposed within 15 minutes. No change in the lipid hydroperoxide content was observed at pH 2.2 in the absence of Fe^{3+}.

Figure 23. Fe^{3+} Catalyzed Decomposition of Phospholipid Hydroperoxides. Liposomal reaction mixtures were argon-saturated and contained 0.2 μmole phospholipid hydroperoxides/ml and 1 μmole lipid phosphate/ml in either 50 mM $HClO_4$, pH 2.2, or 30 mM NaCl, pH 7.0 at 37°C. The following additions were made as indiated: (■-■) no additions in 50 mM $HClO_4$, pH 2.2; (●—●) 0.1 mM Fe^{3+} in 50 mM $HClO_4$, pH 2.2; (▲—▲) 0.1 mM Fe^{3+} in 30 mM NaCl, pH 7.0. Incubations and assays were performed as described in Materials and Methods.


pH 2.2
pH 7.0
Fe+3
200
100
nmole LOOH/ml
pH 2.2
Fe+3
5
10
15
TIME (min)

Initiation of Lipid Peroxidation by Lipid Hydroperoxide Decomposition

As shown in Figure 24, the addition of Fe^{3+} to partially-peroxidized liposomes at pH 2.2 in the presence of dioxygen (air saturated) did not result in a decrease in lipid hydroperoxide content. At an initial lipid hydroperoxide concentration of 0.2 mM, Fe^{3+} caused a rapid increase in the lipid hydroperoxide content within the first 45 seconds. This increase was followed by a gradual decrease to a steady state concentration of 0.2 mM. MDA, a breakdown product of lipid hydroperoxides (Dahle et al., 1962), increased in content linearly up to 3 minutes after which the rate of its formation decreased. Since MDA formation is indicative of lipid hydroperoxide decomposition, the steady state lipid hydroperoxide concentration suggests that lipid hydroperoxides are being generated and decomposed during the course of the reaction. No changes in the lipid hydroperoxide content or in the MDA content were observed in the absence of Fe^{3+}. As with Fe^{3+} catalyzed decomposition of lipid hydroperoxides (Figure 23), the rates of lipid peroxidation shown in Figure 24 were also pH sensitive. The rate of lipid peroxidation as assayed by MDA formation was found to have a pH optimum of 2.2 (Figure 25). Activity decreased rapidly at pH values below and above 2.2.

Effect of Lipid Hydroperoxide and Iron Content on Lipid Peroxidation

The effect of lipid hydroperoxide concentration on the rate of MDA formation is shown in Figure 26. No lipid peroxidation occurred in the absence of added lipid hydroperoxides or Fe^{3+}. In the presence of 0.1 mM Fe^{3+}, the rate of MDA formation increased proportionally to the lipid hydroperoxide concentration up to 0.3 mM. However, at higher peroxide concentrations, the rate of MDA formation decreased. The slope

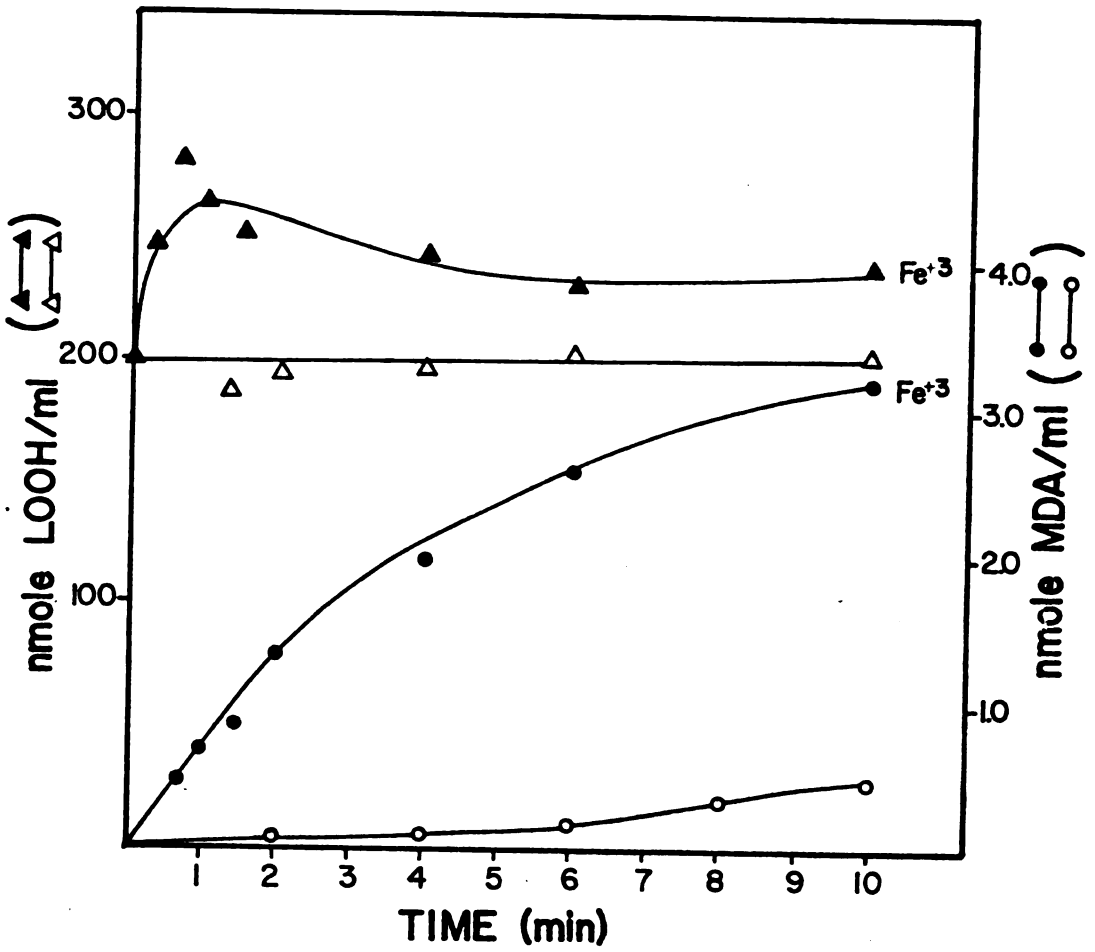


Figure 24. Fe^{3+} Catalyzed Lipid Hydroperoxide-Dependent Lipid Peroxidation. Liposomal reaction mixtures contained 0.2 mM phospholipid hydroperoxides and 1 mM lipid phosphate in 50 mM $HClO_4$, pH 2.2 at 37°C. The MDA content (circles) and the LOOH content (triangles) were measured in reaction mixtures without $FeCl_3$ (open symbols) or containing 0.1 mM $FeCl_3$ (closed symbols). The appropriate reactions were initiated by the addition of Fe^{3+}. Incubations and assays were performed as described in Materials and Methods.

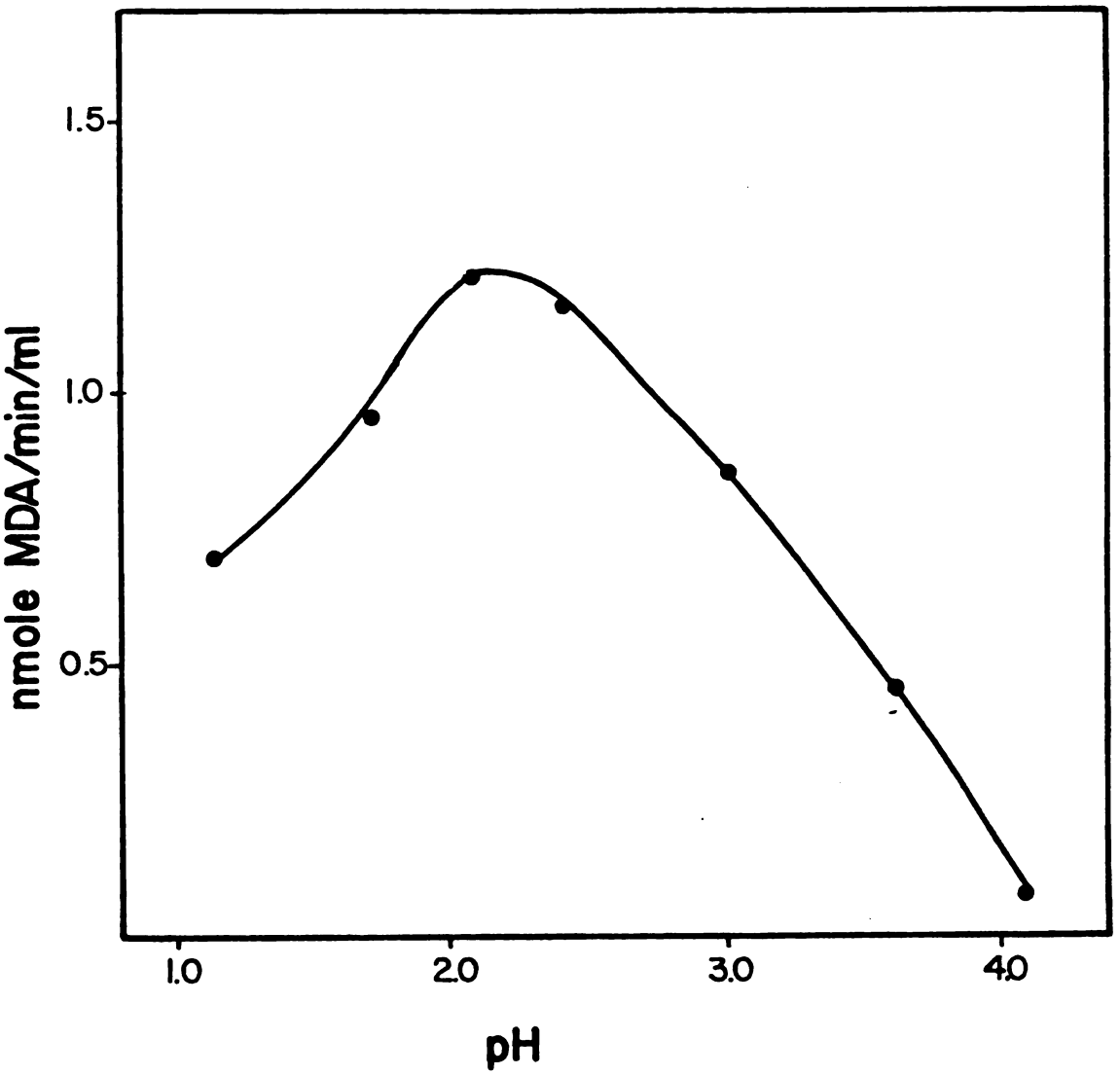


Figure 25. Effect of pH on Lipid Hydroperoxide-Dependent Lipid Peroxidation. Reaction mixtures contained 0.1 mM phospholipid hydroperoxides, 1 mM lipid phosphate and 0.1 mM $FeCl_3$ in 50 mM $HClO_4$, varying pH at 37°C. Reactions were initiated by the addition of $FeCl_3$. Incubations and assays were performed as described in Materials and Methods.

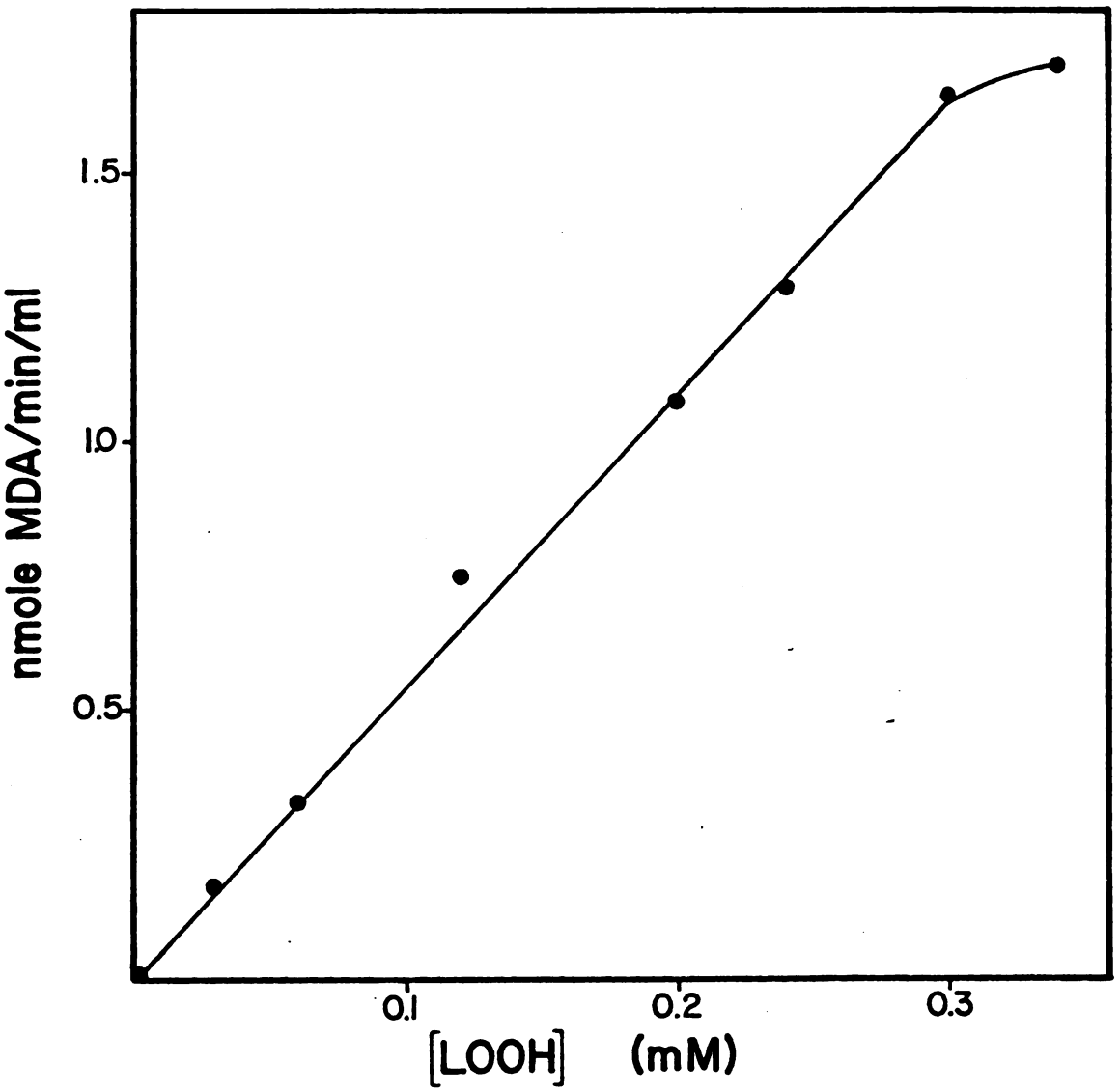


Figure 26. Effect of Lipid Hydroperoxide Content on the Rate of Lipid Peroxidation. Reaction mixtures contained 1 μmole lipid phosphate/ml, 0.1 mM $FeCl_3$ and varying concentrations of phospholipid hydroperoxides in 50 mM $HClO_4$, pH 2.2 at 37°C. Reactions were initiated by the addition of $FeCl_3$. Phospholipid hydroperoxides generated by soybean lipoxidase were mixed with unperoxidized phospholipids to make liposomes of the desired lipid hydroperoxide content. Incubations and assays were performed as described in Materials and Methods.

of the log-log plot was unity demonstrating a first order relationship between the rate of MDA formation and lipid hydroperoxide concentration.

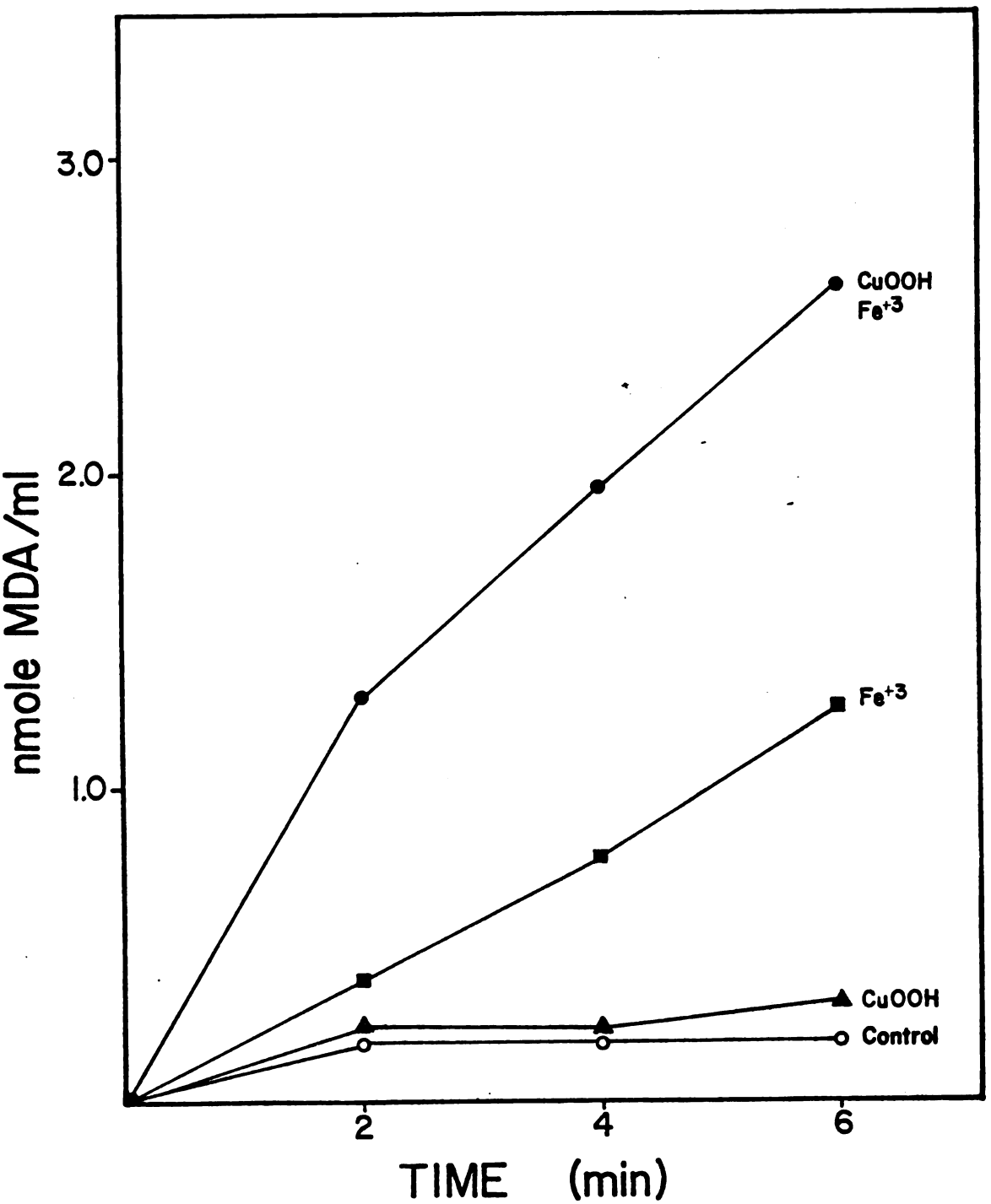
The ability of organic hydroperoxides to initiate lipid peroxidation at low pH was further investigated by utilizing cumene hydroperoxide instead of phospholipid hydroperoxides (Figure 27). Arachidonic acid micelles (5 mg/ml) were used as the substrate for peroxidation. The addition of cumene hydroperoxide (2 mM) to arachidonic acid did not result in lipid peroxidation. The incubation of Fe^{3+} (0.1 mM) alone with arachidonic acid resulted in low levels of MDA formation. This may be indicative of peroxide contamination in the arachidonic acid preparation. The inclusion of 2 mM cumene hydroperoxide in the presence of 0.1 mM Fe^{3+} resulted in a 4 fold enhancement of lipid peroxidation.

The rate of lipid peroxidation was also shown to be dependent on the Fe^{3+} concentration (Figure 28). Increasing the Fe^{3+} concentration up to 0.1 mM at a constant lipid hydroperoxide concentration of 0.2 mM caused a linear increase in the rate of MDA formation. Log-log plots yielded a slope of one indicating that the rate of MDA formation is first order in respect to $FeCl_3$ concentration.

EPR Spin Trapping of Ferric Ion Catalyzed Decomposition of Lipid Hydroperoxides

The mechanism of lipid peroxidation initiated from the Fe^{3+} catalyzed decomposition of lipid hydroperoxides was further investigated by EPR spin trapping experiments with the nitrone spin trap POBN (60 mM). This was done in order to identify the initial radical formed by Fe^{3+} catalyzed decomposition of lipid hydroperoxides. Rather than using phospholipid hydroperoxides, these experiments were performed with

Figure 27. Cumene Hydroperoxide-Dependent Lipid Peroxidation. Reaction mixtures contained 5 mg arachidonate/ml in 50 mM $HClO_4$, pH 2.2 at 37°C. The following additions were made as indicated: (o—o) no additions; (▲—▲) 2 mM cumene hydroperoxide (CuOOH); (■—■) 0.1 mM Fe^{3+}; (●—●) 0.1 mM Fe^{3+} and 2 mM CuOOH. Incubation and assays were performed as described in Materials and Methods.


3.0
2.0
1.0
nmole MDA/ml
CuOOH
Fe+3
Fe+3
CuOOH
Control
2
4
6
TIME (min)

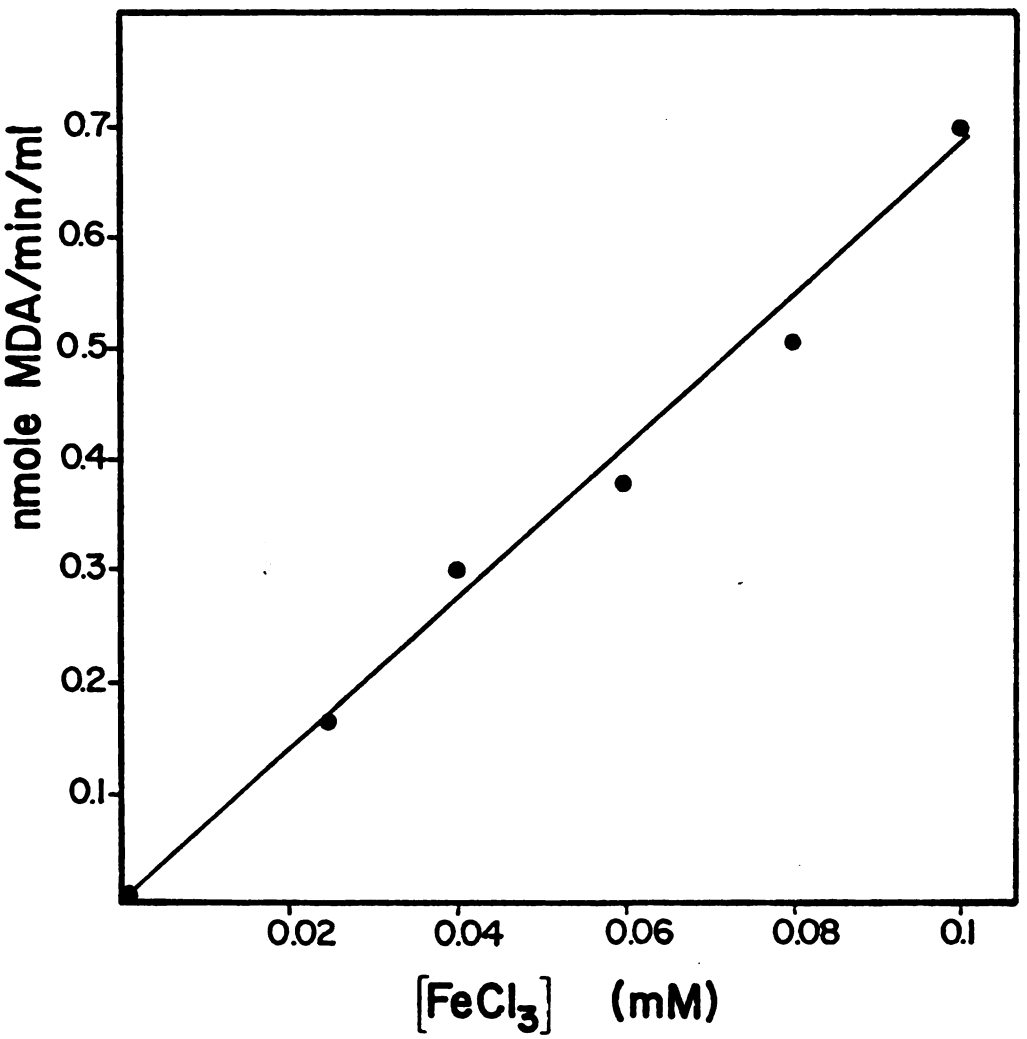


Figure 28. Effect of Fe^{3+} Concentration on Lipid Hydroperoxide-Dependent Lipid Peroxidation. Liposomal reaction mixtures contained 0.2 mM phospholipid hydroperoxides, 1 mM lipid phosphate, and the specified $FeCl_3$ concentration in 50 mM $HClO_4$, pH 2.2 at 37°C. Reactions were initiated by the addition of $FeCl_3$. Incubations and assays were performed as described in Materials and Methods.

purified LAHPO (0.1 mM). An EPR signal was detected from the anaerobic incubation of Fe^{3+} (0.1 mM) and LAHPO (0.1 mM) with 60 mM POBN at pH 2.2 (Figure 29). This signal had a g value of 2.0049, characteristic of a free radical. No EPR signal was observed when LAHPO was incubated with 60 mM POBN in the absence of added Fe^{3+} at pH 2.2. However, the incubation of Fe^{3+} with POBN in the absence of LAHPO did result in an EPR signal. Elsworth and coworkers (1974) reported that low pH enhances the rate of nitrone oxidation by Fe^{3+}. The products of this oxidation (POBN by Fe^{3+}) are EPR active as shown in Figure 29. The EPR signal has a g value of 2.0049 and hyperfine splitting (hps) constants of A_N=14.75 G and A_H=14.75 G. Subtraction of this signal from the EPR signal observed with the incubation of LAHPO and Fe^{3+} in the presence of 60 mM POBN (Figure 29) simplified the signal to one which has a g value of 2.0049 and hps constants of A_N=15.74 G and A_H=2.50 G.

In order to determine the structure of this spin adduct, the EPR spectrum was compared to the spectrum of the lipid alkoxy and lipid peroxy POBN spin adduct. The EPR spectrum of the lipid alkoxy spin adduct (Figure 29) was obtained by reductive cleavage of LAHPO by Fe^{2+} at pH 2.2 in the presence of 60 mM POBN.

$$LOOH + Fe^{2+} \longrightarrow LO\cdot + {}^{-}OH + Fe^{3+}$$

The EPR spectrum of the lipid peroxy spin adduct (Figure 29) was obtained by aerobic incubation of linoleic acid with soybean lipoxidase in the presence of 60 mM POBN. To avoid possible changes in the hps constants by pH, the pH of the lipoxidase incubation mixture was adjusted to 2.2

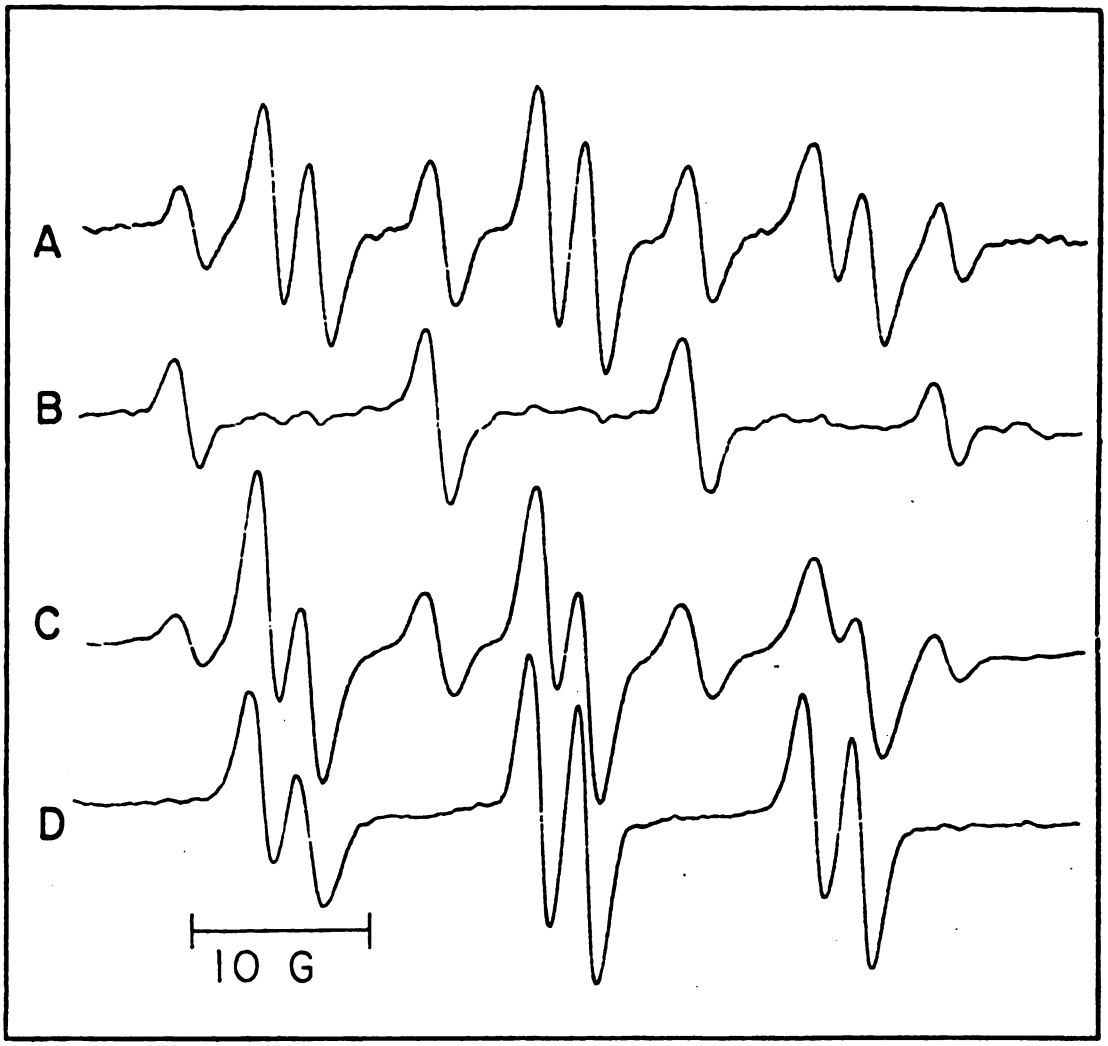


Figure 29. EPR Spectra of POBN Spin Adducts of Lipid Peroxide Radicals. Reaction mixtures contained 60 mM POBN in 50 mM $HClO_4$, pH 2.2 at 25°C. The EPR spectra were obtained by scanning reaction mixtures with the following additions: (A) 0.1 mM LAHPO and 0.1 mM $FeCl_3$. g=2.0049, A_N=15.74 G, A_H=2.50 G; (B) 0.1 mM $FeCl_3$. g=2.0049, A_N=14.75 G, A_H=14.75 G; (C) 0.1 mM LAHPO and 0.1 mM $FeCl_2$. g=2.0051, A_N=15.80 G, A_H=1.95 G; (D) 0.5 mM linoleate and 0.1 mg soybean lipoxidase/ml in 30 mM NaCl, pH 7.0. After a minute preincubation, pH was adjusted to 2.2 by addition of 50 mM $HClO_4$. g=2.0049, A_N=15.73 G, A_H=2.50 G. EPR instrument settings are described in Materials and Methods.

after a 1 minute incubation of 0.5 mM linoleate with 0.1 mg soybean lipoxidase/ml in the presence of 60 mM POBN. Based on the g value and hps constants, the initial free radical formed by lipid hydroperoxides and Fe^{3+} at low pH appears to the lipid peroxy radical.

DISCUSSION

The results of this study demonstrate that unchelated ferric iron can participate in lipid hydroperoxide-dependent lipid peroxidation. The rate of peroxidation of liposomes is dependent on both lipid hydroperoxide content and Fe^{3+} concentration. Other organic peroxides can substitute for endogenous peroxides as demonstrated by peroxidation of arachidonic acid upon addition of Fe^{3+} and cumene hydroperoxide. Under anaerobic conditions, the ability of Fe^{3+} to catalyze the breakdown of lipid hydroperoxides was demonstrated to be pH dependent. Eighty percent of the original lipid hydroperoxides were decomposed within 15 minutes at pH 2.2. At pH 7.0, Fe^{3+} had no affect on lipid hydroperoxides. This result is in agreement with the results of O'Brien (1969) on Fe^{3+} catalyzed decomposition of LAHPO. However, the lowest pH investigated by these workers was 5.5.

EPR spin trapping techniques revealed that free radicals were formed in Fe^{3+} catalyzed decomposition of lipid hydroperoxides. The EPR spectra suggest that the initial free radical formed by LAHPO and Fe^{3+} is the lipid peroxy radical. The g value and hyperfine splitting constants of this signal corresponded to the EPR signal obtained with the POBN lipid peroxy adduct obtained by aerobically incubating linoleic acid with soybean lipoxidase. The radical products of lipid hydroperoxide breakdown catalyzed by Fe^{3+} are capable of participating in propagation of lipid peroxidation. Under aerobic

conditions, the addition of Fe^{3+} to partially peroxidized liposomes caused an initial increase in lipid hydroperoxide content followed by a gradual decrease to steady state concentrations. The increase in MDA content indicated lipid hydroperoxide decomposition occurred in conjunction with lipid hydroperoxide formation.

The rate of lipid hydroperoxide dependent lipid peroxidation has a pH optimum of 2.2. This result is analogous to the results obtained by Barb et al. (1951) for Fe^{3+} catalyzed decomposition of H_2O_2. The lower solubility of unchelated Fe^{3+} in aqueous solutions due to hydrolysis (Barb et al., 1951) could account for the decrease in rates of MDA formation at pH values above 2.2. At pH values below 2.2, the rate of lipid peroxidation shows an inverse dependence on H^+ concentration suggesting that a deprotonation step is involved prior to free radical formation similar to the mechanism of Fe^{3+} catalyzed decomposition of hydrogen peroxide (Walling and Weil, 1974). The results suggest the following scheme:

$$LOOH + Fe^{3+} \longrightarrow LOOFe^{2+} + H^+ \qquad I$$

$$LOOFe^{2+} \longrightarrow LOO\cdot + Fe^{2+} \qquad II$$

$$LOO\cdot + LH \longrightarrow LOOH + L\cdot \qquad III$$

$$L\cdot + O_2 \longrightarrow LOO\cdot \qquad IV$$

$$Fe^{2+} + LOOH \longrightarrow LO\cdot + {}^-OH + Fe^{3+} \qquad V$$

$$LO\cdot + LH \longrightarrow LOH + L\cdot \qquad VI$$

$$2L\cdot \longrightarrow L\text{-}L \qquad VII$$

$$LO\cdot + L\cdot \longrightarrow LOL \qquad VIII$$

$$LOO\cdot + L\cdot \longrightarrow LOOL \qquad IX$$

The redox reaction of lipid hydroperoxides (LOOH) and Fe^{3+} generates the LOO· and Fe^{2+} (I, II). In the presence of unsaturated lipid (LH), LOO· participates in propagation of lipid peroxidation (III). The Fe^{2+} generated from reaction II can initiate another chain of free radical reactions via reductive cleavage of LOOH (V,VI). In the presence of oxygen, the rate of LOOH formation from LOO· (III) is equivalent to the rate of its decomposition resulting in a steady-state concentration of LOOH and increase in MDA content. Under anaerobic conditions, reaction IV is not significant and termination reactions (VII, VIII, IX) are of greater significance resulting in net loss of LOOH (Figure 23). The redox generation of LOO· from LOOH and Fe^{3+} is dependent on H^+ concentration (I). Thus the rate of LOOH breakdown is inversely correlated with H^+ concentration, provided H^+ concentration is sufficiently high to maintain the solubility of Fe^{3+}. These two effects result in a pH optimum of 2.2 for the rate of lipid peroxidation catalyzed by LOOH and Fe^{3+}.

LIST OF REFERENCES

LIST OF REFERENCES

Adams, E.G. and Wardman, P. (1977) in Free Radicals in Biology (Pryor, W.A., ed.) Vol. III, p.53, Academic Press, New York.

Altman, K.I., Gerber, G.B., and Okada, S. (1970) in Radiation Biochemistry, Vol. 1 and 2, Academic Press, New York.

Anbar, M. and Netta, P. (1967) Int. J. Appl. Radiat. Isotop. 18, 493.

Ausloos, P. (1968) in Fundamental Processes in Radiation Chemistry, Wiley-Interscience, New York.

Aust, S.D. and Svingen, B.A. (1981) in Free Radicals in Biology (Pryor, W.A. ed.), in press, Academic Press, New York.

Aust, S.D., Roerig, D.L., and Pederson, T.C. (1972) Biochem. Biophys. Res. Commun. 47, 1133.

Babior, B.M., Kipnes, R.S., and Curnette, J.T. (1973) J. Clin. Invest. 52, 741.

Bacq, Z.M. and Alexander, P. (1966) in Fundamentals of Radiobiology, 2nd Ed., Pergamon, Oxford.

Baldridge, C.W. and Gerard, R.W. (1933) Am. J. Physiol. 103, 235.

Barb, W.G., Baxendale, J.H., George, P., and Hargrave, K.R. Tra.s Faraday Soc. 47, 591.

Bartlett, G.R. (1959) J. Biol. Chem. 234, 446.

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

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

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

Benson, S.W. (1968) in Thermochemical Kinetics, Wiley, New York.

Bergstrom, S. and Holman, R.T. (1948) Nature 161, 55.

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

Biaselle, C.J. and Miller, J.G. (1974) J. Amer. Chem. Soc. 96, 3813.

Boguth, W. and Niemann (1971) Biochim. Biophys. Acta. 248, 121.

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

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

Borg, D.C. (1976) in Free Radicals in Biology (Pryor, W.A., ed), Vo. 1, p. 69, Academic Press, New York.

Buege, J.A. and Aust, S.D. (1978) in Methods in Enzymology, Vol. LII. Biomembranes C-Biological Oxidations (S. Fleischer and L. Packer, eds.) Academic Press, New York.

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

Bus, J.S., Aust, S.D., and Gibson, J.E. (1975) Res. Commun. Chem. Pathol. Pharmacol. 11, 31.

Calderbank, A. (1968) Adv. Pest Control Res. 8, 127.

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

Christopher, J., Pistorius, E., and Axelrod, B. (1970) Biochim. Biophys. Acta. 198, 12.

Czapski, G. (1971) Ann. Rev. Phys. Chem. 22, 171.

Chhabra, R.S., Pohl, R.J., and Fouts, J.R. (1974) Drug Metabol. Dispos. 2, 443.

Chio, K.S. and Tappel, A.L. (1969) Biochemistry, 8, 2827.

Christophersen, B.O. (1968) Biochim. Biophys. Acta. 164, 35.

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

Conney, A.H. (1967) Pharmacol. Rev. 19, 317.

Csallany, A.S. (1971) Int. Z. Vitaminforsch 41, 376.

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

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

DiLuzio, N.R. (1963) Physiologist 6, 169.

DiLuzio, N.R. (1964) Life Sci. 3, 113.

DiLuzio, N.R. and Costales, F. (1965) Exp. Molec. Path. 4, 141.

Diaper, D.G.M. (1973) Oxid. Combust. Rev. 6, 145.

Dignam, J.D. and Stroebel, H.W. (1975) Biochem. Biophys. Res. Commun. 60, 1315.

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

Dorfman, L.M. and Adams, G.E. (1973) in Reactivity of Hydroxyl Radical in Aqueous Solutions, NSRDS-NMS, no. 46, United States Department of Commerce, National Bureau of Standards.

Dolev, A., Rehevedder, W.K., and Dutton, H.J. (1967) Lipids 2, 28.

Dunckley, W.L. and Jennings, W.G. (1951) J. Dairy Sci. 34, 1064.

Eriksson, C.E., Olsson, P.A., and Svensson, S.G. (1971) J. Am. Oil Chem. Soc. 48, 442.

Errera, M. and Forssberg, A. (1960) in Mechanisms in Radiobiology, Vols. 1 and 2, Academic Press, New York.

Farrington, J.A., Ebert, M., Land, E.J., and Fletcher, K. (1973) Bochim. Biophys. Acta. 314, 372.

Fee, J.A. and Valentine, J.S. (1977) in Superoxide and Superoxide Dismutases (Michelson, A.M., McCord, J.M., and Fridovich, I., eds.), p. 19, Academic Press, New York.

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

Finkelstein, E., Rosen, G.M., and Rauckman, E.J. (1980) Arch. Biochem. Biophys. 200, 1.

Floyd, R.A., Soong, L.M., Stuart, M.A., and Reigh, D.L. (1978) Photochem. Photobiol. 28, 857.

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

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

Foote, C.S. (1968) Accts. Chem. Res. 1, 104.

Foote, C.S. (1976) in Free Radicals in Biology (Pryor, W.A., ed.), Vol. II, p. 85, Academic Press, New York.

Foote, S.C., Shook, F.C., and Abakerli, R.A. (1980) J. Am. Chem. Soc. 102, 2503.

Fridovich, I. (1970) J. Biol. Chem. 245, 4053.

Fridovich, I. (1974) Advan. Enzymol. 41, 35.

Fridovich, S.E. and Porter, N.A. (1981) J. Biol. Chem. 256, 260.

Funk, M.O., Isaac, R., and Porter, N.A. (1976) Lipids 11, 113.

Gardner, H.W. (1975) J. Agric. Food. Chem. 23, 129.

Gardner, H.W., Kleinman, R., and Weisleder, D. (1974) Lipids 9, 699.

George, P. (1965) in Oxidases and Related Redox System (King, T.E., Mason, H.S., and Morrison, M., eds.), Vol. I, p. 3, Wiley, New York.

Gillette, J.R., Davis, D.C., and Sasame, H.A. (1972) Ann. Rev. Pharmacol. 12, 57.

Gnosspelius, Y., Thor, H., and Orrenius, S. (1969-1970) Chem-Biol. Interact. 1, 125.

Goodman, J. and Hochstein, P. (1977) Biochem. Biophys. Res. Commun. 77, 797.

Gram., T.E. and Fouts, J.R. (1966) Arch. Biochem. Biophys. 114, 331.

Gregory, E.M. and Fridovich, I. (1973) J. Bacteriol. 114, 543.

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

Gunsalus, I.C., Pederson, T.C., and Sligar, S.G. (1975) Ann. Rev. Biochem. 44, 377.

Haber, F. and Weiss, J. (1934) Proc. R. Soc. Lond, A 147, 332.

Halliwell, B. (1976) FEBS Lett. 72, 8.

Halliwell, B. (1978) FEBS Lett. 92, 321.

Hamilton, G.A. (1969) Advan. Enzymol. 32, 55.

Hamilton, G.A. (1974) in Molecular Mechanisms of Oxygen Activation (Hayaishi, O., ed.), p. 405, Academic Press, New York.

Harbour, J.R. and Bolton, J.R. (1978) Photochem. Photobiol. 28, 231.

Hardwich, T.J. (1957) Can. J. Chem. 35, 428.

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

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

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

Hirokata, Y., Shigematsu, A., and Omura, T. (1978) J. Biochem. 83, 431.

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

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

Holmes, R.S. and Masters, C.J. (1970) FEBS Lett. 11, 45.

Howard, J.A. (1973) in Free Radicals (Kochi, J.K., ed.), Vol. II, p. 3, John Wiley and Sons, New York, NY.

Ilan, Y.A. and Czapski, G. (1977) Biochem. Biophys. Acta. 498, 368.

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

Iyer, G.Y., Islam, M.F., and Quastel, J.H. (1961) Nature (Lond.) 192, 535.

Janzen, E.G. (1980) in Free Radicals in Biology (Pryor, W.A., ed.), Vol. IV, p. 116, Academic Press, New York.

Janzen, E.G. and Blackburn, B.J. (1968) J. Am. Chem. Soc. 90, 5909.

Kalish, G.H. and DiLuzio, N.R. (1966) Science 152, 1390.

Kameda, K., Ono, T., and Imai, Y. (1979) Biochim. Biophys. Acta. 572, 77.

Kamin, H. and Masters, B.S.S. (1968) in Enzymatic Oxidation of Toxicants (Hodgson, ed.), p. 5, North Carolina State University at Raleigh.

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

Kellogg, E.W., III and Fridovich, I. (1977) J. Biol. Chem. 252, 6721.

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

Khon, H.I. and Liversedge, M. (1944) J. Pharmacol. Exp. Therap. 82, 292.

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

Kluge, H., Rasch, R., Brux, B., and Frunder, H. (1967) Biochem. Biophys. Acta. 141, 260.

Koppenol, W.H. (1978) Photochem. Photobiol. 28, 431.

Kosower, N.S., Song, K.R., and Kosower, E.M. (1969) Biochim. Biophys. Acta. 192, 3632.

Lai, C.-S. and Piette, L.W. (1977) Biochem. Biophys. Res. Commun. 78, 51.

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

Lee, I.P., Yamamura, H.I., and Dixon, R.C. (1968) Biochem. Pharmacol. 17, 1671.

Levandoski, N.G., Baker, E.M., and Canham, J.E. (1964) Biochemistry 3, 1465.

Levin, W., Lu, A.Y.H., Jacobson, M., Kuntzman, R., Poyer, J.L., and McCay, P.B. (1973) Arch. Biochem. Biophys. 158, 842.

Lewis, D.H. and Del Maestro, R. (1980) Acta. Physiol. Scand. Supplementum 492.

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

Lu, A.Y.H., Kuntzman, R., West, S., Jacobson, M., and Conney, A.H. (1972) J. Biol. Chem. 247, 1727.

Lyakhovich, V., Mishin, V., and Pokrovksy, A. (1977) Biochem. J. 168, 133.

Mair, R.D. and Hall, R.T. (1971) in Organic Peroxides (Swern, D., ed.), Vol. II, p. 535, Wiley Interscience, New York.

Malkin, R. and Malmstrom, B.G. (1970) Advan. Enzymol. 33, 177.

March, J. (1968) in Advanced Organic Chemistry; Reactions, Mechanisms, and Structure, p. 26, McGraw-Hill, New York.

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

Massey, V., Strickland, S., Mayhew, S.G., Howell, L.G., Engel, P.C., Mathews, R.G., Schuman, M., and Sullivan, P.A. (1969) Biochem. Biophys. Res. Commun. 36, 891.

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

May, H.E. and McCay, P.B. (1968b) J. Biol. Chem. 243, 2296.

McCay, P.B. (1966) J. Biol. Chem. 241, 2333.

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

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

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

McCord, J.M. and Fridovich, I. (1969) J. Biol. Chem. 244, 6049.

McCord, J.M., Keele, B.B., and Fridovich, I. (1971) Proc. Nat. Aca. Sci. U.S. 68, 1024.

Mead, J.F. (1976) in Free Radicals in Biology (Pryor, W.A., ed.), Vol. I, p. 5, Academic Press, Inc., New York, NY.

Mengel, C.E. and Kann, H.E. (1966) J. Clin. Invest. 45, 1150.

Michaelis, L. and Hill, E.S. (1933) J. Am. Chem. Soc. 55, 1481.

Mishin, V., Pokrovsky, A., and Lyakhovich, V.V. (1976a) Biochem. J. 154, 307.

Mishin, V.M., Pospelova, L.N., Pokrovksy, A.G., and Lyakhovich, V.V (1976b) FEBS Lett. 62, 136.

Misra, H.P. and Fridovich, I. (1972) J. Biol. Chem. 247, 188.

Morrison, W.R. and Smith, L.M. (1964) J. Lipid Res. 4, 600.

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

Nakano, M., Takayama, K., Schimizu, Y., Tsuji, Y., Inaba, H., and Migita, T. (1963) J. Am. Chem. Soc. 98, 1974.

Neta, P. and Dorfman, L.M. (1968) Advan. Chem. Ser. 81, 222.

Niehaus, W.G., Jr. and Samuelsson, B. (1968) Eur. J. Biochem. 6, 126.

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

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

Ogryzlo, E.A. (1978) in Singlet Oxygen, Reactions with Organic Compounds and Polymers (Ranby, B., and Rabek, J.F., eds.), p. 4, John Wiley and Sons, New York.

Omura, T. and Sato, R. (1964) J. Biol. Chem. 239, 2370.
Ottolenghi, A., Bernheim, F., and Wilbur, K.M. (1955) Arch. Biochem. Biophys. 56, 157.

Palade, G.E. and Siekevitz, P. (1956) J. Biophys. Biochem. Cytol. 2, 171.

Paul, B.B. and Sbarra, A.J. (1968) Biochim. Biophys. Acta. 156, 168.

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

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

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

Plaa, G.L. and Witschi, H. (1976) Ann. Rev. Pharm. Tox. 16, 125.

Pokorny, J. and Janicek, G. (1968) Nahrung 12, 81.

Porta, E.A. and Hartroft, W.S. (1969) in Pigments in Pathology (Wolman, M., ed.), Chapter 7, Academic Press, NY.

Porter, W.L., Levasseur, L.A., and Henich, A.S. (1972) Lipids 7, 699.

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

Pryor, W.A. (1966) in Free Radicals, McGraw-Hill, NY.

Pryor, W.A. (1971) Chem. Eng. News. 49, 34.

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

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

Pryor, W.A. (1976) in Free Radicals in Biology (W. Pryor, ed.), Vol. 1, p. 1, Academic Press, New York.

Pryor, W.A. (1978) Photochem. Photobiol. 28, 208.

Pryor, W.A. (1980) in Molecular Basis of Environmental Toxicity (Bhatnagar, R.S., ed.), p. 3, Ann Arbor Science, Ann Arbor, Michigan.

Pryor, W..A, Stanley, J.P., and Blair, E. (1976) Lipids 11, 370.

Recknagel, R.O. (1967) Pharmacol. Rev. 19, 145.

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

Riley, C.A., Cohen, G., and Lieberman, M. (1974) Science 183, 208.

Roehm, J.N., Hadley, J.C., and Menzel, D.B. (1971) Arch. Environ. Health 23, 142.

Roels, O.A. (1967) Nutr. Rev. 25, 33.

Root, R.K., Metcalf, J., Oshino, N., and Chance, B. (1975) J. Clin. Invest. 55, 945.

Rotruck, J.T., Pope, A.L., Ganterh, H.E., Swanson, A.B., Hafeman, D.G., and Hoekstra, W.G. (1973) Science 179, 588.

Rotruck, J.T, Pope, A.L., Ganterh, H.E, and Hoekstra, W.G. (1972) J. Nutr. 102, 689.

Roubal, W.T. (1971) Lipids, 6, 62.

Roubal, W.T. annd Tappel, A.L. (1966) Arch. Biochem. Biophys. 113, 5.

Sbarra, A.J. and Karnosky, M.L. (1959) J. Biol. Chem. 234, 1355.

Scholes, G., Ward, J.F., and Weiss, J. (1960) J. Mol. Biol. 2, 379.

Sies, H., Gerstenecker, C., Menzel, H., and Flohe, L. (1972) FEBS Lett. 27, 171.

Sies, H., Gerstenecker, C., Summer, K.H., Menzel, H., and Flohe, L. (1974) in Glutathione (Flohe, J., Benohr, H., Sies, H., Waller, H.D., and Wendel, A., eds.), p. 261, George Thieme, Stuttgart.

Slater, T.F. (1972) in Free Radical Mechanisms in Tissue Injury, p. 4, Pior Limited, London, England.

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

Sugioka, K. and Nakano, M. (1975) Biochem. Biophys. Acta. 423, 203.

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

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

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

Swartz, H.M. (1972) in Biological Applications of Electron Spin Resonance (Swartz, H.M., Bolten, J.R., and Borg, D.C., eds.), Wiley (Interscience), New York.

Swern, D. (1965) in Organic Peroxides, John Wiley and Sons, New York.

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

Tappel, A.L. (1953b) Food Res. 8, 560.

Tappel, A.L. (1955) J. Biol. Chem. 217, 721.

Tappel, A.L. (1962) in Vitamins and Hormones: Advances in Research and Applications (Harris, R.S., ed.), Vol. 20, Academic Press, New York, NY.

Tappel, A.L., Fletcher, B., and Deamer, D. (1973) J. Gerontology 28, 415.

Tappel, A.L. and Zalkin, H. (1959) Arch. Biochem. Biophys. 80, 333.

Taube, H. (1965) J. Gen. Physiol. 49, 29.

Taube, H. (1965) in Oxygen, Proc. Symp., N.Y. Heart Assoc., p. 29, Little, Brown, Boston, Mass.

Thomas, H.V., Meuller, P.K., and Lyman, R.L. (1968) Science 159, 532.

Tien, M., Svingen, B.A., and Aust, S.D. (1981) Fed. Proc. 40, 179.

Turro, N.J. (1965) in Molecular Photochemistry, Benjamin, New York.

Tyler, D.D. (1975) FEBS Lett. 51, 180.

Ugazio, G., Kock, R.R., and Recknagel, R.O. (1973) Exp. Nol. Pathol. 18, 281.

Uri, N. (1961) in Autoxidations and Antioxidants, Vol. I (Lundberg, W.O., ed.), p. 55, Interscience Publishers, New York, NY.

van der Hoeven, T.A. and Coon, M.J. (1974) J. Biol. Chem. 249, 6302.

Vermilion, J.L. and Coon, M.J. (1978) J. Biol. Chem. 253, 8812.

Walling, C. (1975) Acc. Chem. Res. 8, 125.

Walling, C. and Weil, T. (1974) Int. J. Chem. Kinet. 6, 507.

Weening, R.S., Wever, R., and Roos, D. (1975) J. Lab. Clin. Med. 84, 245.

Welton, A.F. and Aust, S.D. (1972) Biochem. Biophys. Res. Commun. 49, 661.

Wertz, J.E. and Bolton, J.R. (1972) in Electron Spin Resonance-Elementary Theory and Practical Applications, McGraw-Hill, New York.

Wills, E.D. (1961) Biochem. Pharmacol. 7, 7.

Wills, E.D. (1975) Biochem. Biophys. Acta. 98, 238.

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

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

Yamazaki, I. (1971) Advan. Biophys. 2, 33.

Yasukochi, Y. and Masters, B.S.S. (1976) J. Biol. Chem. 251, 5337.

Zirlin, A. and Karel, M. (1969) J. Food Sci. 34, 160.

APPENDIX

Publications

Tien, M., Svingen, B.A., and Aust, S.D. (1981) "Superoxide Dependent Lipid Peroxidation", Fed. Proc. 40, 179-182.

Tien, M., and Aust, S.D. "Comparative Aspects of Several Model Lipid Peroxidation Systems", in Proceedings of International Symposium on Lipid Peroxide in Biology and Medicine.

Tien, M., and Aust, S.D. "Initiation of Lipid Peroxidation by Perferryl Complexes", to be published In Proceedings of International Conferences on Oxygen and Oxy-Radicals in Chemistry and Biology (Rodgers, M.A.J., ed.), Academic Press.

Tien, M., and Aust, S.D. "Hydroxyl Radical Involvement in Superoxide-Dependent Lipid Peroxidation" (submitted), Arch. Biochem. Biophys.

Tien, M., and Aust, S.D. "Rabbit Liver Microsomal Lipid Peroxidation" (submitted), Arch. Biochem. Biophys.

Morehouse, L.A., Tien, M., Bucher, J.R., and Aust, S.D. (1981) "The Role of Hydrogen Peroxide in Microsomal Lipid Peroxidation", (submitted), FEBS Lett.

In Preparation

Bucher, J.R., Tien, M., Morehouse, L.A., and Aust, S.D. "Hydroxyl Radical Involvement in NADPH-Dependent Lipid Peroxidation", J. Biol. Chem.

Forney, L.J., Tien, M., and Reddy, C.A. "Radical Initiated Degradation of Lignin by Phanerochaete Chrysosporium".

Abstracts

Tien, M., Svingen, B.A., and Aust, S.D. (1978) "Rabbit Liver Microsomal Lipid Peroxidation", The Pharmacologist 20, 183.

Tien, M., Svingen, B.A., and Aust, S.D. (March 9-13, 1980) "Hydroxyl Radical-Dependent Lipid Peroxidation", Nineteenth Annual Meeting of the Society of Toxicology, Washington, D.C.

Tien, M., Svingen, B.A., and Aust, S.D. (May 25-29, 1980) "Initiation of Lipid Peroxidation by Perferryl Complexes", An International Conference on Oxygen and Oxy-Radicals in Chemistry and Biology.

Aust, S.D., Tien, M., and Svingen, B.A. (1980) "Ferric Iron Dependent Lipid Peroxidation as a Function of pH", J. Am. Oil Chemists Soc. 57, 186.

Tien, M., Svingen, B.A., and Aust, S.D. (1980) "The Mechanism of Initiation of Lipid Peroxidation", Fed. Proc. 39, 1727.

Morehouse, L.A., Tien, M., and Aust, S.D. (May, 8, 1981) "The Effect of Hydrogen Peroxide on Microsomal Lipid Peroxidation", Toxicology in Michigan Today Symposium.

Tien, M., Bucher, J.R., Morehouse, L.A., and Aust, S.D. (July 12-18, 1981) "Spin Trapping of Radicals Involved in Lipid Peroxidation", International Symposium on Spin Trapping and Nitroxyl Radical Chemistry, University of Guelph.

Aust, S.D., and Tien, M. (November 7-8, 1980) "Comparative Aspects of Several Model Lipid Peroxidation Systems", International Symposium on Lipid Peroxide in Biology and Medicine, Nagoya, Japan.