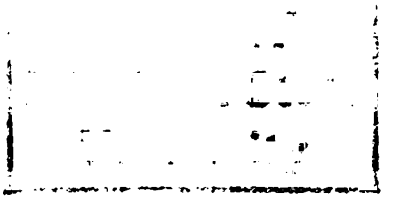

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Lee Alan Morehouse

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ROLE OF CYTOCHROME P450 IN HEPATIC MICROSOMAL MIXED FUNCTION OXIDASE-DEPENDENT SUPEROXIDE PRODUCTION AND LIPID PEROXIDATION

By

Lee Alan Morehouse

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ABSTRACT

ROLE OF CYTOCHROME P450 IN HEPATIC MICROSOMAL MIXED FUNCTION OXIDASE-DEPENDENT SUPEROXIDE PRODUCTION AND LIPID PEROXIDATION

By

Lee Alan Morehouse

NADPH-dependent lipid peroxidation (LP) requires the reduction of ferric ion prior to initiation. This work was undertaken to investigate the role of cytochrome P450 (P450) in this iron reduction step. Two mechanisms for iron reduction by the microsomal electron transport system and its components were examined: superoxide-($O_2^{\cdot-}$)-dependent and direct.

NADPH-Cytochrome P450 reductase (reductase) generated very low rates of $O_2^{\cdot-}$ production, indicating that oxygen is a poor substrate and $O_2^{\cdot-}$ is not the likely reductant. Some ferric chelates (i.e., EDTA-Fe^{3+}) were reduced by the reductase under anaerobic conditions and under aerobic conditions stimulated NADPH oxidation and $O_2^{\cdot-}$ production. Other ferric chelates (i.e., ADP-Fe^{3+}) were not reduced by the reductase, explaining previous results where ADP-Fe^{3+} did not promote LP in a model system dependent upon the reductase. Rat liver microsomes produced more $O_2^{\cdot-}$ than the reductase, but also promoted anaerobic reduction of ADP-Fe^{3+}.

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NADPH-dependent peroxidation of microsomes required only ADP-Fe^{3+} and was not inhibited to any appreciable extent by SOD. Addition of P450 to reductase resulted in a competent electron transport chain that also reduced ADP-Fe^{3+} and stimulated $O_2^{\cdot-}$ production. The degree of stimulation of $O_2^{\cdot-}$ production depended on the particular P450 isozyme; maximum stimulation occurred at P450: reductase of 4 or 5:1. The role of P450 in LP was demonstrated by incorporating P450 isozymes with reductase in phospholipid vesicles. ADP-Fe^{3+} was the only chelate necessary to promote LP, mimicking the intact microsomal system. LP was inhibited by approximately 30-40 percent by SOD, indicating the existence of both $O_2^{\cdot-}$-dependent and independent iron reduction pathways but underscoring the direct reduction pathway as the primary reduction mechanism.

The role of hydrogen peroxide (H_2O_2) in the initiation of microsomal LP was also examined. Ferrous is thought to reduce H_2O_2 generating the hydroxyl radical, the proposed initiating species. However, contrary to the theory, H_2O_2 inhibited LP whenever endogenous or exogenously-added catalase was inhibited by azide.

To

My Parents,

Bobbie

and

Andrew

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LIST OF ABBREVIATIONS

ADP	-	adenosine diphosphate
DLPC	-	dilauroylphosphatidylcholine
DMPO	-	5,5'-dimethyl-1-pyrroline-N-oxide
DMPO·OH	-	hydroxyl radical adduct of DMPO
DMPO·OOH	-	superoxide radical adduct of DMPO
DTPA	-	diethylenetriaminepentaacetate
EDTA	-	ethylenediaminetetraacetate
EPR	-	electron paramagnetic resonance
GSH	-	reduced glutathione
GSSG	-	oxidized glutathione
HBB	-	3,3',4,4',5,5' hexabromobiphenyl
MDA	-	malondialdehyde
MFO	-	mixed function oxidase or mixed function oxygenase
NADH	-	reduced nicotinamide adenine dinucleotide
NADPH	-	reduced nicotinamide adenine dinucleotide phosphate
Reductase	-	NADPH-cytochrome P450 reductase
SOD	-	superoxide dismutase
3-MC	-	3-methylcholanthrene

INTRODUCTION

Although oxygen is an absolute necessity for aerobes, higher partial pressures of oxygen are frequently toxic to organisms. The phenomenon of oxygen toxicity was described many years ago,[1] but the mechanism has remained unresolved at least until the early 1970's, and some researchers would argue even up to the present time.

In the late 1960's, McCord and Fridovich[2] discovered that the one electron reduction of ferricytochrome c by milk xanthine oxidase was mediated via $O_2^{\cdot-}$. Prior to that time, free radical species of any type and particularly $O_2^{\cdot-}$ were generally not considered to be produced in biological systems. Concurrently, they described a specific enzymatic activity for the nearly ubiquitous copper-containing protein erythrocuprein that at the time had no known function. They renamed the protein superoxide dismutase, SOD, in recognition of its ability to catalyze the one electron dismutation or disproportionation reaction of $O_2^{\cdot-}$.[3]

Subsequently, it has been shown that a number of purified enzymes and subcellular organelles generate $O_2^{\cdot-}$. Under the assumption that $O_2^{\cdot-}$ is like other highly reactive free radicals, it has been postulated that organisms must

possess sufficient scavenging capacity to protect against the "normal" fluxes of $O_2^{\cdot-}$ inferred to occur during *in vivo* metabolism. Accordingly, at least one form of SOD has been identified in nearly all aerobes. However, it is postulated that under certain physiological conditions the normal oxygen radical scavenging system is overwhelmed by increased fluxes of $O_2^{\cdot-}$ and/or is compromised in some way. This inability to scavenge oxygen radicals has been postulated to result in toxicity or lethality, and this hypothesis has become known as the superoxide theory of oxygen toxicity.[4]

There have been a number of studies linking excessive $O_2^{\cdot-}$ production to toxicity. However, rarely if at all has elevated $O_2^{\cdot-}$ production *in vivo* been directly demonstrated. Rather, it has usually been inferred from *in vitro* data. Thus, some researchers have expressed skepticism over the theory, pointing out that much of the data are circumstantial. A few have even maintained that $O_2^{\cdot-}$ production is unrelated to oxygen toxicity.[5,6]

However, any disagreement over the mechanism of oxygen toxicity does not lessen the significance of McCord and Fridovich's discoveries, for they have spawned an entirely new area of biological investigation: the role of oxygen radicals in biology and medicine. Over the past decade, $O_2^{\cdot-}$ and the other reduction products of oxygen have been demonstrated or postulated to be involved in numerous physiological, pathological, and toxicological states including phagocytic killing by leukocytes,[7] inflammation,[8]

arthritis,[9] mutagenesis,[10] tumor promotion,[11] diabetes,[12] ischemia,[13] trauma,[14] spinal cord injury,[15] aging,[16] inactivation of enzymes and marking them for proteolysis,[17] peroxidation of membrane lipids,[18] and the toxicity of numerous xenobiotics.[19-23]

This dissertation examines the ability of the NADPH-dependent electron transport in microsomes to generate $O_2^{\cdot-}$ or reduce iron chelate and the relative importance of these two distinct activities in initiating lipid peroxidation, the oxidative degradation of polyunsaturated fatty acids that is proposed to occur in many of the aforementioned states in which oxygen free radicals are proposed to be involved. Chapter I examines the potential of the microsomal electron transport system to reduce ferric chelates commonly used in lipid peroxidation. Previous work in Dr. Aust's laboratory has shown that the NADPH-dependent peroxidation of microsomes requires reduced iron, but iron reduction by microsomes did not appear to be dependent upon $O_2^{\cdot-}$. The results in this chapter provide evidence for an alternate iron reduction pathway.

The potential of rat liver microsomes to generate $O_2^{\cdot-}$ has been known for over a decade. Yet there is considerable disagreement over the amount of $O_2^{\cdot-}$ that is produced. In Chapter II, rates of $O_2^{\cdot-}$ production by rat liver microsomes, and purified NADPH-cytochrome P450 reductase alone or reconstituted with several purified isozymes of

cytochrome P450 are measured using an acetylated cytochrome c reduction assay.

The importance of $O_2^{\cdot-}$ and H_2O_2 in the initiation of microsomal lipid peroxidation promoted by ADP-Fe^{3+} is investigated in Chapter III. According to the iron-catalyzed Haber-Weiss reaction, these partially-reduced oxygen species should be necessary for the generation of ·OH, the proposed initiator of lipid peroxidation. Thus various factors that increase or decrease the production of $O_2^{\cdot-}$ and/or H_2O_2 in microsomes have been tested and their effects on the resultant rates of lipid peroxidation have been measured.

Lastly, in Chapter IV, the role of cytochrome P450 isozymes in the initiation of microsomal lipid peroxidation is examined. Previous studies have shown cytochrome P450 to function in the decomposition of lipid hydroperoxides, but no studies have shown a role in the initiation of lipid peroxidation. The necessity for cytochrome P450 in lipid peroxidation promoted by ADP-Fe^{3+} is demonstrated in a model lipid peroxidation system.

This dissertation is divided into four chapters, each written in a format similar to that of many scientific journals. Each chapter contains an abstract of the work, an introduction to the pertinent literature, material and methods section, results, discussion and references. To avoid unnecessary duplication the material and methods sections in subsequent chapters describe only those methods

or materials not used in preceding chapters. The chapters are preceded by a broad review of the literature on microsomal electron transport, oxygen radical generation and lipid peroxidation.

REVIEW OF LITERATURE

Chemistry of Dioxygen and Its Reduction Products

Dioxygen - Molecular oxygen or dioxygen has two unpaired electrons in its π^* antibonding orbital and, therefore, exists in a triplet ground state. As such, kinetic constraints prevent its direct reaction with most organic compounds that are of singlet multiplicity. Before using oxygen as an oxidant, organisms must activate it in order to overcome this spin restriction.

Activation can occur via several pathways. Photochemical activation can result in the formation of singlet oxygen, a transient species capable of reacting directly with organic molecules. However, the major means by which organisms activate oxygen involves its reduction and/or complexation with metalloenzymes.

Oxygen can be reduced to two molecules of water in four discrete one electron reduction reactions:

$$O_2 + e^- \text{-------} > O_2^{\cdot-} \quad (1)$$

$$O_2^{\cdot-} + e^- \text{-------} > O_2^{2-} \quad (2)$$

$$H_2O_2 + e^- \text{------} > \cdot OH + OH^- \quad (3)$$

$$\cdot OH + e^- \text{------} > OH^- \quad (4)$$

Reduction of oxygen by one, two, or three equivalents results in the formation of $O_2^{\cdot-}$, H_2O_2 or $\cdot OH$, respectively. These three species are collectively referred to as the

partially reduced oxygen species, oxygen radicals, or oxy radicals.

The chemistry of oxygen and its reduction products is complex in part because it can vary drastically with the experimental conditions. As an example, the redox potential of $O_2/O_2^{\cdot-}$ varies from -0.58v in aprotic solvents to -0.33v in water.[24] The reduction potentials of the other oxygen radical species are all positive, so the overall reduction of oxygen to water is exothermic.

Superoxide - The premise of the superoxide theory of oxygen toxicity is that $O_2^{\cdot-}$ is either intrinsically toxic to organisms or gives rise to other reactive species which subsequently initiate oxidative damage. An examination of the chemistry of $O_2^{\cdot-}$ reveals that this implication may be a weak link in the theory. In general, despite its free radical nature, $O_2^{\cdot-}$ is relatively unreactive towards most biological molecules.

The chemistry of $O_2^{\cdot-}$ is, in general, governed by the solvent; it is considerably more reactive in aprotic solvents than in aqueous solutions.[24] In aprotic media it is a good nucleophile and reductant and reacts with many organic compounds. A matter of debate in biology is its purported reactions with biological molecules such as polyunsaturated fatty acids[25], lactate dehydrogenase-bound NADH[26] and α-tocopherol[27] since certain cellular

microenvironments might be sufficiently aprotic to promote such reactions.

In aqueous media $O_2^{\cdot-}$ is considerably less reactive, its predominant reaction being non-enzymatic dismutation to H_2O_2 and oxygen. The rate of dismutation is highly pH dependent, occurring most rapidly at pH 4.8, the pk_a for $O_2^{\cdot-}$.

$$HO_2^{\cdot} + O_2^{\cdot-} \xrightarrow{H^+} H_2O_2 + O_2 \qquad (5)$$

Enzymatic dismutation is catalyzed by a family of SODs. Three types of SOD have been isolated and characterized; each contains a different redox-active metal: manganese, iron or copper. During the catalytic cycle of the enzymatic dismutation, the metal is reduced by the first $O_2^{\cdot-}$ molecule then oxidized by the second. The redox potentials of the $O_2/O_2^{\cdot-}$ (-0.33v) and the $O_2^{\cdot-}/H_2O_2$ (+0.87v)[24] couples are such that $O_2^{\cdot-}$ can act as a reductant towards many metal chelates and an oxidant towards the same reduced chelates. Thus, it is not surprising that a dismutase-like activity has also been ascribed to redox-active complexes containing manganese,[28] iron,[29] and copper.[30] However, these complexes do not have the specificity for $O_2^{\cdot-}$ that characterizes the SODs.[31,32]

The reactions of $O_2^{\cdot-}$ with metal complexes are governed by several factors. First of all the reactions must be sufficiently fast to compete with non-enzymatic dismutation. Second, the redox potential of the metal must be favorable and chelation influences this potential. For example, EDTA chelation of ferrous changes its redox potential from -0.77v

to -0.12v.[33] The effect of this chelation on ferrous autoxidation is dramatic; ferrous autoxidizes extremely slowly at pH 7, whereas EDTA-Fe^{2+} autoxidizes extremely rapidly. Chelation can also affect oxygen-metal redox reactions in a kinetic fashion since inner sphere electron transfer is the typical mechanism by which these reactions occur. Chelators like DTPA, desferrioxamine, and phytic acid inhibit these reactions by occupying all coordination sites of the metal.[34]

Hydrogen Peroxide - Like $O_2^{\cdot-}$, H_2O_2 is relatively unreactive in aqueous solution except in the presence of transition metals. Cleavage of the peroxide bond occurs either homolytically (6) or heterolytically (7), depending upon the oxidation state of the metal:

$$Fe^{2+} + H_2O_2 \text{ ------} > Fe^{3+} + \cdot OH + OH^- \qquad (6)$$

$$Fe^{3+} + H_2O_2 \text{ ------} > Fe^{2+} + HO_2\cdot + H^+ \qquad (7)$$

Reaction (6) is known as Fenton's Reaction.[35] Originally it was demonstrated to be a redox chain reaction with the substrate radical (formed by the $\cdot OH$-mediated oxidation of substrate) reducing ferric to ferrous.[36] However, in biological systems low concentrations of iron probably preclude such a reaction, so the substrate radical is free to oxidize other cellular constituents.

Hydrogen peroxide is scavenged <u>in vivo</u> by two enzymes located at different cellular sites. The hemoprotein catalase is located in the peroxisomes, but due to their

fragility, peroxisomes are often ruptured during cell lysis and traces of catalase activity are often found associated with other subcellular fractions. Catalase catalyzes the two-electron dismutation of H_2O_2 produced by the peroxisomal oxidases.

$$2H_2O_2 \text{ -------> } 2H_2O + O_2 \qquad (8)$$

This reaction occurs in two distinct steps:

$$Fe^{3+} + H_2O_2 \text{ -------> } (FeO)^{3+} + H_2O \qquad (9)$$

$$(FeO)^{3+} + H_2O_2 \text{ ------> } Fe^{3+} + O_2 + H_2O \qquad (10)$$

where Fe^{3+} is the heme iron of catalase. The enzyme-oxygen intermediate is known as Compound I and is an intermediate also common to peroxidases, enzymes that are specific for H_2O_2 as an oxidant but not as a reductant. Many peroxidases such as that purified from horseradish are relatively non-specific, oxidizing numerous hydrogen donor substrates with many of these reactions forming the basis of assays for H_2O_2.[37,38] Catalase is more specific than peroxidases, oxidizing predominantly H_2O_2, but also short chain alcohols.

The selenium-dependent GSH peroxidase is a cytosolic enzyme that catalyzes the reduction of H_2O_2 to H_2O.

$$H_2O_2 + 2GSH \text{ ------> } 2H_2O + GSSG \qquad (11)$$

Reducing equivalents are supplied by GSH, and the product is GSSG. High levels of GSSG can have adverse effects on cells,[39,40] so a necessary complement to this H_2O_2-scavenging system is NADPH-dependent GSH-reductase.

$$GSSG + NADPH \text{ ------> } 2GSH + NADP^+ \qquad (12)$$

Hydroxyl Radical - The ·OH is the most potent oxidizing species proposed to be generated in vivo. Its reactions can be characterized into four general types: abstraction, addition, electron transfer, and radical addition or termination.[41]

$$\cdot OH + C_2H_4 \text{ -------> } C_2H_4OH\cdot \quad (13)$$

$$\cdot OH + C_2H_6 \text{ -------> } C_2H_5\cdot + OH^- \quad (14)$$

$$\cdot OH + [Fe(CN)_6]^{4-} \text{ -----> } [Fe(CN)_6]^{3-} + OH^- \quad (15)$$

$$\cdot OH + \cdot OH \text{ -------> } H_2O_2 \quad (16)$$

Many of the reactions of ·OH with organic molecules proceed with second order rate constants that approach and occasionally even exceed diffusion-controlled rates.[41] Thus, the potential of ·OH to oxidize biological molecules is unquestioned. Numerous studies have demonstrated the oxidation of proteins, enzymes, nucleic acids, and lipids by ·OH generated in vitro. However, there is little evidence for the in vivo generation of ·OH or its subsequent oxidation of biological molecules.

Measurement of Oxygen Radical Production

The superoxide theory of oxygen toxicity holds that an overproduction of $O_2^{\cdot-}$ or a compromised ability to scavenge it renders a cell or organism susceptible to oxidative stress. However, to date, many questions that are raised by this theory have not been answered. What constitutes a damaging flux of $O_2^{\cdot-}$? How much scavenging potential does a cell have? How much auxiliary capacity is present?

These questions have proven difficult to answer because measurement of oxygen radicals *in vivo* has not yet been accomplished. In fact, even the measurement of oxygen radicals *in vitro* has proven to be quite difficult, generally because of several factors. One is the chemical nature of the free radical species themselves. Their intrinsic reactivity usually dictates that the free radicals must be measured indirectly, relying on their reactions with various indicator molecules. Second is the potential for non-specificity in that most of these methods are based on the oxidation or reduction of the indicator molecules, reactions not limited solely to the oxygen radicals of interest. Lastly, there is inherent difficulty in measuring oxygen radical production in a heterogeneous system such as in membranes.

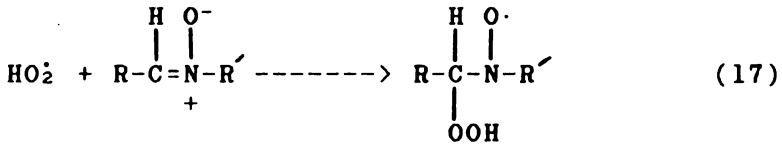
Superoxide -Superoxide has proven to be the most difficult of the partially-reduced oxygen species to quantitate because of its unique reactivity. In aqueous solutions its predominant reaction is disproportionation, so it does not accumulate in aqueous solutions to a concentration sufficient to directly detect it. Most methods used for detection of $O_2^{\cdot-}$ rely on its properties as a reductant or oxidant in aqueous solution. Among the most commonly used methods are the reduction of molecules such as cytochrome c,[42] nitroblue tetrazolium,[43] tetranitromethane,[3] and other

electron acceptors, or the oxidation of compounds like epinephrine,[3] tiron,[44] hydroxylamine, or sulfite.[45]

By far the most preferable assay is the reduction of cytochrome c and it has been used for the detection of $O_2^{\cdot-}$ produced by purified enzymes, subcellular fractions and intact cells. One shortcoming of this assay is that ferricytochrome c can be directly reduced by some flavoproteins and cytochromes, including the reductase.[46] With such enzymes, cytochrome c reduction is not suitable as an assay for $O_2^{\cdot-}$ since it is competing with oxygen for enzymatic reducing equivalents, in addition to scavenging any $O_2^{\cdot-}$ that might be produced.

Several investigators have chemically modified cytochrome c by either acetylation or succinoylation to make its reduction a more broadly applicable assay for $O_2^{\cdot-}$.[46,47] Acylation of cytochrome c decreases its tendency for direct reduction, while usually maintaining its ability to react with $O_2^{\cdot-}$. The suitability of the modified preparations for the measurement of $O_2^{\cdot-}$ production appears to depend upon the percent modification of cytochrome c.

Another commonly used method for $O_2^{\cdot-}$ production is EPR spin trapping. The detection of $O_2^{\cdot-}$ or other free radicals is made possible by their reactions with nitrones or nitroso compounds yielding more stable free radicals that can accumulate to concentrations sufficient to detect them directly with EPR spectroscopy.[48]



(17)

The intensity of the free radical signal is indicative of the concentration of the radical species, and the hyperfine splitting constants of the radical-spin trap adduct signal can also be used as a means to identify which free radical species has been trapped. However, spin trapping of oxygen radicals, in particular $O_2^{\cdot-}$, as a quantitative method has several shortcomings. One of these is the extremely slow reaction of $O_2^{\cdot-}$ with DMPO, the most commonly used nitrone spin trap for oxygen radical detection. Second-order rate constants on the order of 10 to 20 $m^{-1}s^{-1}$ have been reported,[49] thus large concentrations of DMPO are necessary to compete with the other reactions of $O_2^{\cdot-}$ that occur with rate constants many orders of magnitude greater than that of the trapping reaction. Secondly, the DMPO·OOH spin-trap adduct is itself not particularly stable having a half-life estimated to be 27-91 sec.[50] Moreover, decomposition of DMPO·OOH results in the DMPO·OH adduct. Thus spin trapping should really only be considered as a qualitative method for the detection of $O_2^{\cdot-}$.

Hydrogen Peroxide - Quantitation of H_2O_2 is considerably less difficult than $O_2^{\cdot-}$. Nearly all methods rely on the

action of catalase or peroxidases in the coupled oxidation of a variety of hydrogen donor substrates.[37,38]

$$H_2O_2 + Fe^{3+} \text{-------} > H_2O + (FeO)^{3+} \quad (9)$$

$$(FeO)^{3+} + \text{substrate} \text{-----} > Fe^{3+} + \text{product} \quad (18)$$

The compound I of catalase or peroxidase can be observed directly[51] or oxidizes a number of compounds including ethanol, scopoletin, phenol, o-dianisidine to products detected with uv-visible or fluorescence spectroscopy. However, subcellular fractions typically contain traces of catalase activity, so inhibitors of catalase activity such as azide are normally added to incubations containing these fractions.

Hydroxyl Radical - Quantitation of ·OH production is nearly as difficult as $O_2^{\cdot-}$, but for opposite reasons. While the reactivity of $O_2^{\cdot-}$ is limited in aqueous solutions, the ·OH is highly reactive, reacting with most organic compounds. Thus, there is no lack of compounds that can be used to detect ·OH, but as with $O_2^{\cdot-}$ the oxidations of these molecules are not necessarily specific for ·OH. Compounds that have been widely used include methional, dimethyl sulfoxide, benzoate, and salicylate[45] with the yield of products being indicative of the amount of ·OH formed.

EPR spin trapping has also been used to detect ·OH formation <u>in vitro</u>.[49] However, since $O_2^{\cdot-}$ is often produced in systems in which the production of ·OH is being assayed, it is not always possible to determine the fraction of

DMPO·OH signal intensity resulting from the decomposition of the DMPO·OOH. Likewise, there are other factors that may result in a decrease of DMPO·OH signal intensity. Therefore, spin trapping can probably only be considered as a qualitative measure of ·OH formation.

Cellular Sites of Oxygen Radical Generation

Numerous cellular enzymes utilize oxygen, and those characterized as oxidases reduce oxygen with reducing equivalents derived from the oxidation of their substrates. Although mitochondrial cytochrome oxidase reduces oxygen completely to water without the release of intermediate dioxygen reduction products, most other cellular oxidases produce $O_2^{\cdot-}$ and H_2O_2.

It is not known what the consequences of these normal fluxes of $O_2^{\cdot-}$ and/or H_2O_2 are to the cell, but it has been suggested that they might relate to the oxidation of cellular constituents that perhaps is a part of the aging process.[16] From the near ubiquitous nature of the scavenging enzymes in aerobes, it is inferred that $O_2^{\cdot-}$ and H_2O_2 generated during normal metabolism could be toxic, and the scavenging enzymes would therefore serve to protect cells from these potentially deleterious oxygen radicals. The scavenging capacity of these enzymes within the cell is not known, but it is proposed that a variety of conditions can result in overproduction of oxygen radicals to levels exceeding the scavenging potential.

There are several subcellular locales where O_2^- and H_2O_2 are produced. Several cellular oxidases are located in the peroxisomes.[51] Among them are D-amino acid oxidase, urate oxidase, and fatty acyl CoA oxidase, all which generate H_2O_2. Peroxisomes also contain large amounts of catalase, but despite this, some H_2O_2 still appears to diffuse from the peroxisomes.[51] The toxicity of the hyperlipidemic or peroxisomal proliferating drugs is proposed to be the result of the induction of the peroxisomal H_2O_2-producing oxidases and a concomitant increase in peroxisomal H_2O_2 production.[52]

Cytosolic enzymes such as aldehyde oxidase and xanthine oxidase generate $O_2^{\cdot}$ and H_2O_2 during the oxidation of a variety of substrates. Xanthine oxidase is a popular enzyme for use in <u>in vitro</u> studies of oxygen radicals because it is readily obtainable from biological sources and because it generates both $O_2^{\cdot}$ and H_2O_2. However, in the liver and in other tissues the physiological form of the enzyme is proposed to be a dehydrogenase, using NAD^+ rather than oxygen as the oxidant. During an ischemic insult, Ca^{2+}-dependent proteolysis of the enzyme is proposed to occur, converting the dehydrogenase to an oxidase that upon reperfusion can result in an additional source of $O_2^{\cdot}$ and H_2O_2.[13]

Despite the coupled nature of mitochondrial electron transport, a small percent of reducing equivalents appear to "bleed off" the electron transport chain producing $O_2^{\cdot}$ via

the autoxidation of ubisemiquinone.[53] Under conditions such as ischemia or electron transport blockade with azide or cyanide where the mitochondrial electron transport carriers become highly reduced, the concentration of ubisemiquinone increases and its rate of autoxidation increases proportionally. Regardless of the rate of $O_2^{\cdot-}$ production, it appears that only H_2O_2 efflux from mitochondria occurs, presumably because of the efficient scavenging of $O_2^{\cdot-}$ by the mitochondrial Mn-containing SOD.

Polymorphonuclear leukocytes and other phagocytic cells contain a plasma membrane-bound electron transport system consisting of an NADPH dehydrogenase, quinone and a b-type cytochrome.[7] Appropriate stimuli trigger the production of $O_2^{\cdot-}$ by this system, the so-called "oxygen burst." This burst of oxygen radical production contributes to oxidative killing of bacteria, but is also proposed to be involved in the inflammatory response.

Another major source of oxygen free radicals is the microsomal electron transport system. Its function is to catalzye the oxidation of numerous substrates both endogenous and exogenous.

$$NADPH \cdot H^+ + O_2 + SH \text{ ------> } NADPH + H_2O + SOH \quad (19)$$

As shown above, it catalyzes the insertion of one atom of oxygen into the substrate, thus it has monooxygenase activity. The other atom of oxygen is reduced to water, so the system has both oxygenase and oxidase activity and is often called the MFO system. This electron transport system

also generates $O_2^{\cdot}$ and H_2O_2 as is discussed in a subsequent section.

Microsomal Electron Transport System

The electron transport system of microsomes is composed of two distinct electron transport chains, differentiated by the pyridine nucleotide cofactors they use as electron donors. One chain is specific for NADH, consists of the flavoprotein NADH-cytochrome b_5 reductase and cytochrome b_5, and serves as an electron donor to the stearyl-Co A desaturase system. The other electron transport chain is NADPH-specific, consists of the flavoprotein reductase and the various isozymes of cytochrome P450 and functions in the metabolism of various substrates. While these electron transport chains are separate in terms of function, they do interact. There is a well documented NADH synergism of NADPH-dependent mixed function oxidase activity.[54] Others have suggested that the reductase might also reduce cytochrome b_5.[55]

The reductase is an amphipathic molecule having both a globular hydrophilic domain containing its catalytic sites and a hydrophobic membrane-binding domain. Its electron transfer properties are quite unique relative to other flavoproteins. It contains 1 mole FMN and 1 mole FAD per mole of enzyme, distinguishing it from other flavoproteins that usually contain only a single flavin moiety. It utilizes a 2 electron donor in NADPH but reduces cytochrome

P450 and other substrates by 1 electron.[56,57] The variety of substrates it reduces is quite diverse: cytochrome P450 and perhaps cytochrome b_5, cytochrome c, ferricyanide, paraquat, 2,6-dichlorophenolindophenol, nitroso compounds, and numerous naphthoquinones and anthracyclines.

Early purification procedures relied on the solubilization of the catalytic domain from the membrane by treatment of rat liver microsomes with proteases.[58,59] This protease-solubilized reductase retained its ability to reduce numerous exogenously added substrates such as cytochrome c, ferricyanide and 2,6 dichlorophenolindophenol, but its electron acceptor in the microsomal membrane was not identified until later.

The reductase has more recently been purified from microsomal membranes following detergent solubilization.[60,61] Enzyme purified in this manner retains its hydrophobic domain and reduces cytochrome P450 as well as the other electron acceptors. Lu and Coon[62] utilized this detergent-solubilized reductase to reconstitute MFO activity in DLPC micelles also containing cytochrome P450. No activity was observed when the protease-solubilized enzyme was used. Thus, cytochrome P450 isozymes and the reductase incorporated in phospholipid vesicles also form a competent electron transfer chain and exhibit MFO activity.

The orientations of the reductase and cytochrome P450 in the microsomal membrane are such that an interaction between them can easily be postulated even though the

biochemical mechanism of the interaction is not completely understood. Microsomal cytochrome P450 isozymes are integral membrane proteins, whereas the reductase's globular region sits above the plane of the membrane perched on its hydrophobic domain anchored in the membrane. There are approximately 20-30 cytochrome P450 molecules per reductase molecule, and there is some controversy over whether the microsomal proteins are present as preformed clusters or laterally diffusing.[63] Clearly, the structure of the reductase suggests its potential to operate within a cluster of cytochrome P450 isozymes reducing some or all of them by pivoting about its hydrophobic domain.

Cytochrome P450 isozymes metabolize drug substrates by activating molecular oxygen with reducing equivalents generated by the reductase and catalyzing the insertion of one atom of oxygen into the substrate. It does this by a rather complex series of steps that have not been completely delineated. Figure 1 shows the proposed scheme of the redox cycle of cytochrome P450 isozymes.[64] The "resting" state of cytochrome P450 in the membrane is in the ferric oxidation state and mostly low spin (I). However, it exists in an equilibrium between low and high spin and numerous conditions such as temperature, pressure and ionic strength affect this equilibrium.[65-67] The most important effector is the binding of substrate which frequently induces a conversion to high spin. The reductase reduces cytochrome P450 by one electron yielding the ferrous form of the

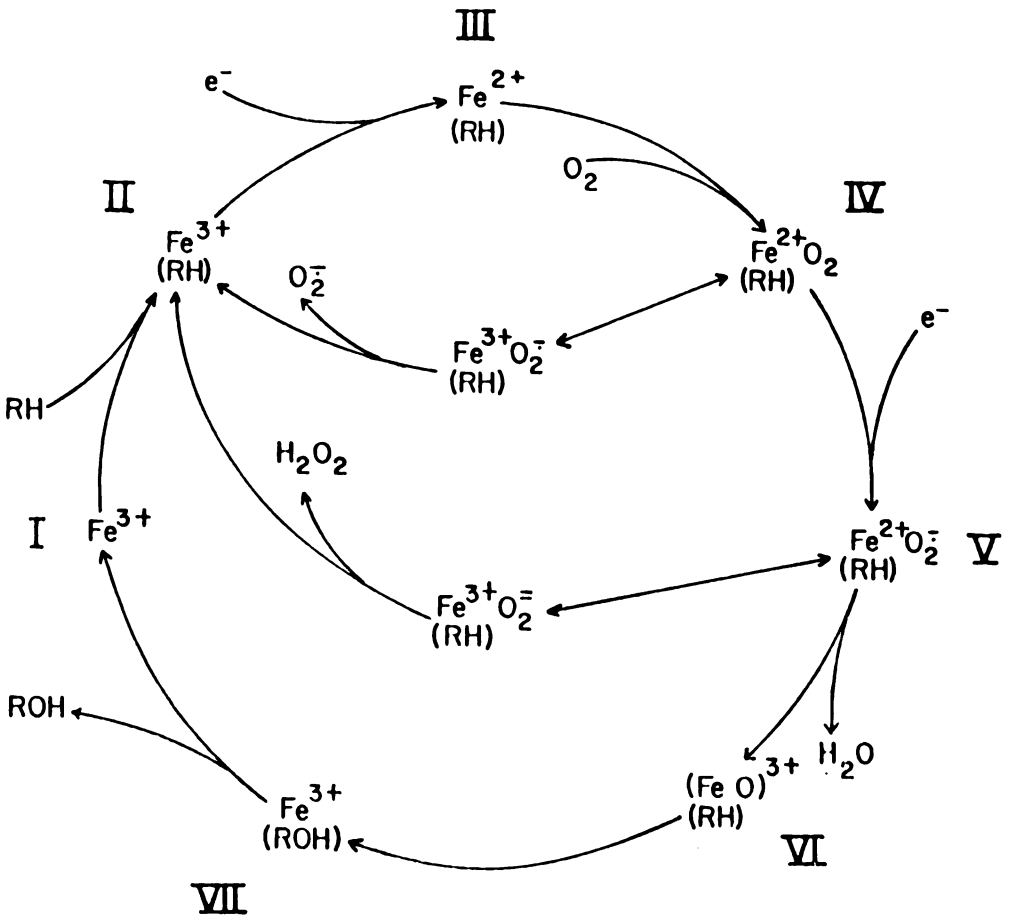


Figure 1: <u>Schematic of Cytochrome P450-Dependent Oxidations:</u>
The substrate is represented by RH and its oxidation product by ROH. The heme iron of cytochrome P450 is represented by Fe^{3+}. Oxidation states of iron and oxygen are assigned to show stoichiometry only.

hemoprotein (II) to which oxygen readily binds (III). The addition of a second electron furnished either by the reductase or cytochrome b_5 yields a heme iron-oxygen complex reduced by two electrons (IV). These two reducing equivalents reduce one atom of oxygen to water, forming an activated complex of unknown identity but apparently having the oxidation state of Compound I of catalase or peroxidase (V). This complex then oxidizes substrate to generate the product (VI) and the ferric form of cytochrome P450 (VII).

Cytochrome P450 can also use reducing equivalents obtained via cleavage of organic hydroperoxides, in much the same way that compound I of catalase is formed with H_2O_2.[64] The activated iron-oxygen intermediate may be the same as that generated by the sequential NADPH-dependent reductions. However, cleavage of the hydroperoxide yields a radical species that can alkylate and inactivate the enzyme.

Uncoupling of Microsomal Electron Transport

The degree of uncoupling exhibited by the microsomal electron transport system is considerably greater than that of the mitochondria at least *in vitro*, although oxygen radical production by the endoplasmic reticulum *in vivo* has not been unequivocally demonstrated. Microsomal uncoupling occurs with the production of $O_2^{\cdot}$, H_2O_2, or additional H_2O with the following stoichiometries:

$$NADPH \cdot H^+ + O_2 \text{ -------} > 2\ HO_2^{\cdot} + NADP^+ \quad (20)$$

$$NADPH \cdot H^+ + O_2 \text{ -------} > H_2O_2 + NADP^+ \quad (21)$$

$$2NADPH \cdot H^+ + O_2 \text{ ------} > 2H_2O + 2NADP^+ \quad (22)$$

Since microsomal metabolism is often accompanied by greater NADPH and O_2 consumption than predicted in (19) and NADPH is consumed in the absence of substrates, the uncoupling reactions listed above appear to occur both in the presence and absence of substrate. It is not clear whether the $O_2^{\cdot}$ and H_2O_2 produced by microsomes in the absence of substrate is due to the reduction of that fraction of cytochrome P450 present in the high spin conformation.

Some have proposed that the reductase generates $O_2^{\cdot}$ via autoxidation of a reduced flavin moiety,[68,69] while others have proposed that very little if any is generated.[56,57] However, for several reasons cytochrome P450 is postulated to have a more important role in microsomal $O_2^{\cdot}$ and H_2O_2 production. Microsomal oxygen radical production is greater in the presence of many substrates than in their absence[70,71]. Cytochrome P450 inhibitors also inhibit microsomal $O_2^{\cdot}$ and H_2O_2[72,73] production although inhibition is not usually complete. Microsomes isolated from animals treated with inducers of cytochrome P450 exhibit different rates of $O_2^{\cdot}$ and H_2O_2 production.[70]

The mechanism by which uncoupling occurs is unclear. It is generally considered that the oxy-ferrous (IV) and ferrous superoxo (V) complexes of cytochrome P450 dissociate to $O_2^{\cdot}$ and H_2O_2, respectively. It is hypothesized that the

bulk of H_2O_2 generated by microsomes originates from $O_2^{\cdot}$[74] suggesting that the transfer of the second electron to the oxy-ferrous complex of cytochrome P450 might be a limiting step in drug metabolism and/or oxygen reduction. The observation of a four electron uncoupling reaction[75,76] is intriguing because this suggests that the activated cytochrome P450 complex (V) might subsequently be reduced by two electrons to generate additional water.

Induction of Cytochrome P450

Another area of active current research in the biochemistry of the cytochrome P450 system that may have significance with respect to oxygen radical production is the inducibility of cytochrome P450 isozymes in response to various xenobiotics.[77] Two major classes of inducers have been described. One class of inducers is typified by 3-MC and is termed 3-MC type inducers. Other polyhalogenated aromatic hydrocarbons that can assume planar conformations such as dioxins, naphthalenes, β-naphthoflavone, and certain congeners of polychlorinated and polybrominated biphenyls, and 3-MC all bind to a cytosolic receptor protein called the Ah receptor. The receptor-ligand complex translocates to the nucleus, binds to chromatin, and induces the transcription of several genes including two isozymes of cytochrome P450. These are termed P450c and d in the nomenclature of Levin et al.*[78] The other major class of inducing agent is typified by the barbituate phenobarbital

and is characterized by the induction of several forms of cytochrome P450, the major isozyme being cytochrome P450b in the Levin nomenclature.[79] Induction of the reductase also occurs upon treatment of animals with phenobarbital. The mechanism by which phenobarbital induction occurs is still not known.

Comparatively very little work has been done on the oxidase activities of the various isozymes. The investigations that have been conducted have mostly focused on oxygen radical production by microsomes from phenobarbital-treated animals.[70,74,80,81] The major phenobarbital-inducible isozyme has been tested for its ability to produce $O_2^{\cdot-}$ and especially H_2O_2 in reconstituted systems.[71,75,81,82] Other studies have investigated the autoxidation of several purified chemically-reduced cytochrome P450 isozymes, and it appears that ferrocytochrome P450 do autoxidize producing $O_2^{\cdot-}$ and/or H_2O_2.[83,84]

Lipid Peroxidation

Biological membranes have a critical role in maintaining cellular integrity and function. This fact,

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*Other nomenclatures are based on relative mobility of isozymes on SDS slab gels, sequence of elution from DEAE columns or on type of inducing ligand used, but that of Levin is the simplest and will be used throughout this dissertation.

when taken with the chemical nature of phospholipids, makes the oxidation of membrane phospholipids a potentially deleterious phenomenon, perhaps a more severe insult to biological organisms than damage to proteins or even to DNA. Due to the free radical nature of the oxidation of phospholipids, initiation of the chain reaction can lead to the oxidation of neighboring phospholipids. Therefore, the formation of a relatively small amount of oxidants can lead to the oxidation of many phospholipids resulting in loss of membrane fluidity, rupture of cells and/or organelles, activation and/or inactivation of membrane-associated enzymes, altered membrane permeability, and ionic gradients, and loss of Ca^{2+} homeostasis. In addition, by-products of lipid peroxidation such as MDA, 4-hydroxynonenal and lipid hydroperoxides have been shown to have cytotoxic properties.[85-87]

The chemistry of lipid peroxidation was detailed in the 1950's, mostly by oil and food chemists investigating rancidity in fats. The process consists of three distinct phases: initiation, propagation, and termination.[88] In initiation reactions, abstraction of allylic or methylene hydrogens from polyunsaturated fatty acids (LH) by an initiating species (I·) results in lipid alkyl radicals (L.).

$$LH + I\cdot \text{ -------} > L\cdot + IH \qquad (23)$$

These radicals then enter into propagation reactions where the net number of free radical species is conserved. Alkyl

radicals react with dioxygen to form lipid peroxyl radicals (LOO·) that abstract methylene hydrogens from neighboring polyunsaturated fatty acids resulting in lipid hydroperoxides and new alkyl radicals:

$$L\cdot + O_2 \text{ -------> } LOO\cdot \qquad (24)$$

$$LOO\cdot + L'H \text{ -----> } LOOH + L'\cdot \qquad (25)$$

Secondary initiation reactions involving the cleavage of lipid hydroperoxides by transition metals are also important. These reactions have been termed lipid hydroperoxide dependent initiations[89] and are quite analogous to those described for H_2O_2.

$$Fe^{2+} + LOOH \text{ -------> } Fe^{3+} + LO\cdot + OH^- \qquad (26)$$

$$Fe^{3+} + LOOH \text{ -------> } Fe^{2+} + LOO\cdot + H^+ \qquad (27)$$

Cleavage of lipid hydroperoxides is generally facilitated by acid pH, although heme and certain other ferric chelates rapidly catalyze the decomposition of peroxides at neutral pH.[90]

Termination reactions result in the net consumption of free radicals.

$$LOO\cdot + L\cdot \text{ -------> } LOOL \qquad (28)$$

From a practical standpoint, termination reactions also occur when free radical species abstract hydrogen from compounds that form more stable free radicals that are unable to participate subsequent propagation reactions. The lipid-soluble antioxidant vitamin E or α-tocopherol and sulfhydryl compounds are proposed to act in this manner.

The degree or extent of lipid peroxidation can be assayed in a number of ways. One of the most popular methods involves the condensation of MDA with thiobarbituric acid to form a Schiff base with an absorbance maximum at 535 nm. Other methods of assessing lipid peroxidation include assays for lipid hydroperoxides, disappearance of polyunsaturated fatty acids, conjugated diene formation, direct HPLC assays for malondialdehyde, release of ^{51}Cr, lactate dehydrogenase and other cytosolic enzymes from cells in culture, and ethane and pentane expiration in animals.[91] The choice of assay methodology is usually based on the particular system of study.

Cellular Antioxidant Defenses

As previously mentioned cells contain SOD, catalase, and GSH peroxidase that scavenge the oxygen radicals produced during cellular metabolism. In addition to reducing H_2O_2, GSH peroxidase also catalyzes the reduction of lipid hydroperoxides to the corresponding alcohols. However, besides these enzymes, cells have several additional antioxidant defense mechanisms. Perhaps the most studied of these is α-tocopherol or vitamin E.

Vitamin E is a lipid-soluble antioxidant present at different concentrations in various tissues. It is a component of membranes and is proposed to terminate the free radicals chain reaction of lipid peroxidation. The importance of vitamin E is clearly evident from the

nutritional studies that demonstrate the protective effects that vitamin E supplementation or the deleterious effects vitamin E deficiency has on organisms subjected to oxidative stress.[92]

Other species like GSH, and protein or non-protein sulfhydryls may act in a similar manner to vitamin E by reacting with lipid peroxyl radicals to yield less reactive radicals that can not abstract ·H from neighboring polyunsaturated fatty acids. Whether in this capacity or as a cofactor for GSH peroxidase, GSH-S-transferase or in some other role, GSH has been shown to be a critical factor in maintaining cellular antioxidant defenses.[93,94]

Phospholipase A_2 is also proposed to have a function in protecting membranes against lipid peroxidation. The enzyme has enhanced activities towards oxidized fatty acids esterified to phospholipids.[95] By hydrolyzing the ester bond linking the fatty acid to the phospholipid, the oxidized fatty acid is removed from the membrane and prevented from participating in further propagation reactions within the membrane bilayer.

Initiator Formation

Once initiated, lipid peroxidation is a self-propagating event. Thus, the bulk of scientific investigations in this field have dealt with the factors important to the initiation process, the identities of the initiating species, and means to inhibit their formation.

There are two major pathways by which oxidizing agents capable of initiating lipid peroxidation are proposed to be formed. The first of these involves the metabolism of certain xenobiotics to free radical species that directly abstract methylene hydrogens and initiate lipid peroxidation. The classical example of such a compound is carbon tetrachloride;[96] it is metabolized by the hepatic microsomal MFO system to the trichoromethyl radical ($\cdot CCl_3$). This radical abstracts hydrogen from a polyunsaturated fatty acid to initiate lipid peroxidation. Of particular relevance to oxygen toxicity is the other major route for the initiation of lipid peroxidation and involves the participation of $O_2^{\cdot-}$ and H_2O_2 and their subsequent redox reactions.

Haber-Weiss Reaction - Superoxide and H_2O_2 are usually considered to be toxic by virtue of their reaction to form more reactive species capable of oxidizing cellular macromolecules such as polyunsaturated fatty acids. The most often proposed initiator of lipid peroxidation is $\cdot OH$ formed by the Haber-Weiss reaction:[97]

$$O_2^{\cdot-} + H_2O_2 \text{ ------> } O_2 + OH^- + \cdot OH \qquad (29)$$

This reaction clearly provides a mechanism for the proposed toxicity of excessive O_2^- and H_2O_2 production. However, what was initially overlooked by the first investigators who proposed it was the requirement for metal ion catalysis. It was not until 1978 when several groups of scientists

demonstrated that the rate of the uncatalyzed Haber-Weiss reaction was extremely slow, probably too slow to account for the biological damage attributed to $O_2^{\cdot-}$ and H_2O_2 production. Since then, the Haber-Weiss reaction has become known as the iron-catalyzed Haber-Weiss reaction, not because catalysis of this reaction is a property exclusive to iron, but rather in recognition of the biological abundance of iron and its propensity to undergo one electron oxidation-reduction reactions.

$$O_2^{\cdot-} + Fe^{3+} \text{-------} > Fe^{2+} + O_2 \qquad (30)$$

$$O_2^{\cdot-} + HO_2^{\cdot} \text{-------} > H_2O_2 + O_2 \qquad (5)$$

$$Fe^{2+} + H_2O_2 \text{------} > Fe^{3+} + OH^- + \cdot OH \qquad (6)$$

This reaction sequence has been most often invoked as the causative factor in oxygen toxicity and most of the physiological, toxicological and pathological states mentioned previously.

It is generally difficult to determine the participation of $O_2^{\cdot-}$ and H_2O_2 in the reactions leading to the formation of an initiating species. The most widely used method for determining $O_2^{\cdot-}$ and H_2O_2 participation is the addition of SOD or catalase, respectively. In many cases <u>in vitro</u> lipid peroxidation is inhibited by SOD or catalase, but rarely is inhibition complete. Thus, one cannot discount the possibility that other substances can substitute for $O_2^{\cdot-}$ or H_2O_2 and perhaps produce other initiating species besides $\cdot OH$. Likewise, it has proven extremely difficult to ascertain to what extent and when

·OH-mediated oxidation of biological molecules has occurred. This can be attributed at least in part to the intrinsic reactivity of ·OH which readily reacts with most molecules. Thus, it may often be difficult to achieve sufficient concentrations of detector molecules to compete with the spectrum of organic molecules with which ·OH can react, especially *in vivo.*

Objections to the Haber-Weiss Reaction - A closer consideration of the iron-catalyzed Haber-Weiss reaction has led some investigators to question its nearly universal acceptance in explaining oxidative damage to biomolecules. The purpose of $O_2^{\cdot-}$ in the iron-catalyzed Haber-Weiss reaction is two-fold: reduction of ferric (30) and a source of H_2O_2 via dismutation (5). Thus, the requirement for $O_2^{\cdot-}$ can be met by supplying alternate reductants and sources of H_2O_2. Fee has recognized this and has termed the iron-catalyzed Haber-Weiss reaction "superoxide-driven Fenton's chemistry."[98] His contention is that $O_2^{\cdot-}$ is only one of numerous cellular reductants and is present at miniscule concentrations relative to other reducing agents such as ascorbate or glutathione. Numerous *in vitro* studies have demonstrated that these and other reducing agents can support lipid peroxidation, and in these systems lipid peroxidation is not inhibited by SOD.

Other objections to the Haber-Weiss reaction are based on a different premise, that being the chemistry of iron-

oxygen reactions. The iron-catalyzed Haber-Weiss reaction is merely a reduction of ferric followed by the oxidation of the ferrous. As such, the tendency of the iron chelates to undergo reduction and/or oxidation is determined by the redox potential of the chelate, not by the presence of $O_2^{\cdot-}$ or H_2O_2. Thus, there are examples of ferrous chelates that rapidly autoxidize such as EDTA, citrate, pyrophosphate[99] and ones that are not oxidized by either oxygen or H_2O_2 such as o-phenanthroline, dipyridyl, and bathophenanthroline sulfonate.[100] Therefore, it will be important to identify biologically-relevant iron chelates and characterize their redox chemistry to determine their tendency to catalyze the Haber-Weiss reaction or other reactions leading to strong oxidizing species in order to ascertain the extent to which the iron-catalyzed Haber-Weiss reaction might occur <u>in vivo</u>.

A chelator of special note is ADP. It was the chelator used by Hochstein and Ernster[101] when they observed microsomal lipid peroxidation. A comparison of the data in the literature indicates that ADP-iron promotes greater rates of lipid peroxidation than other iron chelates, especially when phospholipids are present as bilayers (i.e., liposomes, microsomes, vesicles). Moreover, although ADP-iron can catalyze $\cdot OH$ formation, the yield of $\cdot OH$ is lower than with other iron chelates.[102] In fact, when ADP-Fe^{3+} is used to promote lipid peroxidation, it appears that production of $\cdot OH$ and initiation of lipid peroxidation may be inversely related.[103]

A final consideration in the debate over the significance of the Haber-Weiss reaction *in vivo* relates to the bioavailability of iron. While iron is an abundant element in biology, it is not readily available; most iron is present in high molecular weight proteins.[104] Very low amounts of what is termed low molecular weight iron has been isolated from cells,[104] and recent studies have begun to assess the conditions under which the high molecular weight iron proteins will release iron.[103-107] Thus, the metal catalyst for the Haber-Weiss reaction or other oxygen radical reactions may well be the limiting factor in the formation of strong oxidizing species *in vivo*. Therefore, perhaps the rigid control of iron metabolism by the cell should also be considered as a major antioxidant defense mechanism.

Alternate Initiators - A growing number of investigations have demonstrated that SOD and catalase did not always inhibit lipid peroxidation or other oxidative damage. This is an indication that the ·OH may not be the only initiations species. Some investigators have referred to a crypto-·OH as being the initiating species.[108] This species is proposed to possess equal or nearly equal reactivity to free ·OH but be kinetically distinct from ·OH. Others have suggested various iron-oxygen complexes as being responsible for initiation including perferryl ion,[109] an adriamycin complex with ADP-Fe^{3+},[110] ferryl iron,[111] an activated

methemoglobin or metmyogoblin complex,[112] and a ferrous-dioxygen-ferric complex.[113] Lipid peroxidation initiated by these proposed initiators was not inhibited by SOD, catalase, or ·OH scavengers.

Just as the ability of SOD, catalase, or ·OH traps to inhibit lipid peroxidation is not definitive evidence for ·OH participation, the inability of these scavengers to inhibit does not discount the possibility that $O_2^{\cdot-}$, H_2O_2, or ·OH were involved. In fact, the studies in which alternate initiators were proposed have been criticized on the grounds that the data can be explained by an inaccessibility of the scavengers to the site(s) of oxygen radical generation.[114,115] To settle this question it will be necessary to characterize these alternative initiating species.

NADPH-Dependent Lipid Peroxidation

Hochstein and Ernster were the first to report that rat liver microsomes incubated with NADPH underwent peroxidation.[101] They characterized the process, finding a requirement for ADP-Fe^{3+}.[116] Lipid peroxidation was not inhibited by SOD, catalase, or ·OH traps and a perferryl ion ($Fe^{2+}O_2$) was proposed to be the initiating species.[109]

However, perhaps the most thorough study on microsomal lipid peroxidation was done by McCay and his associates. They characterized the process with regard to the alterations of phospholipid, oxygen uptake, polyunsaturated

fatty acid loss, MDA formation, phospholipid peroxides as intermediates and the enzymatic nature of the process.[117-121] Pederson and Aust were the first to demonstrate that the enzyme responsible for catalyzing NADPH oxidation as a precedent to lipid peroxidation was the reductase.[122] They did this by inhibiting microsomal lipid peroxidation with Fab fragments prepared from rabbit IgG raised against purified reductase and by developing a model lipid peroxidation system containing the purified enzyme, microsomal phospholipid liposomes and iron chelates.

Whereas ADP-Fe^{3+} was the only iron chelate needed to promote lipid peroxidation in the intact microsomal system, Pederson and Aust noted an additional requirement for EDTA-Fe^{3+} in their reconstituted system. A previous study had indicated that the direct addition of ferrous to microsomes resulted in peroxidation even in the absence of NADPH,[123] so these data taken together suggested that the role of NADPH and the reductase in the microsomal system was to reduce ADP-Fe^{3+} in the microsomal system. In addition there appeared to be some substrate specificity to the reduction in that the purified enzyme seemed to reduce EDTA-Fe^{3+} but not ADP-Fe^{3+}.

Roles of Cytochrome P450 in Lipid Peroxidation

Cytochrome P450 is a group of isozymes having broad and overlapping substrate specificities. To date, the relative ability of individual forms of cytochrome P450 or the

complement of isozymes induced by xenobiotics to promote the initiation of lipid peroxidation has not been demonstrated. Perhaps one of the reasons for this can be attributed to the controversy over how microsomal lipid peroxidation is initiated and what factors contribute to the formation of an initiating species. Previous results have shown that xanthine oxidase could promote the ADP-Fe^{3+}-dependent peroxidation of microsomal phospholipids where $O_2^{\cdot-}$ was required for iron reduction.[124] Therefore, the reconstitution of the reductase with cytochrome P450, that has been shown to generate $O_2^{\cdot-}$, would be predicted to promote peroxidation also. However, in only one instance has a reconstituted MFO system been shown to initiate lipid peroxidation.[125] In this case, there was no apparent requirement for iron, although iron chelators did inhibit peroxidation.

Whereas the role of cytochrome P450 in initiation of peroxidation is questionable, it can apparently function in lipid hydroperoxide-dependent initiation reactions. Cytochromes P450 can utilize lipid hydroperoxides or other organic hydroperoxides as a means to produce activated oxygen capable of oxidizing MFO substrates.[64] They catalyze a homolytic cleavage of the peroxide bond generating an alkoxyl radical and an activated heme iron intermediate that may be related to the Compound I intermediate of catalase or species (VI) of cytochrome P450 (Figure 1).

$$Fe^{3+} + LOOH \text{ -------} > (FeO)^{3+} + LO\cdot \qquad (27)$$

The alkoxyl radical is proposed to be capable of abstracting hydrogens from neighboring fatty acids to continue the free radical chain reaction of lipid peroxidation. Svingen et al.[89] have shown that cytochrome P450 could promote the peroxidation of phospholipids liposomes containing lipid hydroperoxides in the absence of additional iron or reducing equivalents. A similar type of study comparing different cytochrome P450 contents of tumor microsomes to their susceptibility to peroxidation initiated by a xanthine-xanthine oxidase system demonstrated that the extent of peroxidation in microsomes containing more cytochrome P450 was greater.[126]

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CHAPTER I

Ferric Chelates Stimulate NADPH Oxidation by NADPH-Cytochrome P450 Reductase: A Superoxide-Independent Iron Reduction Activity

ABSTRACT

Rat liver microsomes and purified components of the microsomal electron transport system catalyzed increased rates of NADPH oxidation in the presence of certain ferric chelates. NADPH oxidation by purified NADPH-cytochrome P450 reductase was stimulated by EDTA-Fe^{3+} and DTPA-Fe^{3+} but not by ADP-Fe^{3+} or desferrioxamine-Fe^{3+}. Stimulation was dependent upon iron, since neither DTPA nor EDTA affected rates of NADPH oxidation. Microsomal NADPH oxidation was also stimulated by EDTA-Fe^{3+} and DTPA-Fe^{3+}, but to a lesser extent by ADP-Fe^{3+}. Increasing concentrations of EDTA-Fe^{3+} and DTPA-Fe^{3+} resulted in increasing rates of NADPH oxidation by microsomes or the purified reductase.

These results suggested that EDTA-Fe^{3+} and DTPA-Fe^{3+} were substrates for the reductase. This was demonstrated with EPR spin trapping experiments in which NADPH-dependent reduction of EDTA-Fe^{3+} and subsequent $\cdot OH$ formation was not inhibited by SOD. That EDTA-Fe^{3+} and DTPA-Fe^{3+} were indeed substrates for the reductase was confirmed by monitoring the NADPH-dependent reduction of these chelates in anaerobic incubations containing microsomes, purified reductase, or reductase reconstituted with cytochrome P450 in DLPC micelles. These results are consistent with the NADPH oxidation data in that EDTA-Fe^{3+} and DTPA-Fe^{3+} are directly

reduced primarily by the reductase and perhaps also by cytochrome P450 but to a much lesser extent. The purified reductase was unable to reduce ADP-Fe^{3+} but microsomes or the reconstituted electron transport system reduced ADP-Fe^{3+} in addition to EDTA-Fe^{3+} and DTPA-Fe^{3+}.

INTRODUCTION

The NADPH-dependent peroxidation of microsomal lipids was first described by Hochstein et al.[1] They characterized this process with respect to its requirements for NADPH and ADP-Fe^{3+}.[2] Subsequent studies indicated that the addition of ferrous to microsomes resulted in peroxidation in the absence of NADPH.[3] Thus, it was inferred that NADPH was the source of reducing equivalents for the reduction of iron and subsequently the reductase was shown to be (one of) the microsomal enzyme(s) necessary for iron reduction.[4]

That iron must be first reduced is implicit in virtually all mechanisms proposed for the initiation of lipid peroxidation.[5-10] However, the mechanism by which iron is reduced by the purified reductase or microsomes has remained a subject of controversy. Researchers have theorized that the reductase generates $O_2^{\cdot-}$ as the first step towards the formation of $\cdot OH$ by a $O_2^{\cdot-}$-driven iron-catalyzed Haber-Weiss reaction.[7,8,10]

$$O_2^{\cdot-} + Fe^{3+} \longrightarrow Fe^{2+} + O_2 \qquad (1)$$

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

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

Superoxide dismutase should scavenge $O_2^{\cdot-}$ and prevent its reduction of ferric (1). However, SOD has not consistently been reported to inhibit NADPH-dependent lipid peroxidation. One group of investigators has postulated

that although SOD was not inhibitory in their system, reduction of ferric was still via $O_2^{\cdot-}$;[7] others have postulated that the reason that SOD failed to inhibit lipid peroxidation might be that it is inaccessible to the site(s) of $O_2^{\cdot-}$ generation.[10,11] This is clearly a difficult argument to disprove, or for that matter to prove.

Other investigators have hypothesized that the inability of SOD to inhibit lipid peroxidation is because the mechanism of iron reduction is not dependent upon $O_2^{\cdot-}$; they postulated that iron reduction occurs via a direct transfer of an electron to ferric from the NADPH-dependent electron transport system.[5,6,12] Microsomes have been shown to possess both NADH- and NADPH-dependent ferricyanide reductase activities catalyzed by cytochrome b_5 reductase[13] and the reductase,[14] respectively, so there is a precedent for such an activity. Inherent in this direct reduction hypothesis is that the ferric chelate is in fact a substrate for an electron transport protein and presumably its binding to the protein should stimulate NADPH oxidation by the reductase. This is what occurs following the addition of ferricyanide to microsomes.

There may be a second consideration that could be inferred from the existence of a direct iron reduction pathway in microsomes. One might predict some specificity for particular ferric chelates that would not be exhibited by $O_2^{\cdot-}$-dependent reduction systems. This has not been directly observed, but some indirect evidence that this is

indeed the case is based on the ability of ADP-Fe^{3+} to promote the NADPH-dependent lipid peroxidation in a reconstituted lipid peroxidation system in the presence of EDTA-Fe^{3+} but not in its absence.[12] It was postulated that EDTA-Fe^{3+} but not ADP-Fe^{3+} was reduced by the reductase (in a $O_2^{\cdot}$-independent process). More recent results seem to support this interpretation since ADP-Fe^{3+} has been shown to promote xanthine oxidase-dependent peroxidation of phospholipid liposomes in the absence of EDTA-Fe^{3+} and in a process completely inhibited by SOD.[15]

Previous studies had shown that EDTA-Fe^{3+} stimulated NADPH oxidation by the purified reductase,[6,16] however the ability of ferric chelates commonly used in the study of oxygen radical-mediated oxidations to act as electron acceptors in NADPH-dependent electron transport has not been extensively studied. Therefore, we have investigated the ability of microsomal MFO components to reduce several ferric chelates and concomitantly oxidize NADPH.

MATERIALS AND METHODS

Chemicals: Cytochrome c (Type VI), SOD, bromelain, xanthine oxidase, NADPH, ADP, DTPA, DLPC, o-phenanthroline, and xanthine were all obtained from Sigma. Desferrioxamine under the proprietary name desferal was obtained from CIBA-Geigy and Aldrich was the source for DMPO which was vacuum distilled before use. Catalase was a product of Millipore and EDTA was purchased from Mallinckrodt Chemical Company. All other reagents were of analytical grade and used without further purification. Solutions intended for use in EPR, NADPH oxidation, or iron reduction assays were treated with Chelex 100 (Bio Rad) to remove contaminating transition metal ions.

Preparation of Microsomes: Rat liver microsomes were isolated from male Sprague-Dawley rats (250-300g) (Charles River) by the method of Pederson and Aust.[17] Microsomal pellets were resuspended in 10 mM EDTA, 1.15% KCl pH 7.0 and centrifuged at 105,000 xg for 60 min. Pellets were resuspended in 50 mM NaCl previously passed through a Chelex 100 and washed twice to remove residual EDTA. Microsomes were used immediately or stored at -20°C in 50 mM Tris pH 7.5 containing 50% glycerol.

Purification of Enzymes: Cytochrome P450b and the reductase were isolated from liver microsomes of rats pretreated with 0.1% phenobarbital in their drinking water for 10 days prior to sacrifice. Protease-solubilized reductase was isolated using the procedure of Pederson et al.[17] with minor modifications. Fractions from the Sephadex G-100 column (Pharmacia) were pooled on the basis of cytochrome c reductase activity, and instead of ion exchange chromatography, the pooled fractions were subjected to affinity chromatography on 2'-5' ADP Agarose[18] (PL Biochemicals) using buffers containing no dithiothreitol nor detergent. Specific activities of purified preparations ranged between 40 and 66 units/mg.

Detergent-solubilized reductase was solubilized from microsomes by treating them with Emulgen-911 (Kao Atlas, Japan) to 1.5% final concentration and chromatographing on DEAE-Sephadex A-25 (Pharmacia) by the method of Dignam and Strobel.[19] The fractions having cytochrome c reductase activity were pooled and diluted two-fold with 20% glycerol and 0.1% Emulgen-911 and subjected to affinity chromatography on 2'-5' ADP-Agarose as per Yasukochi and Masters.[18] Specific activities ranged from 40-60 units/mg protein.

Cytochrome P450b was purified from the flow-through of the DEAE Sephadex A-25 column using the procedure of Waxman and Walsh.[20] Briefly this consists of DEAE-Cellulose

chromatography (Whatman) as described by West and Lu[21] with subsequent chromatography on hydroxyapatite and CM-Sepharose (Pharmacia). Purified preparations had specific contents of 10-14 nmol/mg protein.

Assays: Purified preparations of reductase and commercial xanthine oxidase were chromatographed on Sephadex G-25 (Pharmacia) to remove buffer salts and contaminating metal ions, and assayed for their ability to reduce cytochrome c. Cytochrome c reduction was monitored spectrophotometrically at 550 nm with 1 unit of enzyme activity defined as the amount of enzyme reducing 1 μmol cytochrome c/min/ml at 25°C using an extinction coefficient of 21 x 10^3 M^{-1} sec^{-1}. Cytochrome P450 was assayed by the procedure of Omura and Sato.[22] NADPH oxidation was monitored at 340 nm using an extinction coefficient of 6.22 x 10^3 M^{-1} sec^{-1}. Catalase activity was assayed using the method of Beers and Sizer,[23] and SOD was assayed as per McCord and Fridovich.[24] Protein was assayed either by the method of Lowry[25] or the bicinchoninic acid method[26] adapted for use with microtiter plates.[27]

EPR Measurements: Electron paramagnetic resonance spin trapping experiments were performed using a Varian Century-112 EPR spectrometer at 20°C. Spectrometer settings were 3320 G magnetic field, 9.412 GHz, 1000KHz modulation

frequency, 15mW microwave power, 0.63 modulation amplitude, 1 sec time constant and 8 min scan time.

Iron Reduction Assays: Iron reduction in incubations containing microsomes or purified enzymes was monitored spectrophotometrically at 510 nm, observing the absorbance of the o-phenanthroline-Fe^{2+} complex. Aliquots of anaerobic incubations (0.5 or 1.0 ml) were quenched in 0.2% o-phenanthroline (1 ml) and the protein precipitated by the addition of 0.5 ml of 20% TCA. The o-phenanthroline-Fe^{2+} chelate was extracted from the acidified mixture with 2 ml of n-amyl alcohol and the absorbance of the organic phase was measured. Standard curves were prepared using known ferric chelate concentrations reduced with excess thioglycolate.

RESULTS

Stimulation of NADPH-Cytochrome P450 Reductase-Dependent NADPH Oxidation by Ferric Chelates: The rate of NADPH oxidation catalyzed by the reductase in aerobic solution is shown in Table 1. The ability of EDTA-Fe^{3+} and DTPA-Fe^{3+} to markedly stimulate basal NADPH oxidation rates is also shown. The stimulation by these chelates was dependent upon iron as evidenced by the inability of the chelating agents to stimulate NADPH oxidation in the absence of added iron. Neither ADP-Fe^{3+} nor desferrioxamine-Fe^{3+} stimulated NADPH oxidation by the reductase to any significant extent.

From these data it could not be determined whether EDTA-Fe^{3+} and DTPA-Fe^{3+} were actually substrates for the flavoprotein reductase or simply acted in a catalytic fashion to induce the reductase to generate $O_2^{\cdot-}$ via autoxidation of reduced flavins. Therefore, the effect of varying concentrations of DTPA-Fe^{3+} and EDTA-Fe^{3+} on NADPH oxidation was investigated and the results are shown in Figure 2. Both DTPA-Fe^{3+} and EDTA-Fe^{3+} appeared to act as substrates for the reductase having apparent Km's of 540 μM and 170 μM respectively.

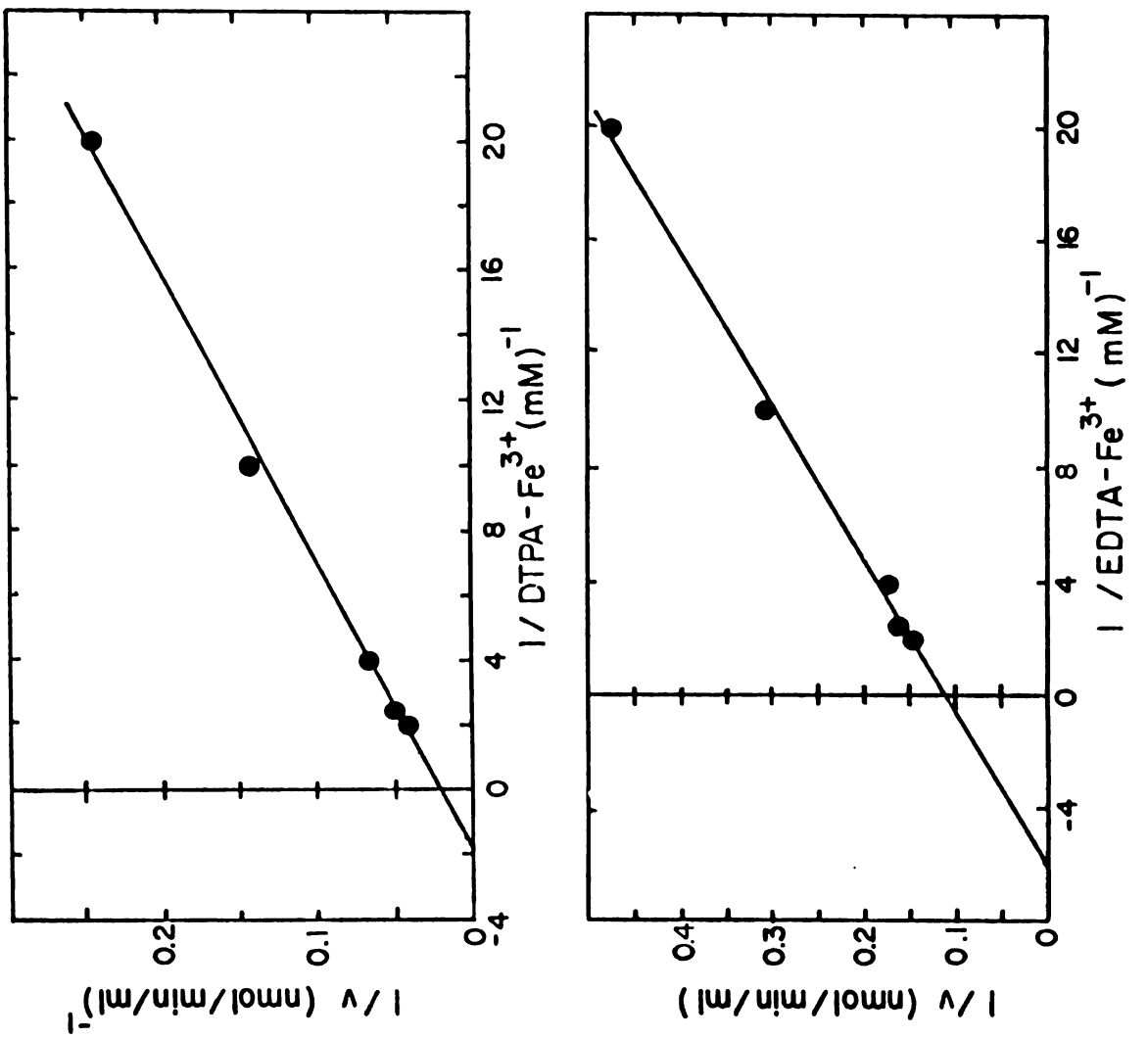
EPR Spin Trapping: Evidence suggesting the direct reduction of EDTA-Fe^{3+} by the reductase was also obtained with EPR spin trapping. The ability of a xanthine-xanthine oxidase

Table 1. Effect of Ferric Chelates on the Rate of NADPH Oxidation by NADPH-Cytochrome P450 Reductase

	NADPH oxidation (nmol/unit)
Complete System	1
+EDTA	1
+EDTA-Fe^{3+}	130
+DTPA	2
+DTPA-Fe^{3+}	220
+ADP	2
+ADP-Fe^{3+}	1
+desferrioxamine	1
+desferrioxamine-Fe^{3+}	1

The complete system contained NADPH (0.1mM), purified reductase (0·007u/ml) and where indicated, chelator or ferric chelates in 0.3M NaCl pH 7.0 at 37°C. The concentration of chelators and chelates was 0.11 mM chelator and 0.1 mM $FeCl_3$ except for ADP which was 0.5 mM. NADPH oxidation was expressed per unit of cytochrome c reductase activity.

Figure 2: Stimulation of NADPH-Cytochrome P450 Reductase-Dependent NADPH Oxidation by EDTA-Fe^{3+} and DTPA-Fe^{3+}.
Assay conditions were the same as those described in Table 1. Initial rates of NADPH oxidation (nmol/min/ml) at each ferric chelate concentration were plotted against the ferric chelate concentrations.


1 / v (nmol/min/ml)
0
0.1
0.2
0.3
0.4
-4
0
4
8
12
16
20
1 / EDTA-Fe3+ (mM)-1
1 / v (nmol/min/ml)-1
0.1
0.2
1 / DTPA-Fe3+ (mM)-1

system to form ·OH via Fentons reaction (3) was compared to the reductase system. The production of $O_2^{\cdot-}$ by xanthine oxidase is shown in Figure 3A. The addition of SOD inhibited the formation of the DMPO·OOH adduct signal (Figure 3B). In the presence of EDTA-Fe^{3+}, the DMPO·OOH is not readily formed, since the reaction of $O_2^{\cdot-}$ with EDTA-Fe^{3+} has a rate constant many orders of magnitude greater than its reaction with DMPO (6.5×10^5 vs. 10-20 $M^{-1}S^{-1}$).[28,29] What was observed was the DMPO adduct of ·OH presumably formed via an iron-catalyzed Haber-Weiss reaction (Figure 3C). The DMPO·OH signal intensity was almost completely inhibited by SOD (Figure 3D), evidence that the reduction of EDTA-Fe^{3+} was dependent upon $O_2^{\cdot-}$. Catalase also inhibited DMPO·OH formation consistent with the formation of ·OH by the Fentons reaction (3).

The results of the analogous experiment with NADPH and the reductase are shown in Figure 4. Very little DMPO·OOH was detectable during the oxidation of NADPH by the reductase (Figure 4A) and no discernable inhibition by SOD was observed (Figure 4B). The addition of EDTA-Fe^{3+} to this system resulted in the formation of DMPO·OH adduct (Figure 4C) just as with the xanthine oxidase system shown previously. However, SOD when added to this incubation did not inhibit EDTA-Fe^{3+}-dependent ··OH formation (Figure 4D) strongly suggesting that reduction of this ferric chelate is not dependent upon $O_2^{\cdot-}$. These same experiments can not be

Figure 3: Effect of Superoxide Dismutase on Xanthine Oxidase-Dependent Hydroxyl Radical Formation.
All incubations contained xanthine oxidase (0.01 u/ml), xanthine (0.33 mM), DMPO (3 mM) in 0.3 M NaCl pH 7.0. The following additions were made to generate the corresponding EPR spectra: A. none; B. SOD (200 u/ml); C. EDTA-Fe^{3+} (0.022 mM EDTA: 0.022 mM $FeCl_3$); D. EDTA-Fe^{3+} and SOD; E. EDTA-Fe^{3+} and catalase (750 u/ml).

Figure 4: Effect of Superoxide Dismutase on NADPH-Cytochrome P450 Reductase-Dependent Hydroxyl Radical Formation:

All incubations contained purified reductase (0.1 u/ml), NADPH (0.14 mM), and DMPO (3 mM) in 0.3 M NaCl, pH 7.0. The following additions were made to generate the corresponding EPR spectra: A. none; B. SOD (200 u/ml); C. EDTA-Fe^{3+} (0.022 mM EDTA: 0.02 mM $FeCl_3$); D. EDTA-Fe^{3+} and SOD; E. EDTA-Fe^{3+} and catalase (750 u/ml).

done using DTPA-Fe^{3+} since this chelate is not able to efficiently catalyze the Haber-Weiss reaction.

Anaerobic Iron Reduction by NADPH-Cytochrome P450 Reductase: The data presented are clearly indicative of EDTA-Fe^{3+} and DTPA-Fe^{3+} being substrates for the reductase. Therefore, the enzyme should be capable of reducing these chelates, and under anaerobic conditions the ferrous chelates should accumulate. The data showing the reduction of EDTA-Fe^{3+} and DTPA-Fe^{3+} by the reductase are presented in Table 2. No evidence for iron reduction with either DTPA-Fe^{3+} or EDTA-Fe^{3+} was observed, again consistent with the requirement for iron in the stimulation of NADPH oxidation. Neither ADP-Fe^{3+} nor desferrioxamine-Fe^{3+} addition to the reductase resulted in ferrous formation (Table 2).

Stimulation of Microsomal NADPH Oxidation by Ferric Chelates: Rat liver microsomes also oxidized NADPH in aerobic solution. As shown in Table 3, the rate of NADPH oxidation was 23 nmol/unit cytochrome c reductase activity, considerably greater than that exhibited by the purified reductase. Expressing NADPH oxidation as a function of their cytochrome c reductase activities allows such a comparison to be made. Presumably, the bulk of NADPH oxidation by the reductase in microsomes was a result of the presence of cytochrome P450. Despite the higher basal level of microsomal NADPH oxidase activity, a clear stimulation by

Table 2. Rates of Reduction of Ferric Chelates by NADPH-Cytochrome P450 Reductase under Anaerobic Conditions

	nmol Fe^{2+}/unit
Complete system	0
-NADPH	0
-Reductase	0
+DTPA	0
+DTPA-Fe^{3+}	340
+EDTA	0
+EDTA-Fe^{3+}	85
+ADP-Fe^{3+}	0
+Desferrioxamine-Fe^{3+}	0

The complete system contained NADPH (2.5 mM) and purified reductase (0.06 U/ml) in 0.3 M NaCl, pH 7.0. The concentration of chelators or ferric chelates added to the complete system was 0.11 mM chelator +/- 0.1 mM $FeCl_3$. The ADP-Fe^{3+} concentration was 0.5 mM ADP, 0.1 mM $FeCl_3$.

Table 3. Effect of Ferric Chelates on Rates of NADPH Oxidation by Rat Liver Microsomes

	NADPH Oxidation (nmol/unit)
Complete system	23
+EDTA	22
+EDTA-Fe^{3+}	190
+DTPA	27
+DTPA-Fe^{3+}	260
+ADP	22
+ADP-Fe^{3+}	39
+Desferrioxamine	22
+Desferrioxamine-Fe^{3+}	16

The complete system contained microsomes (0.05 mg/ml) and NADPH (0.1 mM) in 0.3 M NaCl, pH 7.0, and was preincubated at 37°C for 5 min. A second aliquot of NADPH was added (to 0.2 mM final concentration) and, where indicated, chelator (0.11 mM) or ferric chelate (0.11 mM chelator, 0.1 mM $FeCl_3$) was also added, except ADP which was 0.5 mM (+/-) 0.1 mM $FeCl_3$. NADPH oxidation was expressed per unit of cytochrome c reductase activity present in microsomes.

EDTA-Fe^{3+} and DTPA-Fe^{3+} was still readily apparent. The addition of ADP-Fe^{3+} also resulted in an approximate 50% stimulation in the rate of NADPH oxidation, but desferrioxamine-Fe^{3+} was without effect.

The kinetics of NADPH oxidation stimulated by varying concentrations of DTPA-Fe^{3+} and EDTA-Fe^{3+} are shown in Figure 5. The apparent Kms for DTPA-Fe^{3+} and EDTA-Fe^{3+} and were 102 μM and 75 μM, respectively. The kinetics of ADP-Fe^{3+} reduction by microsomes is shown in Figure 6. Although the apparent Km for NADPH oxidation stimulated by ADP-Fe^{3+} (3μM) was considerably lower than DTPA-Fe^{3+} or EDTA-Fe^{3+}, the V_{max} was also considerably lower, indicating that the capacity of microsomes to reduce ADP-Fe^{3+} was considerably less than that for EDTA-Fe^{3+} and DTPA-Fe^{3+}.

Anaerobic Iron Reduction by Microsomes and a Reconstituted Mixed Function Oxidase System: These results were also suggestive of these iron chelates being substrates for the microsomal electron transport system. The anaerobic reduction of EDTA-Fe^{3+}, DTPA-Fe^{3+}, and ADP-Fe^{3+} by microsomes is shown in Table 4. Reduction of EDTA-Fe^{3+} and DTPA-Fe^{3+} occurred at much greater rates than that of ADP-Fe^{3+}, consistent with their relative abilities to stimulate microsomal NADPH oxidation. No desferrioxamine-Fe^{3+} reduction by microsomes was detected. The rate of ADP-Fe^{3+} reduction was also shown to increase with increasing microsomal protein (Figure 7), further evidence that

Figure 5: Stimulation of Rat Liver Microsomal NADPH Oxidation by Ferric Chelates.
Assay conditions were the same as those described in Table 2. Initial rates of NADPH oxidation (nmol/min/ml) at each ferric chelate concentration were plotted against the ferric chelate concentrations.

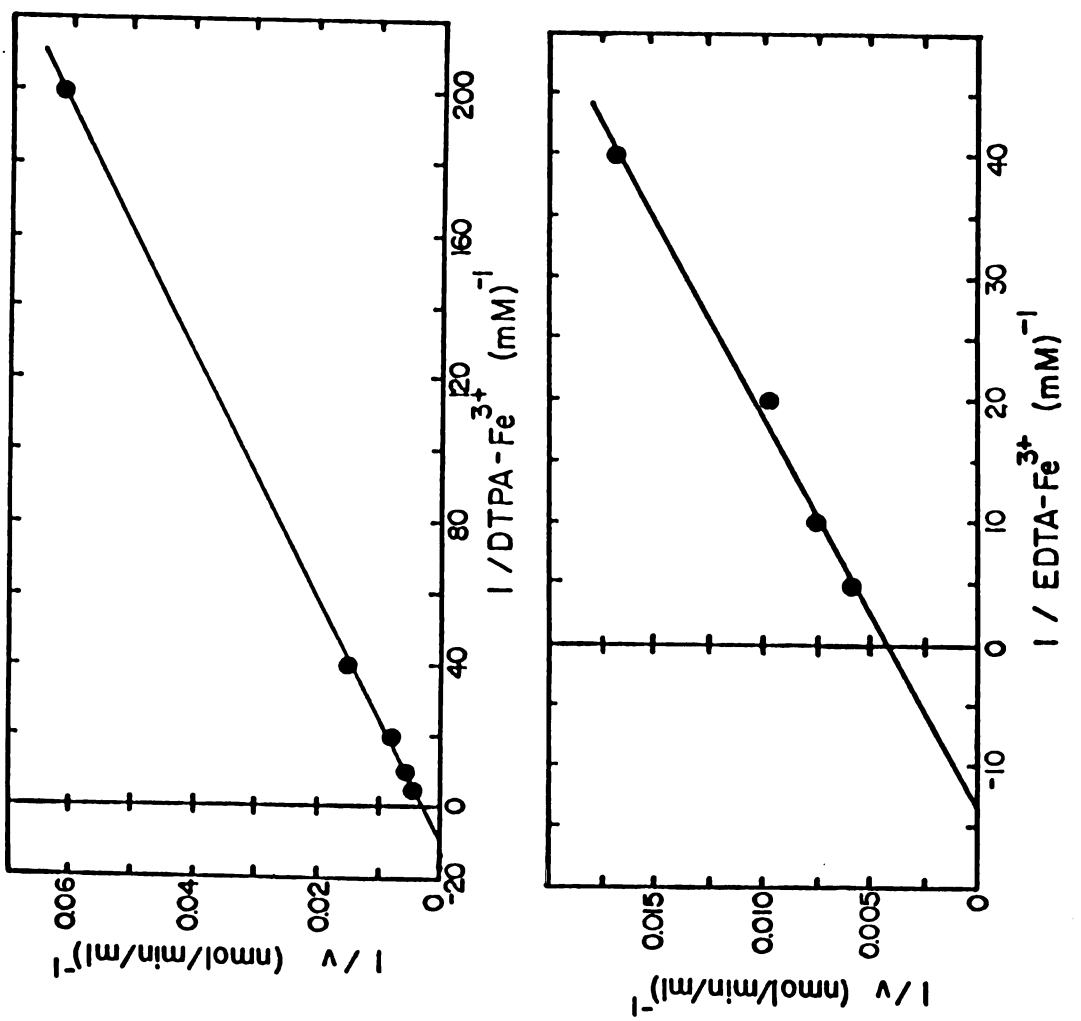

1 / v (nmol/min/ml)$^{-1}$
0.06
0.04
0.02
0
-20
0
40
80
120
160
200
1 /DTPA-Fe^{3+} $(mM)^{-1}$
1 / v (nmol/min/ml)$^{-1}$
0.015
0.010
0.005
0
-10
0
10
20
30
40
1 / EDTA-Fe^{3+} $(mM)^{-1}$

Figure 6: Stimulation of Rat Liver Microsomal NADPH Oxidation by ADP-Fe^{3+}.
Assay conditions were the same as described in Table 2. Initial rates of NADPH oxidation at each ADP-Fe^{3+} concentration were plotted against the concentration of ADP-Fe^{3+}.

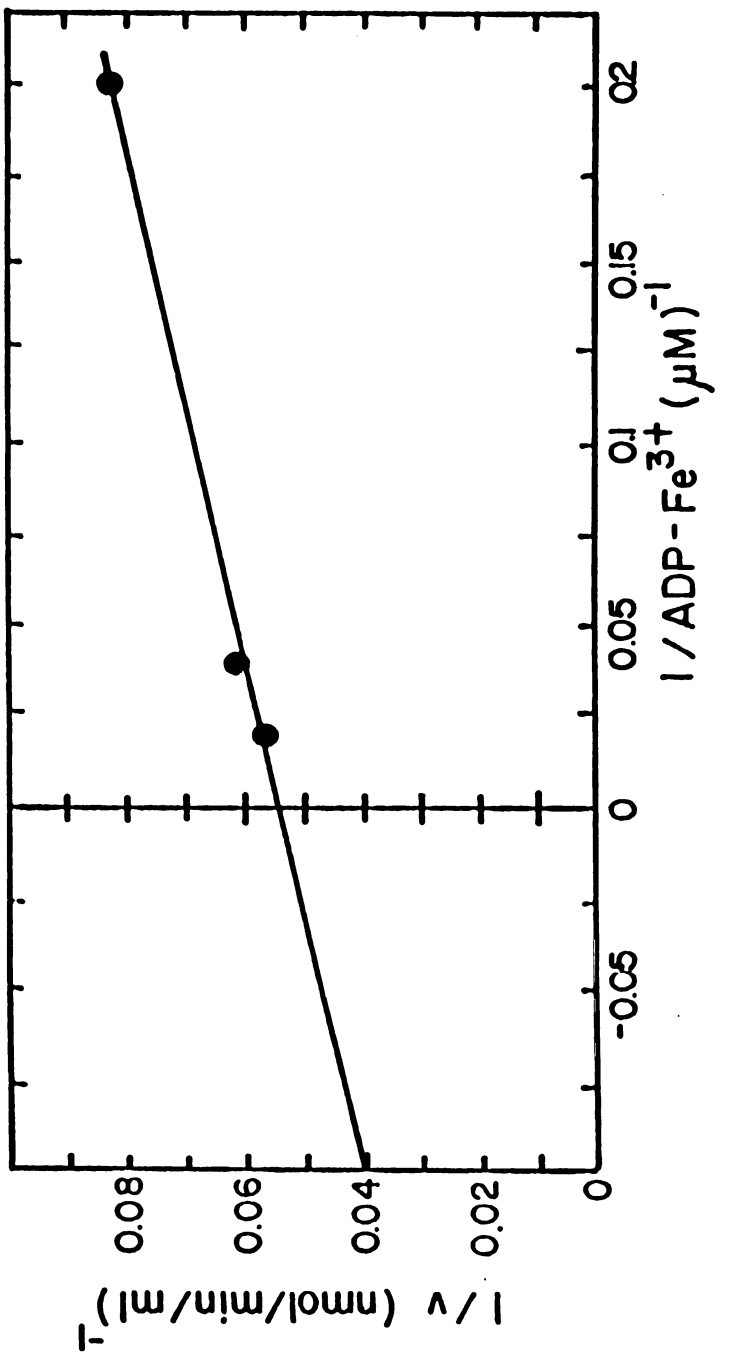
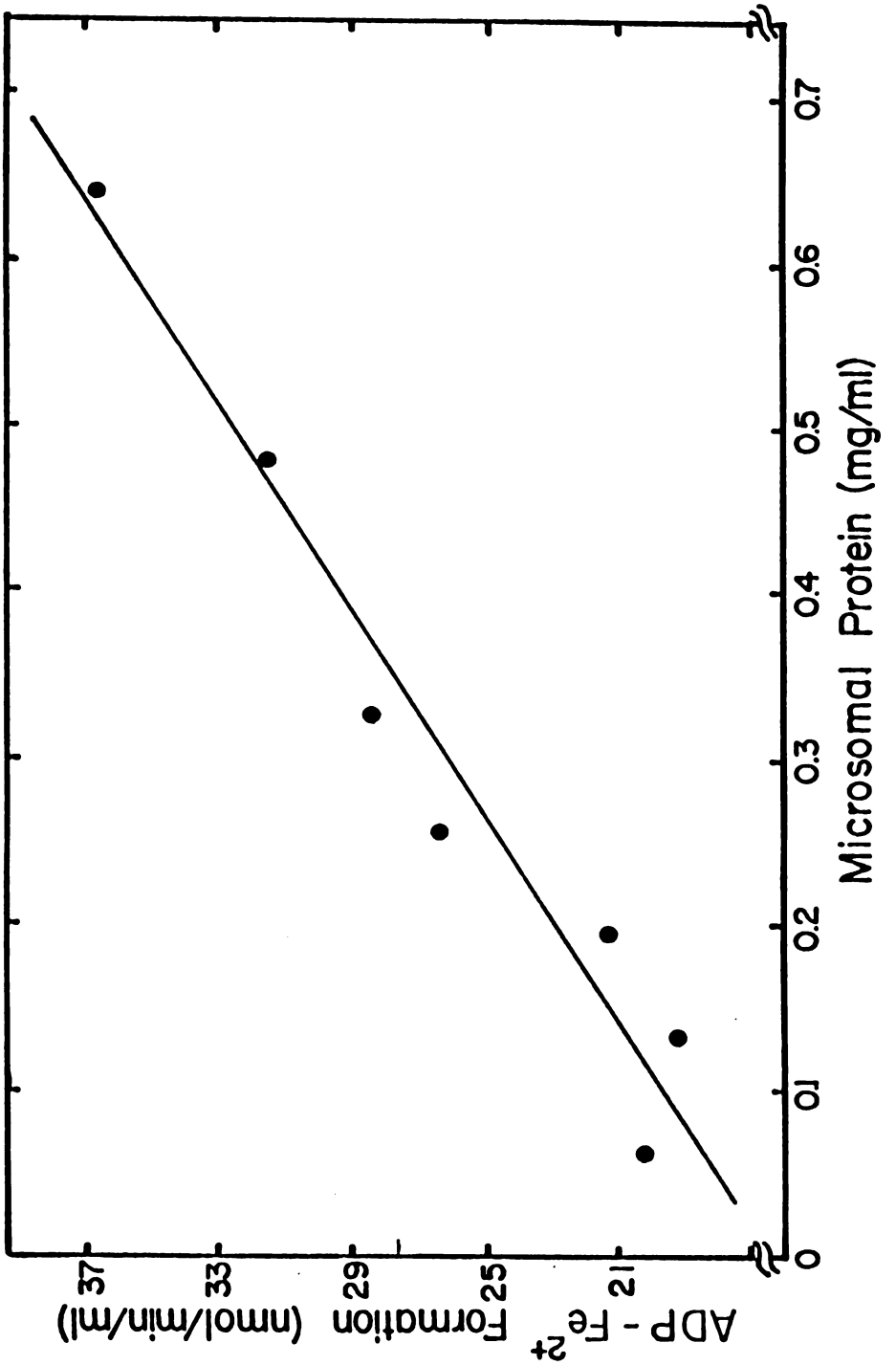

1 / v (nmol/min/ml)$^{-1}$
0.08
0.06
0.04
0.02
0
-0.05
0
0.05
0.1
0.15
0.2
1 / ADP-Fe^{3+} (μM)$^{-1}$

Table 4. Rates of Reduction of Ferric Chelates by Rat Liver Microsomes under Anaerobic Conditions

Chelate	nmol reduced/min/ml
EDTA-Fe^{3+}	70
DTPA-Fe^{3+}	56
ADP-Fe^{3+}	10

Anaerobic incubations contained NADPH (1mM), ferric chelate (0.55 mM chelator; 0.5 mM $FeCl_3$) and rat liver microsomes (0.5 mg/ml) in chelexed 0.05 M NaCl pH 7.0 at 37°C. The concentration of ADP-Fe^{3+} was 2.5 mM ADP, 0.5 mM $FeCl_3$.

Figure 7: Effect of Microsomal Protein Concentration on NADPH-Dependent ADP-Fe^{3+} Reduction.
Incubations contained ADP-Fe^{3+} (17 mM ADP: 1 mM $FeCl_3$) and NADPH (1 mM) with various concentrations of microsomes (isolated from rats pretreated with phenobarbital) in 50 mM NaCl, pH 7.0.


ADP - Fe^{2+} Formation (nmol/min/ml)
37
33
29
25
21
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Microsomal Protein (mg/ml)

microsomes reduce ADP-Fe^{3+} albeit slowly relative to EDTA-Fe^{3+} or DTPA-Fe^{3+}. The ADP:Fe^{3+} ratio also appeared to affect the rate of iron reduction with lower ADP:Fe^{3+} ratios being reduced more efficiently than higher ratios (17:1) (Figure 8).

A reconstituted MFO system consisting of detergent-solubilized reductase and cytochrome P450b incubated in DLPC micelles was also tested for its ability to reduce the same ferric chelates (Table 5). Both EDTA-Fe^{3+} and DPTA-Fe^{3+} were readily reduced by the MFO system whereas ADP-Fe^{3+} was also reduced, but to a lesser extent, just as was the case with microsomes.

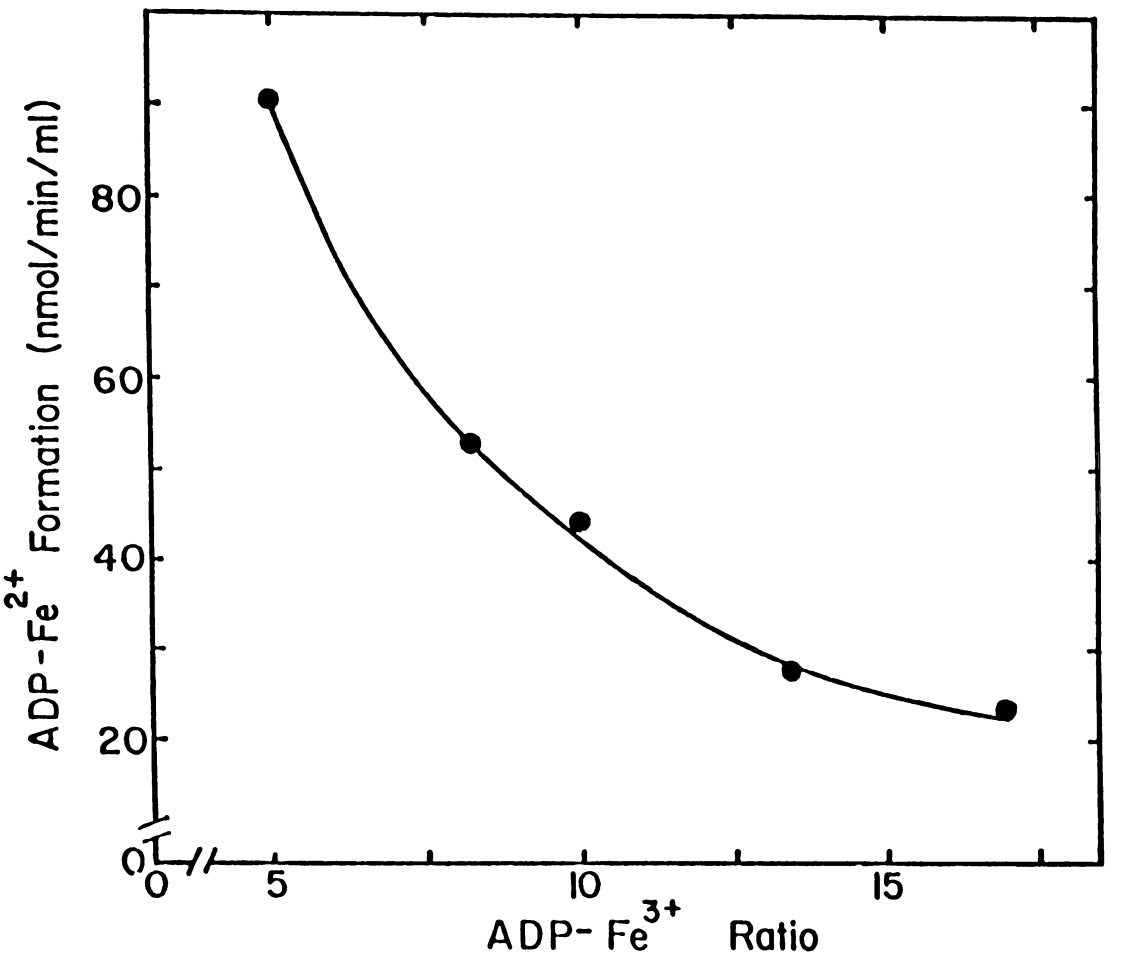


Figure 8: Effect of Various ADP:Fe Ratios on the Rate of ADP-Fe^{3+} Reduction by Microsomes.
Anaerobic incubations contained liver microsomes (0.32 mg/ml) from animals pretreated with phenobarbital, NADPH (1 mM) and ferric iron (1 mM) chelated by different concentrations of ADP.

Table 5. Rates of Reduction of Ferric Chelates by the Reconstituted Mixed Function Oxidase System under Anaerobic Conditions

Chelate	nmol Fe^{3+} reduced/min/ml
EDTA-Fe^{3+}	120
DTPA-Fe^{3+}	121
ADP-Fe^{3+}	6.8

Anaerobic incubations contained NADPH (1mM), ferric chelate (0.55 mM chelator: 0.5 mM $FeCl_3$), and a reconstituted MFO system (0.8 nmol cytochrome P450b, 0.25 nmol reductase, and 48 nmol DLPC in 0.05 M NaCl pH7 at 37°C. The concentration of ADP-Fe^{3+} was 2.5 mM ADP, 0.5 mM $FeCl_3$.

DISCUSSION

The NADPH-dependent peroxidation of lipids has been shown to require reduced iron.[3,5,6,10] However, the mechanism of iron reduction has been a matter of some controversy. Investigators have proposed that $O_2^{\cdot-}$ generation from the purified reductase or microsomes is responsible for iron reduction as an initial step towards the formation of an initiating species of NADPH-dependent lipid peroxidation in reconstituted systems[7,8] or in microsomes,[10,12] respectively.

Some investigators have postulated that the inability of SOD to inhibit lipid peroxidation was suggestive of an alternative, $O_2^{\cdot-}$-independent means for reducing iron.[5,6,12] This would appear to be the case when considering the results of the reconstituted system of Pederson et at.[12] Whereas they and other laboratories had shown that ADP-Fe^{3+} could promote the NADPH dependent peroxidation of microsomes, the reconstituted system required both EDTA-Fe^{3+} and ADP-Fe^{3+}. This is not what one would predict if reduction was $O_2^{\cdot-}$dependent, since studies using xanthine oxidase had demonstrated the peroxidation of microsomes or phospholipid liposomes could be promoted by ADP-Fe^{3+} with no EDTA-Fe^{3+} requirement.[15]

This study extends the results of Pederson et al. by demonstrating what they had postulated, a direct reduction

of EDTA-Fe^{3+} by the reductase. In addition, the direct reduction of two other ferric chelates commonly used in oxygen radical research, DTPA-Fe^{3+} and ADP-Fe^{3+} by the purified reductase and rat liver microsomes, respectively has been demonstrated.

Both EDTA-Fe^{3+} and DTPA-Fe^{3+} greatly stimulate NADPH oxidation by the purified reductase, whereas basal rates of oxidation in the absence of ferric chelates are quite low. This would suggest that O_2 is an extremely poor substrate for this flavoprotein, in contrast to other flavoproteins previously tested.[30] In air-saturated buffer, NADPH oxidation is extremely slow, and as will be demonstrated in succeeding chapter, the rate of $O_2^{\cdot-}$ production is correspondingly slow.

The direct reduction of EDTA-Fe^{3+} by the reductase was also examined using EPR spin trapping, testing the ability of SOD to inhibit the iron-catalyzed formation of DMPO·OH. The ·OH is formed in an iron-catalyzed Haber-Weiss reaction:

$$O_2^{\cdot-} + \text{EDTA-}Fe^{3+} \text{ ------> } \text{EDTA-}Fe^{2+} + O_2 \qquad (1)$$

$$2O_2^{\cdot-} + 2H^+ \text{ -------> } H_2O_2 \qquad (2)$$

$$\text{EDTA-}Fe^{2+} + H_2O_2 \text{ -----> } \text{EDTA-}Fe^{3+} + \cdot OH + OH^- \qquad (3)$$

Superoxide dismutase readily inhibits iron reduction by $O_2^{\cdot-}$ (1). Therefore, in the xanthine oxidase system that serves as a control, SOD completely inhibits the DMPO·OH signal intensity, whereas when EDTA-Fe^{3+} reduction is via NADPH and the purified reductase, SOD has no apparent effect on the DMPO·OH signal intensity.

The direct reduction of ferric chelates was demonstrated by incubating the reductase, rat liver microsomes or a reconstituted MFO system with NADPH under anaerobic conditions. Under these conditions, it is unlikely the observed rate of iron reduction is in fact the actual rate, since it is extremely difficult to completely prevent ferrous chelate oxidation during the incubation and handling of the sample. Nevertheless, ferrous chelate formation under these conditions did occur, demonstrating the potential of microsomes to directly reduce several iron chelates commonly used in lipid peroxidation.

From a thermodynamic standpoint reduction of most ferric chelates by $O_2^{\cdot-}$ should be a favorable process since the redox potential of the $O_2/O_2^{\cdot-}$ couple is -0.33v[31] in aqueous solution. Most iron chelates have negative redox potentials. The redox potential of hydrated ferrous is-0.77v,[32] but it is difficult to test the reduction of ferric since it is highly insoluble at neutral pH. Chelators maintain ferric in a soluble form, permitting iron reduction to occur, but alter the redox potential of the iron.

However, from a kinetic standpoint, chelators can also affect iron redox activity. In general oxygen radical redox reactions tend to be inner-sphere electron transfer processes, so chelators that hinder the access of $O_2^{\cdot-}$ to the metal can inhibit its reduction. This was indeed the case with desferrioxamine as well as several other ferric chelators tested by Graf *et al.*[33] In agreement with their

results, desferrioxamine was not reduced by the microsomal electron transport system in this investigation.

In contrast to $O_2^{\cdot}$-dependent reduction, the direct reduction of ferric chelates might be expected to exhibit some substrate specificity, and indeed this is what is observed in the present study. The purified reductase reduces both EDTA-Fe^{3+} and DTPA-Fe^{3+} but not ADP-Fe^{3+} whereas rat liver microsomes reduce ADP-Fe^{3+} as well as the other two ferric chelates. The reduction of EDTA-Fe^{3+} and DTPA-Fe^{3+} by microsomes would appear to occur at the flavoprotein reductase, but the site of ADP-Fe^{3+} reduction is not clear. The reduction of ADP-Fe^{3+} by a reconstituted MFO system occurs as well, so this would suggest that cytochrome P450 might be the microsomal enzyme ultimately responsible for reducing ADP-Fe^{3+}. In addition, per unit of reductase activity, microsomes oxidize more NADPH (in the presence of EDTA-Fe^{3+} or DTPA-Fe^{3+}) than the purified reductase, suggesting that EDTA-Fe^{3+} and DTPA-Fe^{3+} may also be reduced at other sites on the electron transport chain, perhaps at cytochrome P450.

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CHAPTER II

Superoxide Production by Mixed-Function Oxidase Components

ABSTRACT

Superoxide production by purified reductase, rat liver microsomes, and reconstituted MFO system containing reductase, DLPC, and one of several purified cytochrome P450 isozymes, was determined using an acetylated cytochrome c reduction assay. The reductase generated only a very low rate of $O_2^{\cdot-}$ production (0.2% of its reduction capacity towards cytochrome c) but $O_2^{\cdot-}$ production was stimulated by EDTA-Fe^{3+} and DTPA-Fe^{3+}, the ferric chelates that were previously shown to stimulate NADPH oxidation and be anaerobically reduced by the reductase. Microsomes generated considerably more $O_2^{\cdot-}$ than the reductase alone, presumably due to the presence of cytochrome P450 isozymes.

Several cytochrome P450 isozymes were tested for their potential to reduce oxygen to $O_2^{\cdot-}$ in the presence of the reductase and DLPC. Cytochrome P450d generated slightly more $O_2^{\cdot-}$ than did cytochrome P450b and both generated more than cytochrome P450c. Superoxide production required the same components necessary for successful reconstitution of MFO activity, detergent-solubilized reductase, DLPC and cytochrome P450. Superoxide production in the reconstituted system was confirmed using EPR spin trapping with DMPO.

The ability of microsomes isolated from rats pretreated with various inducers of the MFO system to oxidize NADPH and produce $O_2^{\cdot-}$ and H_2O_2 was also examined. In agreement with

the literature, microsomes from rats induced with phenobarbital generated more $O_2^{\cdot-}$ and H_2O_2 than microsomes from untreated or HBB-treated animals. Despite the ability of HBB to induce cytochrome P450d the isozyme which stimulated $O_2^{\cdot-}$ production in the reconstituted system, microsomes from HBB-induced animals did not generate appreciably more $O_2^{\cdot-}$ and H_2O_2 than microsomes from phenobarbital-induced animals.

INTRODUCTION

There are numerous enzyme and enzyme systems that have been shown to generate $O_2^{\cdot-}$ and H_2O_2.[1,2] However, as of yet it has not been possible to determine the significance of the production of oxygen radicals and in most situations an accurate quantitation of oxygen radical production *in vivo* has not been made. Thus, the quantitation of oxygen radical production *in vitro* is a crucial first step towards the eventual understanding of the potential significance of oxygen radical production *in vivo*.

One of the enzyme systems that has received a great deal of attention with respect to oxygen radical generation is the microsomal MFO system. This electron transport chain catalyzes the monooxygenation of numerous substrates, both endogenous and exogenous. During oxidation of drug substrates and even in the absence of substrates, this electron transport system has been shown to produce $O_2^{\cdot-}$ and H_2O_2. Based on initial studies where SOD, and copper complexes having dismutase activity were purported to inhibit MFO activity,[3,4] it was suggested that the reduction of cytochrome P450 might be via $O_2^{\cdot-}$ generated by the reductase.[4] However, more recent studies have demonstrated that the reductase reduces cytochrome P450 directly.

Significant disagreement still exists over the ability of the reductase to generate $O_2^{\cdot-}$ *in vitro*.[5-8] The previous

results in Chapter I indicated that EDTA-Fe^{3+}, DTPA-Fe^{3+} and perhaps other iron chelates may significantly enhance NADPH oxidation by the reductase, and this may be a factor in rates of $O_2^{\cdot-}$ measured in various systems. Moreover, previous reports in which the epinephrine assay was used to quantitate $O_2^{\cdot-}$ production have since been shown to be suspect.[9-11]

Regardless of the ability of the reductase to generate $O_2^{\cdot-}$, several lines of evidence indicate that the isozymes of cytochrome P450 are directly responsible for the bulk of microsomal oxygen radical generation. Higher rates of H_2O_2 production have been observed with microsomes isolated from rats pretreated with cytochrome P450 inducers.[12] Many chemicals that are metabolized by the hepatic MFO system stimulate H_2O_2 production.[12] Inhibitors of cytochrome P450 such as carbon monoxide[13] and metyrapone,[14] also inhibit microsomal oxygen radical production, and purified oxyferrous complexes of cytochrome P450 isozymes have also been shown to autoxidize or dissociate generating $O_2^{\cdot-}$ and H_2O_2.[15-17]

There have been several studies in which the production of $O_2^{\cdot-}$ and H_2O_2 by microsomes have been studied. However, it is difficult to make many generalizations about the results for a number of reasons. First of all, the measurement of H_2O_2 and especially $O_2^{\cdot-}$ is inherently difficult due to the transient nature of these partially reduced oxygen species.

As previously mentioned, the type of assay can dramatically affect the results that are obtained. Cytochrome c reduction is usually considered as the most reliable assay for $O_2^{\cdot-}$ production, but it cannot be used for quantitation of microsomal $O_2^{\cdot-}$ production since it is an excellent substrate for the reductase. Thus, investigators have modified cytochrome c by acetylation or succinoylation to reduce its tendency for direct reduction by the reductase but yet maintain its potential for reduction by reductants such as $O_2^{\cdot-}$.[18-20]

Secondly, SOD and catalase frequently contaminate microsomal preparations and scavenge $O_2^{\cdot-}$ and H_2O_2, yielding erroneous results.[21,22] Many investigators have added EDTA, or DTPA[5,8,20,23] which (if iron was present) could have lead to artificially high rates of NADPH oxidation as was shown in Chapter I and $O_2^{\cdot-}$ production as will be demonstrated in this chapter. Also, recent results have shown that the production of H_2O_2 varies as the ratio of NADPH to substrate is altered.[24] Thus, the method of preparation of microsomes and purified enzymes as well as reaction conditions may also affect the observed rates of $O_2^{\cdot-}$ production.

One aspect of particular interest and one in which very little information is available is the potentials or tendencies of the various cytochrome P450 isozymes to generate $O_2^{\cdot-}$. Cytochrome P450 isozymes exist in equilibrium mixtures containing both high and low spin forms, and several isozymes are isolated from microsomal membranes

predominantly in the high spin state,[25-28] the spin state induced by the binding of many substrates[29] and the conformation favored for the reduction of heme iron by the reductase.[30] Reduction of heme iron by the reductase precedes the binding of molecular oxygen and its subsequent activation, so greater rates of oxygen activation could be predicted to occur under conditions where more high spin cytochrome P450 is present.

The treatment of animals with various xenobiotics results in the induction of different complements of cytochrome P450, isozymes. The toxicity exhibited by these xenobiotics could conceivably be due, at least in part, to their induction of isozymes with increased tendencies for uncoupled reduction of oxygen. However, it is not clear whether the rate of $O_2^{\cdot-}$ production by microsomes correlates with the presence of particular isozymes or with the fraction of isozymes in the high spin conformation.

Relatively few studies have addressed the oxidase activities of purified cytochrome P450 isozymes. A few studies have investigated $O_2^{\cdot-}$ production by partially purified preparations of MFO components.[31,32] A greater number of investigators have been concerned with oxygen radical production by microsomes and the overall percentage of NADPH oxidation that results in the uncoupled reduction of oxygen to H_2O_2 or water in microsomal or reconstituted MFO assays.[20,33-35] Thus, the following studies were initiated to better characterize the uncoupling of the

microsomal MFO system and the experimental factors that influence the measurement of oxygen radical production by microsomes or the reconstituted system.

MATERIALS AND METHODS

Purification of Cytochrome P450c and P450d: Male Sprague Dawley rats (150-250 g) were pretreated with HBB (10 μmol/kg) given by gavage and sacrificed 3 days later. Liver microsomes were isolated as previously described in Chapter I and solubilized with sodium cholate added to a final concentration of 0.8% (w/v). Solubilized microsomal protein was subjected to affinity chromatography on N-octylamino Sepharose.[36] The cytochrome P450 fraction eluting at 0.3% sodium cholate, 0.1% Lubrol PX (Sigma) was dialyzed and then applied to a DEAE-Sepharose column and chromatographed by the method of Ästrom and DePierre.[36] The pools of fractions containing cytochrome P450c and P450d were each subjected to hydroxyapatite chromatography, washing with 2 column volumes of 30, 90, and 180 mM phosphate buffer. Purified cytochrome P450c eluted with 90 mM phosphate whereas cytochrome P450d eluted with 180 mM phosphate. Preparations of these cytochrome P450 isozymes were homogeneous on SDS gel electrophoresis and had specific contents of 12-18 nmol/mg protein.

Acetylation of Cytochrome c: Cytochrome c (Sigma) was acetylated using the procedure of Wada and Okunuki.[37] A 1 mM cytochrome c solution in half-saturated sodium acetate on ice was treated with a 20-fold molar excess of acetic

anhydride over the constitutive lysine residues. The constantly stirred solution was maintained at pH 7.0 over the course of reaction through the dropwise addition of 1 N NaOH. After 30 min, the reaction mixture was dialyzed against 200 volumes of distilled H_2O at 4°C (three changes). The dialyzed protein solution was centrifuged at 10,000xg for 20 min to remove any insoluble material. The resulting supernatant was adjusted to pH 7.5 and applied to a 1 x 20-cm column of DEAE-cellulose (Whatman, Clifton, N.J.) previously equilibrated with 5 mM KH_2PO_4, pH 7.0. The column was first washed with 150 ml of equilibration buffer before the bulk of the acetylated cytochrome c was eluted with 0.2 M KH_2PO_4, pH 7.0. Acetylated cytochrome c was dialyzed against distilled H_2O before lyophilization. Prior to use, the lyophilized preparation was dissolved in 0.3 M NaCl, and EDTA was added to 10 mM final concentration. The use of EDTA was intended to chelate any divalent cations loosely associated with the acetylated protein. This solution was incubated on ice for 1 hr. The solution was centrifuged at low speed to remove any insoluble material prior to chromatography on Sephadex G-25. The percentage acetylation of cytochrome c ranged from 95-99% using picrylsulfonic acid assay.[19]

Acetylated Cytochrome c Reduction. The reduction of acetylated cytochrome c was monitored spectrophotometrically at 550 nm with a Cary 219 recording spectrophotometer. The

cuvette chamber was maintained at 37°C. The initial rate of acetylated cytochrome c reduction was monitored in each instance, and a value of 21 $mM^{-1}cm^{-1}$ for the extinction coefficient of acetylated cytochrome c was used.

When $O_2^{\cdot-}$ production by a reconstituted system was monitored, cytochrome P450 isozymes were preincubated with the reductase and DLPC for 5 min prior to the addition of the other reagents. When no preincubation was performed, initial rates of NADPH oxidation and $O_2^{\cdot-}$ production were not maximal indicating that efficient complex formation had not occurred in the diluted solution.

Hydrogen Peroxide Production: Hydrogen peroxide was measured by a modification of the procedure employed by Hildebrandt and Roots.[12] Microsomes (0.2 mg/ml) were incubated with NADPH (0.5 mM) in the presence of azide (3mM) and desferrioxamine (1 mM) at 37° C in a shaking water bath. Azide was added to prevent catalatic decomposition of H_2O_2 and desferrioxamine was used to inhibit non-heme iron-catalyzed decomposition of H_2O_2. Aliquots from the incubation mixture were quenched in ice cold 3% TCA and allowed to stand on ice for 20 min before they were centrifuged at low speed for 10 min to pellet precipitated material. Supernatants (0.66 ml) were neutralized with 2 M triethanolamine (0.06 ml). Finally, o-dianisidine and horseradish peroxidase (1 μg/ml) were added and the H_2O_2-dependent oxidation of o-dianisidine[38] was monitored at

460 nm and compared to a standard curve prepared with known concentrations of H_2O_2.

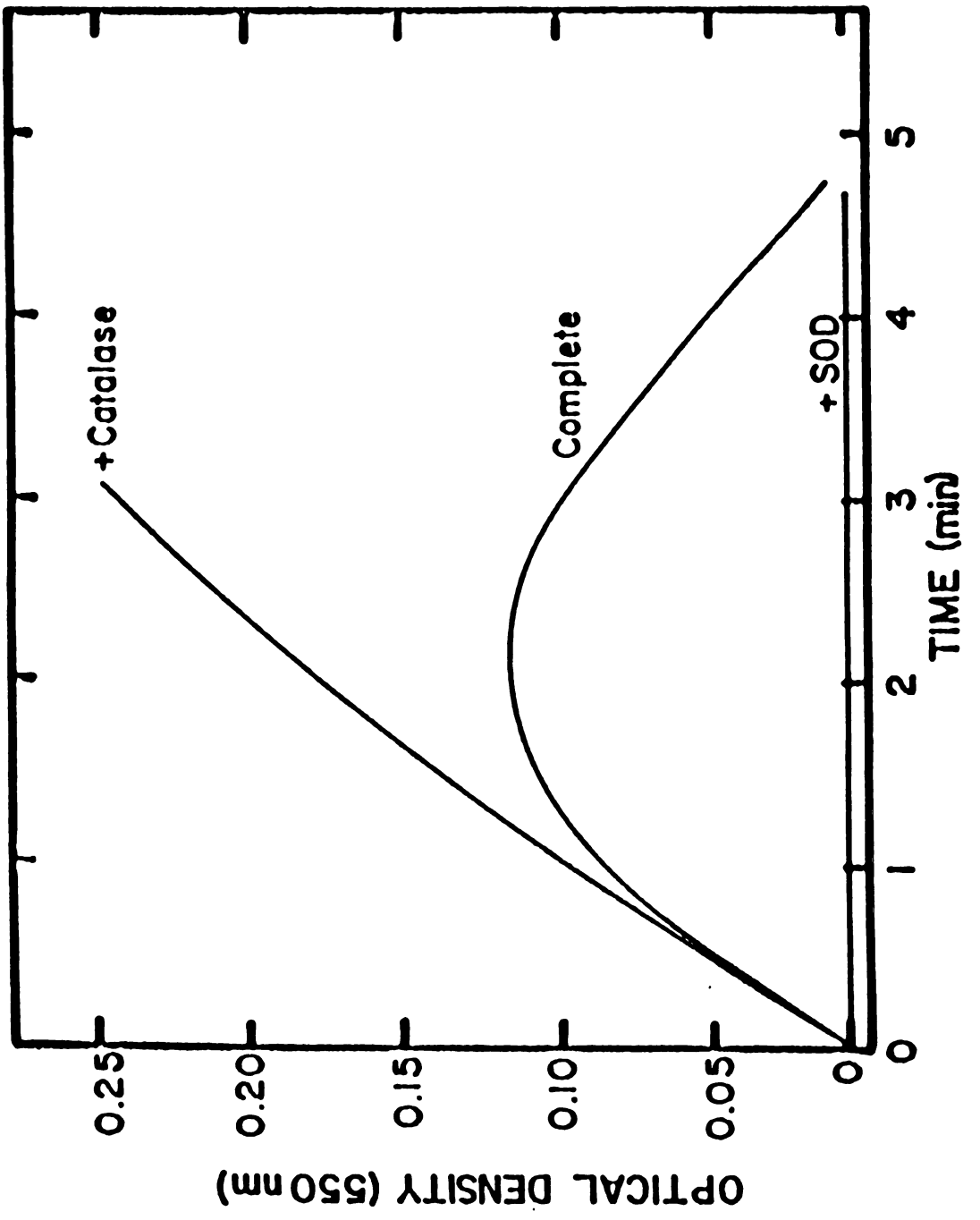
RESULTS

Reduction of Acetylated Cytochrome c by Xanthine Oxidase and NADPH Cytochrome P450 Reductase: The reduction of acetylated cytochrome c by $O_2^{\cdot-}$ produced by xanthine oxidase is shown in Figure 9. The rate of reduction was linear for at least 5 min when catalase was included in the incubation mixture. No reduction of acetylated cytochrome c was observed when SOD was added. Purified reductase also catalyzed acetylated cytochrome c reduction (Figure 10). However, the rate of reduction was linear either in the presence or absence of catalase and was inhibited only slightly by SOD. The difference between the rate of acetylated cytochrome c reduction in the presence and in the absence of SOD was used as the measure of $O_2^{\cdot-}$ generation.

Superoxide Generation by Xanthine Oxidase: Effect of Iron Chelates: The effects of several chelators and ferric chelates on the production of $O_2^{\cdot-}$ by xanthine oxidase are shown in Table 6. Superoxide production was calculated as the fraction of the acetylated cytochrome c reduction rate inhibited by SOD per unit of unmodified cytochrome c reduction. Using this assay, approximately 66% (658 nmol/unit) of the $O_2^{\cdot-}$ produced by xanthine oxidase could be detected, although by either increasing the acetylated cytochrome c concentration or decreasing the xanthine

Figure 9: Reduction of Acetylated Cytochrome c by Xanthine Oxidase

Incubations contained xanthine oxidase (0.02 u/ml), xanthine (0.17 mM), and acetylated cytochrome c (1.6 mg/ml) in 0.3 M NaCl, pH 7.0. Some incubations also contained SOD (230 u/ml) or catalase (460 u/ml).


+Catalase
Complete
+SOD
0.25
0.20
0.15
0.10
0.05
0
0
1
2
3
4
5
TIME (min)
OPTICAL DENSITY (550 nm)

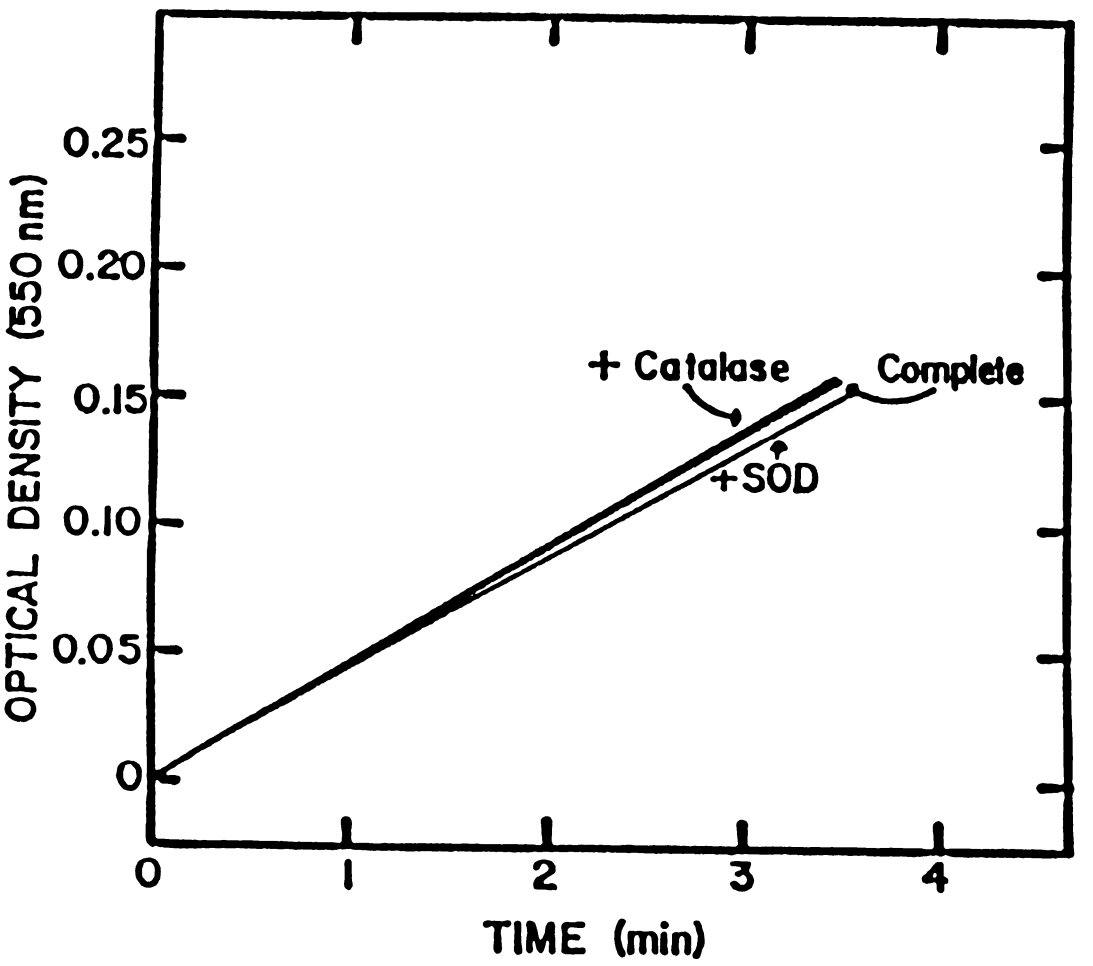


Figure 10: Reduction of Acetylated Cytochrome c by NADPH-Cytochrome P450 Reductase

Incubations contained purified reductase (0.015 u/ml), NADPH (0.1 mM), and acetylated cytochrome c (1.6 mg/ml) in 0.3 M NaCl, pH 7.0. Some incubations also contained SOD (230 u/ml) or catalase (460 u/ml).

Table 6. Superoxide Production by Xanthine Oxidase

	Acetylated Cytochrome c Reduction (nmol/unit)	Superoxide Production (nmol/unit)
Complete system	660	
		660 (100)
+SOD	0	
+EDTA	580	
		580 (88)
+EDTA + SOD	0	
+EDTA-Fe^{3+}	530	
		530 (80)
+EDTA-Fe^{3+} + SOD	0	
+ADP	570	
		570 (86)
+ADP + SOD	0	
+ADP-Fe^{3+}	550	
		550 (83)
+ADP-Fe^{3+} + SOD	0	
+DTPA	600	
		600 (91)
+DTPA + SOD	0	
+DTPA-Fe^{3+}	620	
		620 (94)
+DTPA-Fe^{3+} + SOD	0	
+Desferrioxamine	650	
		650 (98)
+Desferrioxamine + SOD	0	
+Desferrioxamine-Fe^{3+}	620	
		620 (94)
+Desferrioxamine-Fe^{3+}+SOD	0	

Incubations contained xanthine oxidase (0.001 u/ml), xanthine (0.17 mM), acetylated cytochrome c (1.6 mg/ml), and catalase (460 u/ml) in 0.3 M NaCl, pH 7.0. Incubations also included SOD (230 u/ml), chelator (0.11 mM), or ferric chelate (0.11 mM chelator, 0.1 mM $FeCl_3$) as indicated. ADP and ADP-Fe^{3+} concentrations were 0.5 mM and 0.5 mM; 0.1 mM $FeCl_3$, respectively. Values in parentheses refer to the percentage of $O_2^{\cdot}$ production in the complete system.

oxidase concentration, greater percentages of $O_2^{\cdot}$ were detected. Any one of the chelators or ferric chelates tested caused a modest inhibition of apparent $O_2^{\cdot}$ production by xanthine oxidase, but under no circumstances did acetylated cytochrome c reduction occur in the presence of SOD.

Superoxide Production by Purified NADPH Cytochrome P450 Reductase: Effect of Iron Chelates. Acetylated cytochrome c reduction and $O_2^{\cdot}$ production by the purified reductase are shown in Table 7. The significant differences between xanthine oxidase and reductase-dependent reduction of acetylated cytochrome c apparent in Figures 9 and 10 are also evident from the data comprising Table 7. The addition of SOD to the incubations containing the reductase did not completely inhibit acetylated cytochrome c reduction. Superoxide production by the reductase was 2.3 nmol/unit cytochrome c reductase activity (0.2% of its ability to reduce unmodified cytochrome c). In contrast to their inhibition of xanthine oxidase-dependent acetylated cytochrome c reduction, all iron chelates that were tested enhanced reductase-dependent acetylated cytochrome c reduction. Ferric chelates enhanced both $O_2^{\cdot}$-dependent and $O_2^{\cdot}$-independent rates of acetylated cytochrome c reduction. Particularly noteworthy was the stimulation of apparent $O_2^{\cdot}$ production by the reductase when either EDTA-Fe^{3+} or DTPA-Fe^{3+} was included in the assay, for these iron chelates are

Table 7. Superoxide Production by Purified NADPH-Cytochrome P450 Reductase

	Acetylated Cytochrome c Reduction (nmol/unit)	Superoxide Production (nmol/unit)
Complete system	9.7	2.3 (100)
+SOD	7.4	
+EDTA	13	4.8 (209)
+EDTA + SOD	8.2	
+EDTA-Fe^{3+}	93	42 (1826)
+EDTA-Fe^{3+} + SOD	51	
+ADP	16	7.1 (309)
+ADP + SOD	8.9	
+ADP-Fe^{3+}	24	12 (522)
+ADP-Fe^{3+} + SOD	12	
+DTPA	9.4	1.3 (56)
+DTPA + SOD	8.1	
+DTPA-Fe^{3+}	140	20 (870)
+DTPA-Fe^{3+} + SOD	120	
+Desferrioxamine	13	3.1 (135)
+Desferrioxamine + SOD	9.9	
+Desferrioxamine-Fe^{3+}	11	3.0 (130)
+Desferrioxamine-Fe^{3+} + SOD	8.0	

Incubations contained NADPH (0.1 mM), purified reductase (0.014/ml) and acetylated cytochrome c (1.6 mg/ml) in 0.3 M NaCl pH 7.0 with SOD (230 u/ml), chelator (0.11 mM) or ferric chelate (0.11 mM:0.1 mM $FeCl_3$) added as indicated. The concentration of ADP was 0.5 mM. Values in parentheses refer to the percentage of $O_2^{\cdot-}$ production in the complete system.

often used in EPR spin trapping, lipid peroxidation, and reconstituted drug metabolism systems. These chelates were shown to be substrates for the reductase in the previous chapter. In the absence of reductase, ferrous chelates were shown to reduce unmodified cytochrome c as well as acetylated cytochrome c (data not shown). It was also observed that EDTA-Fe^{3+} or DTPA-Fe^{3+} did not affect the rate of unmodified cytochrome c reduction.

The stimulation by EDTA-Fe^{3+} or DTPA-Fe^{3+} of acetylated cytochrome c reduction by the purified reductase was concentration dependent in that increasing concentrations of ferric present as either the DTPA or EDTA chelate resulted in increasing rates of acetylated cytochrome c reduction (Figure 11). Relatively little stimulation of acetylated cytochrome c reduction in the presence of ADP-Fe^{3+} or desferrioxamine-Fe^{3+} was observed.

Superoxide Generation by Rat Liver Microsomes: Effect of Iron Chelates: NADPH-dependent acetylated cytochrome c reduction and $O_2^{\cdot-}$ production by rat liver microsomes is shown in Table 8. The rate of $O_2^{\cdot-}$ production was 15.6 nmol/unit unmodified cytochrome c reductase activity. Therefore, $O_2^{\cdot-}$ production accounted for only about 1.5% of the total NADPH-dependent reduction capacity of microsomes. Chelating agents (without added iron) had little effect on microsomal acetylated cytochrome c reduction or $O_2^{\cdot-}$ production. However, ferric chelates, especially EDTA-Fe^{3+}

Figure 11: Stimulation of Acetylated Cytochrome c Reduction by Ferric Chelates

Incubations contained purified reductase (0.01 u/ml), NADPH (0.1 mM), acetylated cytochrome c (1.6 mg/ml) and varying concentrations of ferric chelates in 0.3 M NaCl pH 7.0. The chelator: iron ratios were all 1.1:1 except ADP-Fe^{3+} which was 5:1. The chelates used were: (●), DTPA-Fe^{3+}; (■), EDTA-Fe^{3+}; (▼), ADP-Fe^{3+}; (○), desferrioxamine-Fe^{3+}.

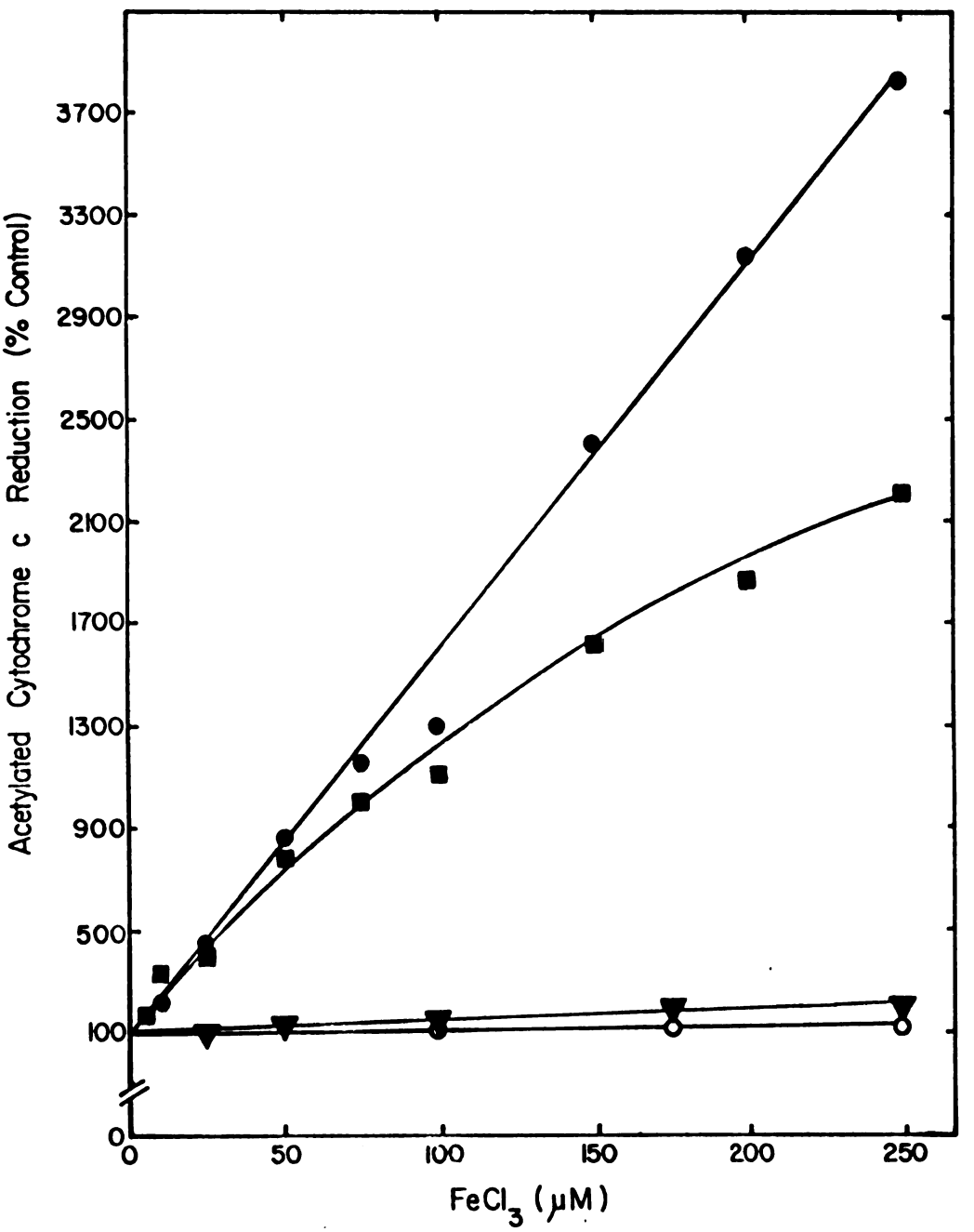

Acetylated Cytochrome c Reduction (% Control)
3700
3300
2900
2500
2100
1700
1300
900
500
100
0
0
50
100
150
200
250
FeCl3 (μM)

Table 8. Superoxide Production by Rat Liver Microsomes

	Acetylated Cytochrome c Reduction (nmol/unit)	Superoxide Production (nmol/unit)
Complete system	28	15 (100)
+SOD	13	
+EDTA	30	15 (100)
+EDTA + SOD	15	
+EDTA-Fe^{3+}	140	70 (467)
+EDTA-Fe^{3+} + SOD	70	
+ADP	26	9.0 (60)
+ADP + SOD	17	
+ADP-Fe^{3+}	61	33 (220)
+ADP-Fe^{3+} + SOD	28	
+DTPA	27	11 (73)
+DTPA + SOD	16	
+DTPA-Fe^{3+}	260	30 (200)
+DTPA-Fe^{3+} + SOD	230	
+Desferrioxamine	24	10 (67)
+Desferrioxamine + SOD	14	
+Desferrioxamine-Fe^{3+}	28	10 (67)
+Desferrioxamine-Fe^{3+} + SOD	18	

Incubations contained NADPH (0.1 mM), rat liver microsomes (0.05 mg/ml) and acetylated cytochrome c (1.6 mg/ml) in 0.3 M NaCl pH 7.0 with SOD (230 u/ml), chelator (0.11 mM), or ferric chelate (0.11 mM; 0.1 mM $FeCl_3$) added as indicated. The concentration of ADP was 0.5 mM and ADP-Fe^{3+} was 0.5 mM ADP, 0.1 mM $FeCl_3$. Values in parentheses refer to the percentages of $O_2^{\cdot}$ production in the complete system.

and DTPA-Fe^{3+}, stimulated the rate of acetylated cytochrome c reduction measured either in the presence or absence of SOD. Thus, ferric chelates appeared to enhance both $O_2^{\cdot-}$-dependent and $O_2^{\cdot-}$-independent pathways for acetylated cytochrome c reduction.

Superoxide Production by the Reconstituted Mixed Function Oxidase System: The previous results demonstrated that microsomes generated more $O_2^{\cdot-}$ than did the purified reductase when normalized to equal reductase activities. This, along with reports from the literature indicated that increased $O_2^{\cdot-}$ production by microsomes was probably due to the presence of cytochrome P450. This was confirmed by the results of experiments shown in Figure 12 in which purified cytochrome P450 isozymes were reconstituted with detergent-solubilized reductase in DLPC micelles, the standard reconstituted system shown by researchers to form a competent electron transport chain capable of oxidizing various MFO substrates. The addition of increasing cytochrome P450 isozyme concentrations resulted in increased $O_2^{\cdot-}$ production. Cytochrome P450d produced slightly more $O_2^{\cdot-}$ than did cytochrome P450b and either produced more than cytochrome P450c. Superoxide production by all isozymes increased until the molar ratio reached about 4-5 nmol cytochrome P450/nmol reductase. Beyond that ratio the rate of $O_2^{\cdot-}$ production remained the same suggesting that the

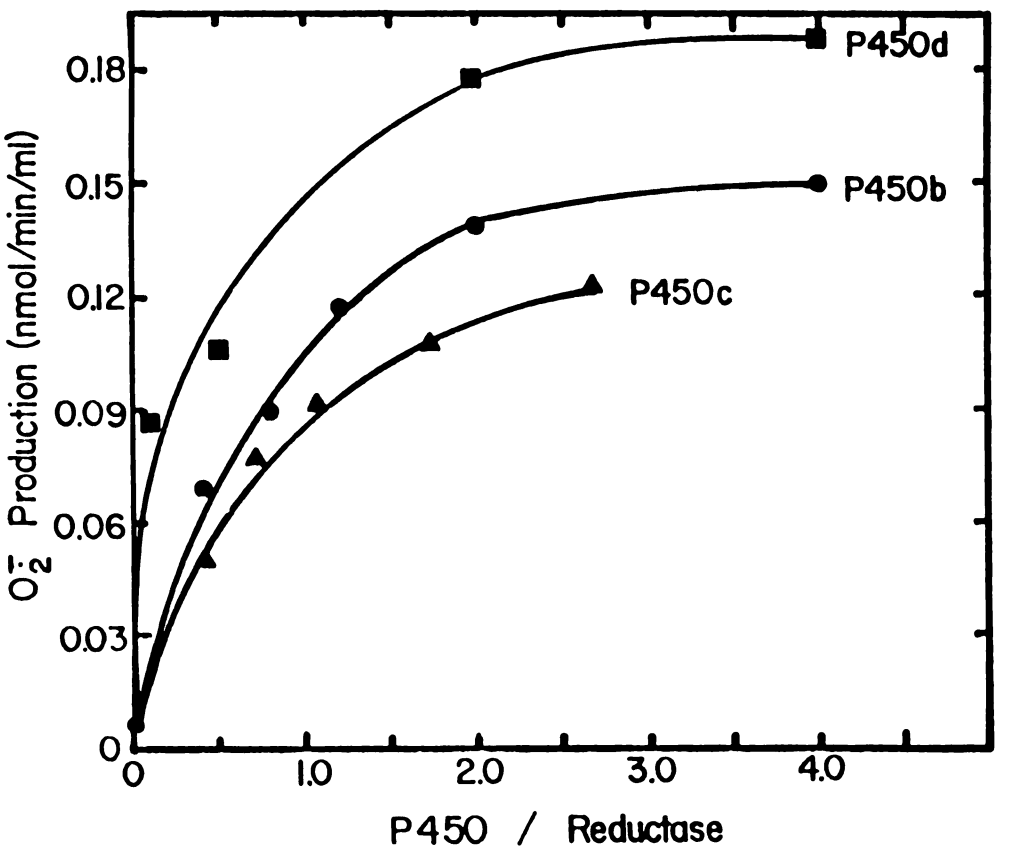


Figure 12: <u>Effect of Increasing Cytochrome P450: NADPH Cytochrome P450 Reductase Ratios on Superoxide Production in the Reconstituted System:</u>
Incubations contained (0.012 nmol reductase), NADPH (0.1 mM), acetylated cytochrome c (1.6 mg/ml), catalase (500 u/ml), DLPC (20 μg/ml), desferrioxamine (0.5 mM) and varying amounts of cytochrome P450 isozymes in 0.05M Tris pH 7.5. Duplicate incubations containing SOD (150 u/ml) were also monitored and $O_2^{\cdot-}$ production was calculated as before. The cytochrome P450 isozymes used were: (■), cytochrome P450d; (●), cytochrome P450b; (▲), cytochrome P450c.

reduction of cytochrome P450 by the reductase was becoming rate-limiting.

However, increasing the concentration of reductase relative to cytochrome P450 isozymes resulted in a continual increase in $O_2^{\cdot-}$ production (Figure 13), presumably due to the potential of the reductase to generate $O_2^{\cdot-}$, regardless of the presence of cytochrome P450 isozymes.

The effects of omitting various reagents from the acetylated cytochrome c reduction assay on the observed rate of $O_2^{\cdot-}$ production is shown in Table 9. Conditions necessary to reconstitute MFO activity (cytochrome P450, detergent-solubilized reductase and DLPC) are also necessary for $O_2^{\cdot-}$ production. On the other hand, the omission of desferrioxamine or catalase, added as precautionary measures to prevent iron-stimulated $O_2^{\cdot-}$ production or H_2O_2-dependent ferroacetylated cytochrome c oxidation respectively, generally had lesser effects on the observed rates of $O_2^{\cdot-}$ production.

These results were confirmed using EPR spin trapping (Figure 14-16). The DMPO·OOH adduct was observed in incubations containing purified cytochrome P450 isozyme, detergent-solubilized, and DLPC. However, the adduct signal was not as unequivocal as that observed in the xanthine oxidase system (Chapter I, Figure 3); one can clearly observe both DMPO·OOH and the appearance of the DMPO·OH adduct over the time of the scan. That the formation of DMPO·OH was primarily due to the decomposition

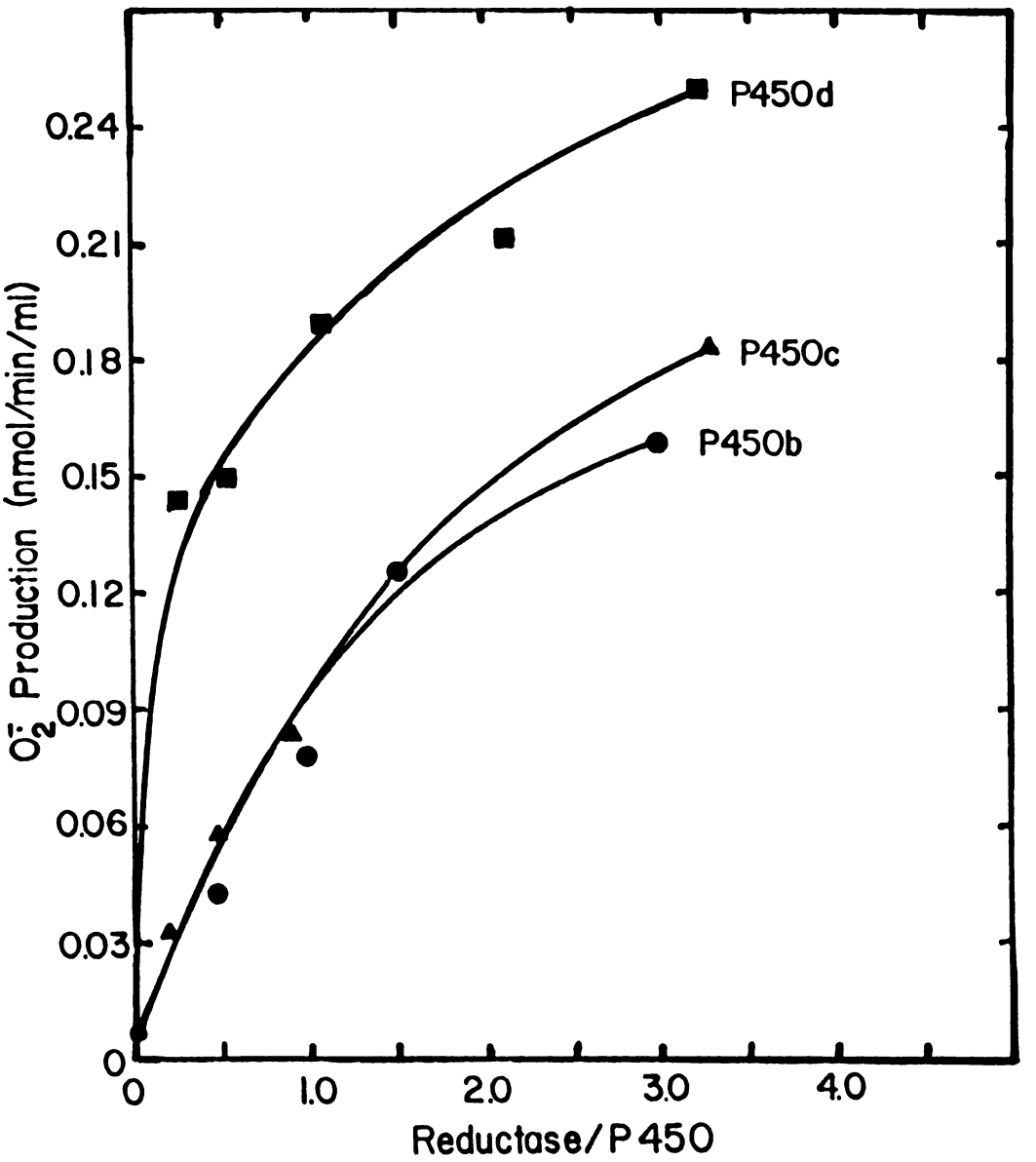


Figure 13: Effect of Increasing NADPH-Cytochrome P450 Reductase: Cytochrome P450 Ratios on Superoxide Production in the Reconstituted System:
Incubations contained cytochrome P450 isozymes: (■), cytochrome P450d; (●), cytochrome P450b, and (▲), cytochrome P450c, 0.012 nmol/ml, NADPH, acetylated cytochrome c, catalase, DLPC, desferrioxamine and SOD at the concentrations specified in Figure 12. The concentration of reductase was adjusted to obtain the indicated ratios.

Table 9. Requirements for Superoxide Production by the Reconstituted Mixed Function Oxidase System

	P450b	P450c	P450d
Complete system	100%	100%	100%
-P450 isozyme	11%	25%	8%
-DLPC	18%	51%	37%
-Catalase	130%	100%	93%
-Desferrioxamine	58%	97%	75%
+Protease-solubilized reductase	6%	22%	3%

The complete system contained DLPC (20 μg/ml), desferrioxamine (0.5 mM), acetylated cytochrome c (1.6 mg/ml), catalase (500 u/ml) NADPH (0.1 mM), cytochrome P450 isozymes (0.1 nmol/ml) and reductase (0.025 nmol/ml) in 50 mM NaCl pH 7.0 at 37°C. Duplicate incubations with SOD (150 u/ml) were also monitored. Superoxide production was calculated from the differences in the rate of acetylated cytochrome c reduction in incubations with and without SOD and expressed as a percent of the complete system.

Figure 14: EPR Spectra of Superoxide Producedd by Cytochrome P450b in the Reconstituted System:
Incubations contained cytochrome P450b (0.17 nmol/ml) reductase (0.038 nmol/ml), DLPC (30 μg/ml), desferrioxamine (0.75 mM), catalase (750 u/ml) and DMPO (60 mM) in 50 mM NaCl pH 7. Spectrometer settings were the same as outlined in Chapter I except the microwave power was 100 mW.

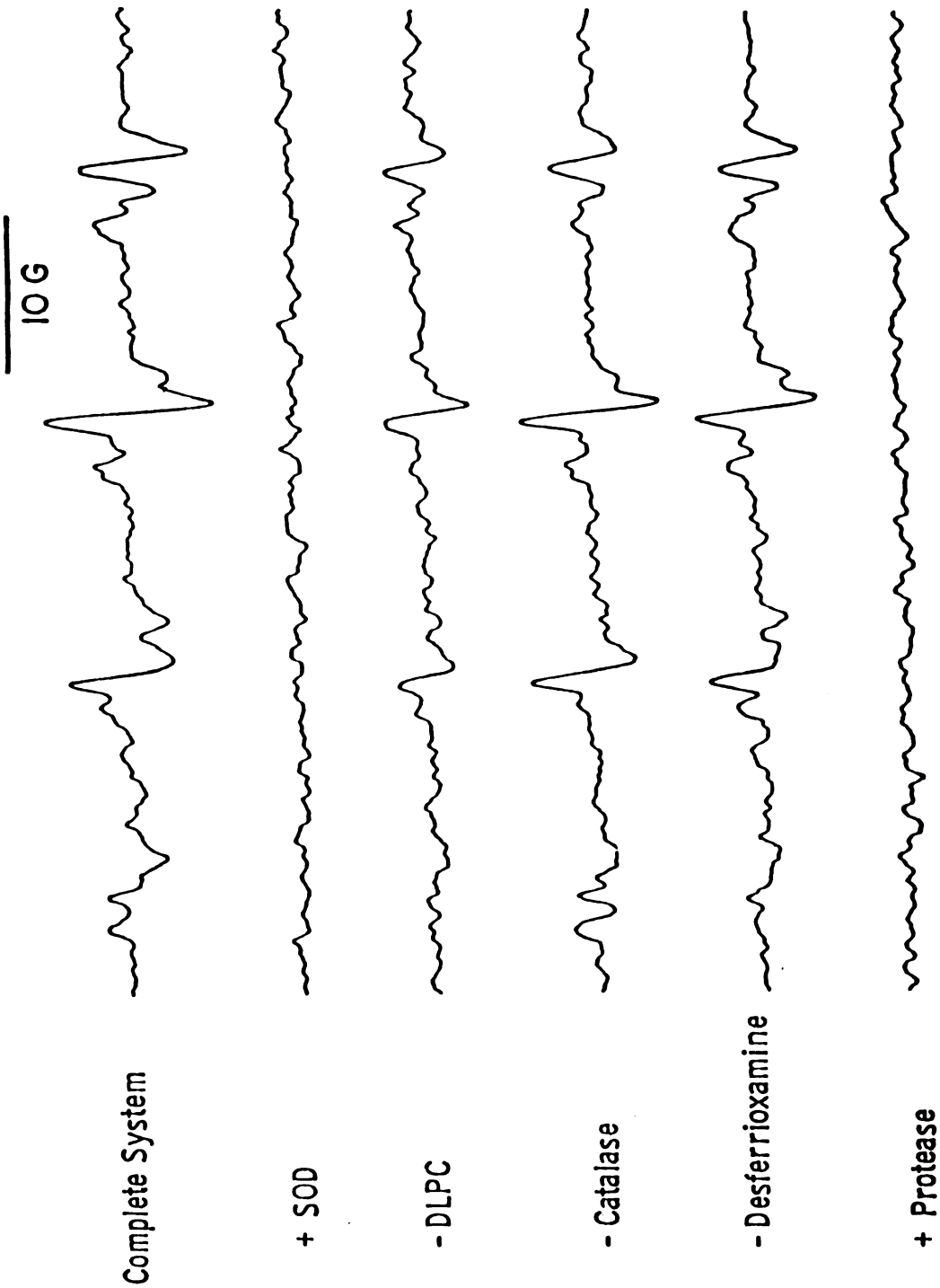

10 G
Complete System
+ SOD
- DLPC
- Catalase
- Desferrioxamine
+ Protease Solubilized Reductase

Figure 15: EPR Spectra of Superoxide Produced by Cytochrome P450c in the Reconstituted System:
Incubations are the same as detailed in Figure 14 with the exception that cytochrome P450c (0.17 nmol/ml) was added.

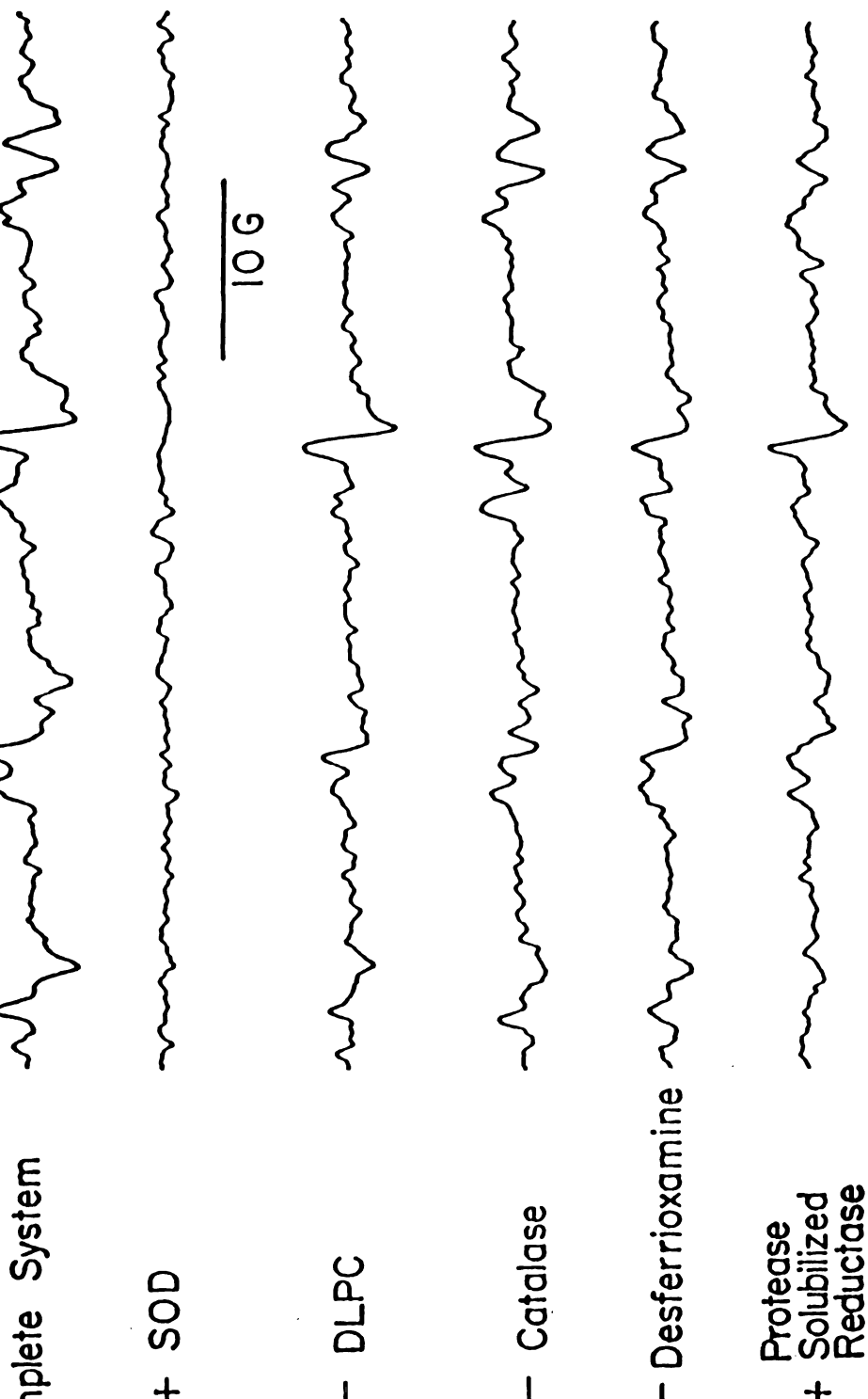
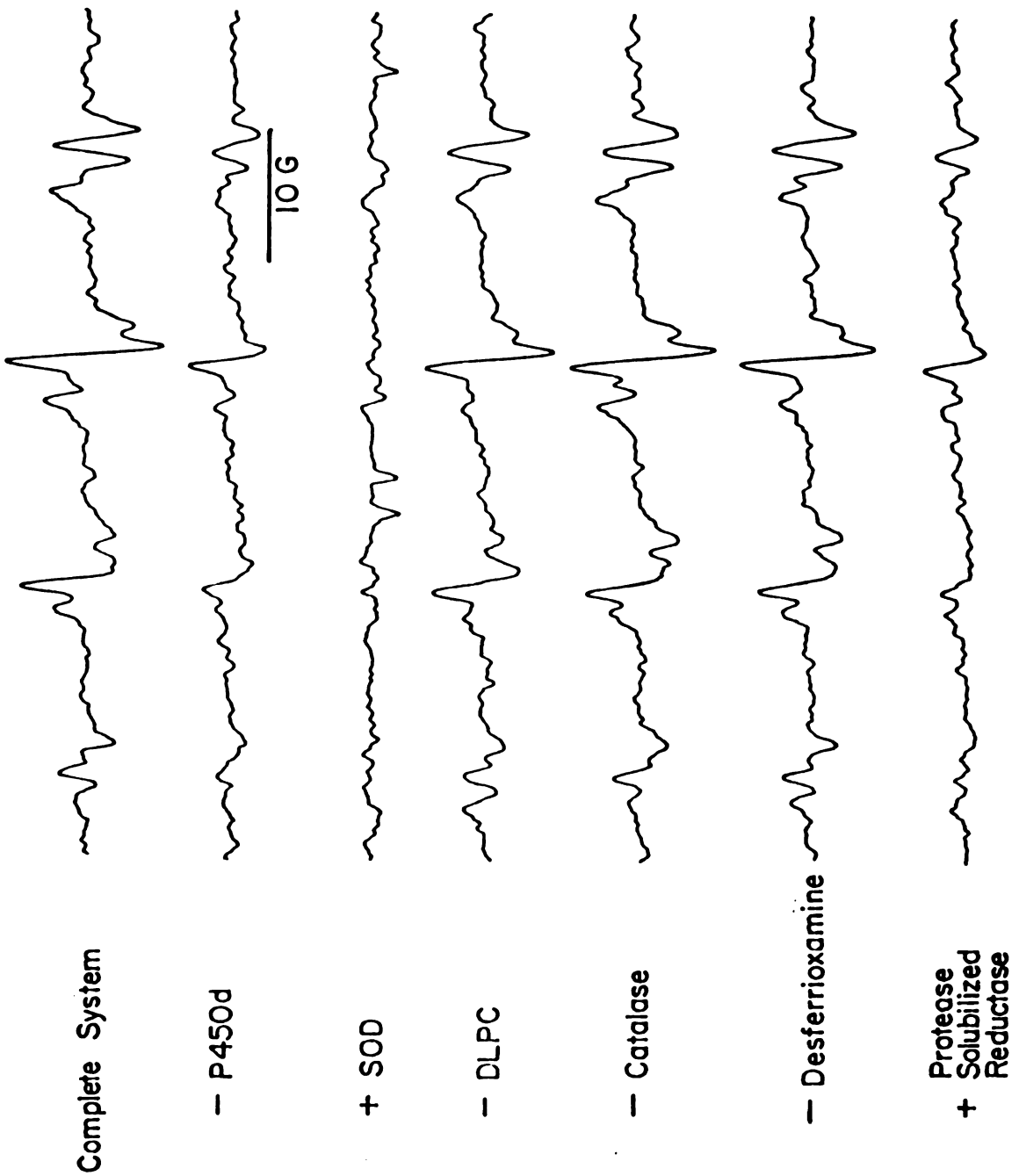

Complete System
+ SOD
10 G
- DLPC
- Catalase
- Desferrioxamine
Protease
+ Solubilized
Reductase

Figure 16: EPR Spectra of Superoxide Produced by Cytochrome P450d in the Reconstituted System:
Incubations are the same as detailed in Figure 14 with the exception that cytochrome P450d (0.17 nmol/ml) was added.


Complete System
- P450d
10 G
+ SOD
- DLPC
- Catalase
- Desferrioxamine
Protease
+ Solubilized
Reductase

of the DMPO·OOH was demonstrated by showing that the addition of SOD inhibited both the DMPO·OOH and DMPO·OH signals. The inability of desferrioxamine, which inhibits iron-catalyzed ·OH formation[39] to inhibit the DMPO·OH signal intensity is further indication that DMPO·OH arose mainly from DMPO·OOH decomposition.

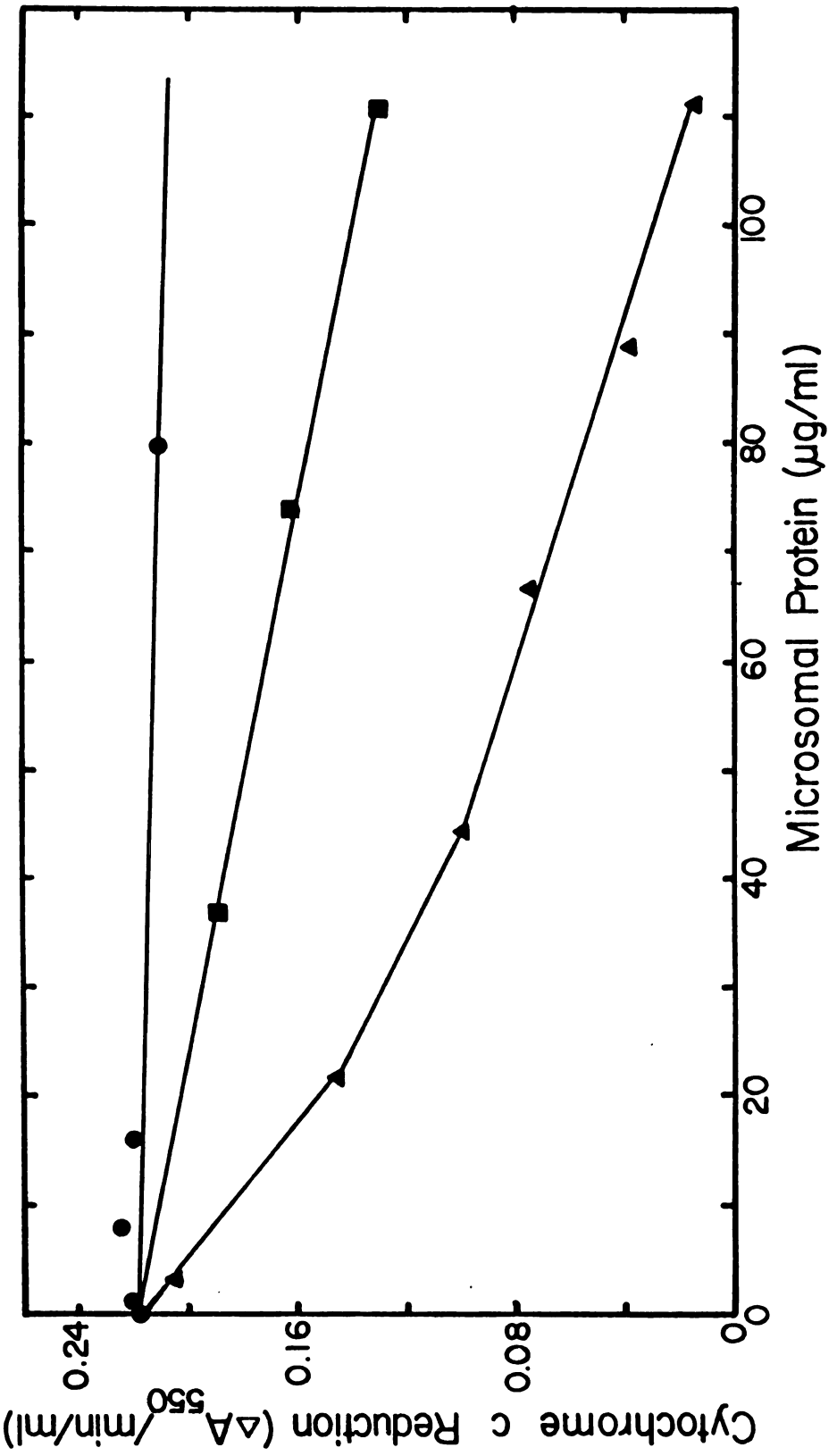
Pitfalls in Measuring Oxygen Radical Production by Microsomes: Although it is of interest to determine $O_2^{\cdot-}$ production by individual forms of cytochrome P450, of perhaps greater importance is the production of $O_2^{\cdot-}$ by the complement of cytochrome P450 isozymes present in hepatic microsomes. However, oxygen radical quantitation is inherently difficult, but in reconstituted MFO systems, there is usually no problem with inadvertent scavenging of $O_2^{\cdot-}$ or H_2O_2. Quantitation of oxygen radical production by microsomes is considerably more difficult however, and depending upon the isolation procedure, microsomes may contain varying amounts endogenous SOD activity, catalase activity and even ferritin which also has been shown to scavenge $O_2^{\cdot-}$.[22] Some investigators have attempted to account for microsomal scavenging of $O_2^{\cdot-}$ by measuring the decrease in $O_2^{\cdot-}$ production by the xanthine-xanthine oxidase system in the presence of microsomes and obtaining a correction factor which they applied to NADPH-dependent $O_2^{\cdot-}$ production to obtain the "true" value for O_2^{-} production.[20]

A similar approach was used to examine several microsomal preparations for their ability to inhibit $O_2^{\cdot-}$-dependent cytochrome c reduction by xanthine oxidase (Figure 17). Rat liver microsomes isolated by the routine differential centrifugation procedure dramatically inhibited cytochrome c reduction. Further washing of the microsomes with 1.15% KCl resulted in a preparation that inhibited cytochrome c reduction to a considerably lesser extent. Subsequent chromatography of the KCl-washed microsomes on Sepharose 2B resulted in microsomes having essentially no inhibitory effect on cytochrome c reduction.

Similar effects of washing and chromatography on the catalase activity in microsomes were also noted (See Chapter III). However, even chromatography was not sufficient to remove the last traces of catalatic activity from microsomes. Therefore, azide, a potent catalase inhibitor, has been used to inhibit the remaining activity present in microsomes.[12] The effect of azide on microsomal H_2O_2 production is shown in Figure 18. In the initial stages of the incubations, H_2O_2 was formed. However, in incubations without azide the final amount of H_2O_2 recovered from the incubations was not indicative of the total amount of H_2O_2 produced due to the catalase activity in microsomes. This was true either for basal rates of H_2O_2 production, or in incubations where H_2O_2 production was stimulated by the redox cycling agent paraquat. In the presence of azide, catalatic decomposition of H_2O_2 was prevented, and H_2O_2

Figure 17: Inhibition of Superoxide-Dependent Cytochrome c Reduction by Microsomes

All incubations contained xanthine (0.33 mM), xanthine oxidase (0.01 u/ml) and cytochrome c (80 μM), in 50 mM Tris, pH 7.5. In addition, incubations also included various concentrations of rat liver microsomes isolated by differential centrifugation (▲), microsomes washed with 1.15% KCl (■), or washed microsomes chromatographed on a Sepharose 2B column (●).


Cytochrome c Reduction (ΔA_{550}/min/ml)
0.24
0.16
0.08
0
0
20
40
60
80
100
Microsomal Protein (µg/ml)

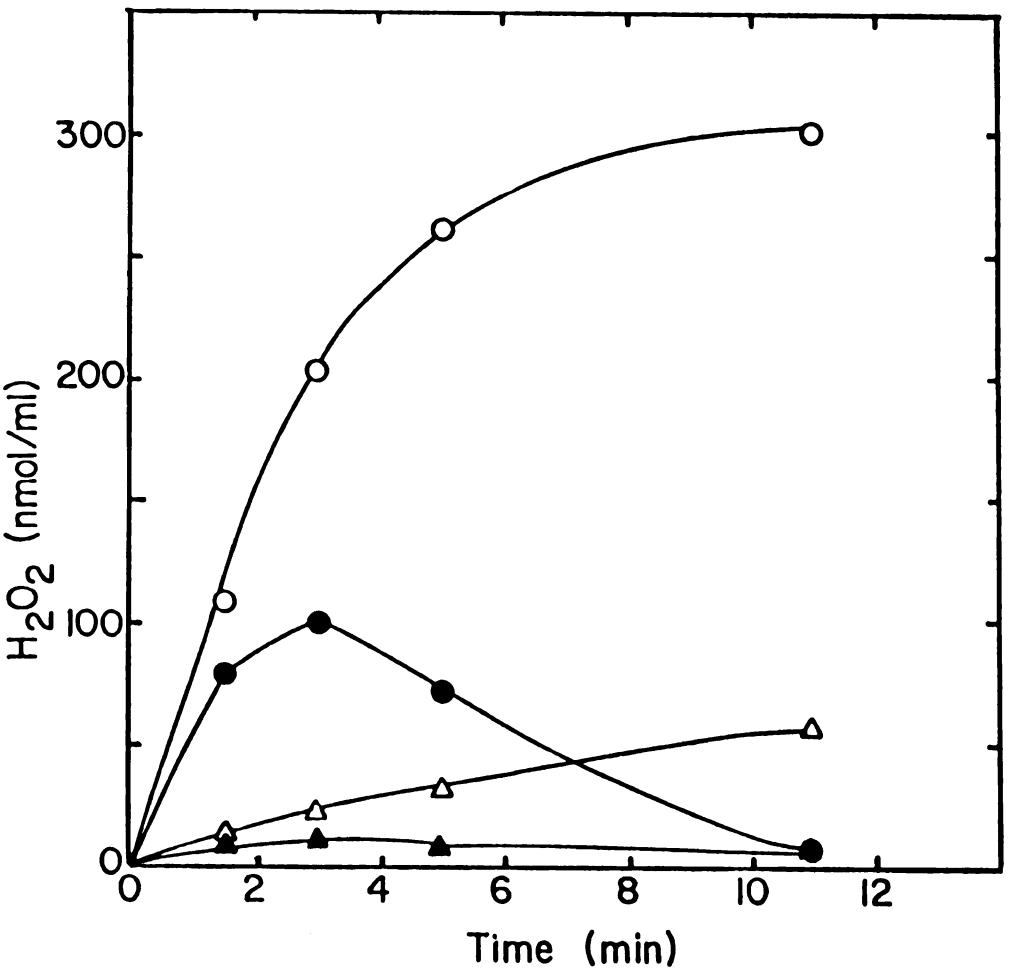


Figure 18: Time Course of Microsomal Hydrogen Peroxide Formation

Incubations contained microsomes (0.25 mg/ml), and NADPH (0.5 mM) in 0.05 M NaCl pH 7. Additions to the incubations were as follows: none, (▲); azide, (3 mM), (△); paraquat, (0.1 mM), (●); paraquat plus azide, (○). Hydrogen peroxide was assayed as detailed in Material and Methods.

accumulated over the entire time course of the of the experiment.

The effect of several ferric chelates on H_2O_2 production by microsomes was also investigated. Most ferric chelates that were tested had an inhibitory effect on microsomal H_2O_2 production (Figure 19). This was attributed to iron-catalyzed decomposition of H_2O_2, a property characteristic of numerous ferrous or ferric chelates. Of the iron chelates tested, only desferrioxamine-Fe^{3+} had no effect on the accumulation of H_2O_2 relative to control incubations. Therefore, desferrioxamine was added to all subsequent assays to prevent the decomposition of H_2O_2 during the incubation.

Stoichiometry of Microsomal Oxygen Radical Production:

The rates of NADPH oxidation and $O_2^{\cdot-}$ production by liver microsomes isolated from untreated rats or rats pretreated with phenobarbital or HBB are compared in Table 10. Significantly higher rates of NADPH oxidation and $O_2^{\cdot-}$ occurred in liver microsomes from phenobarbital- and HBB-pretreated animals than in untreated animals. This corresponded to an increase in cytochrome P450 content in these microsomes relative to control. Hydrogen peroxide production was assayed and the results are also shown in Figure 10. Again rates of H_2O_2 were greater in microsomes isolated from phenobarbital- or HBB-pretreatd rats than in those of control animals.

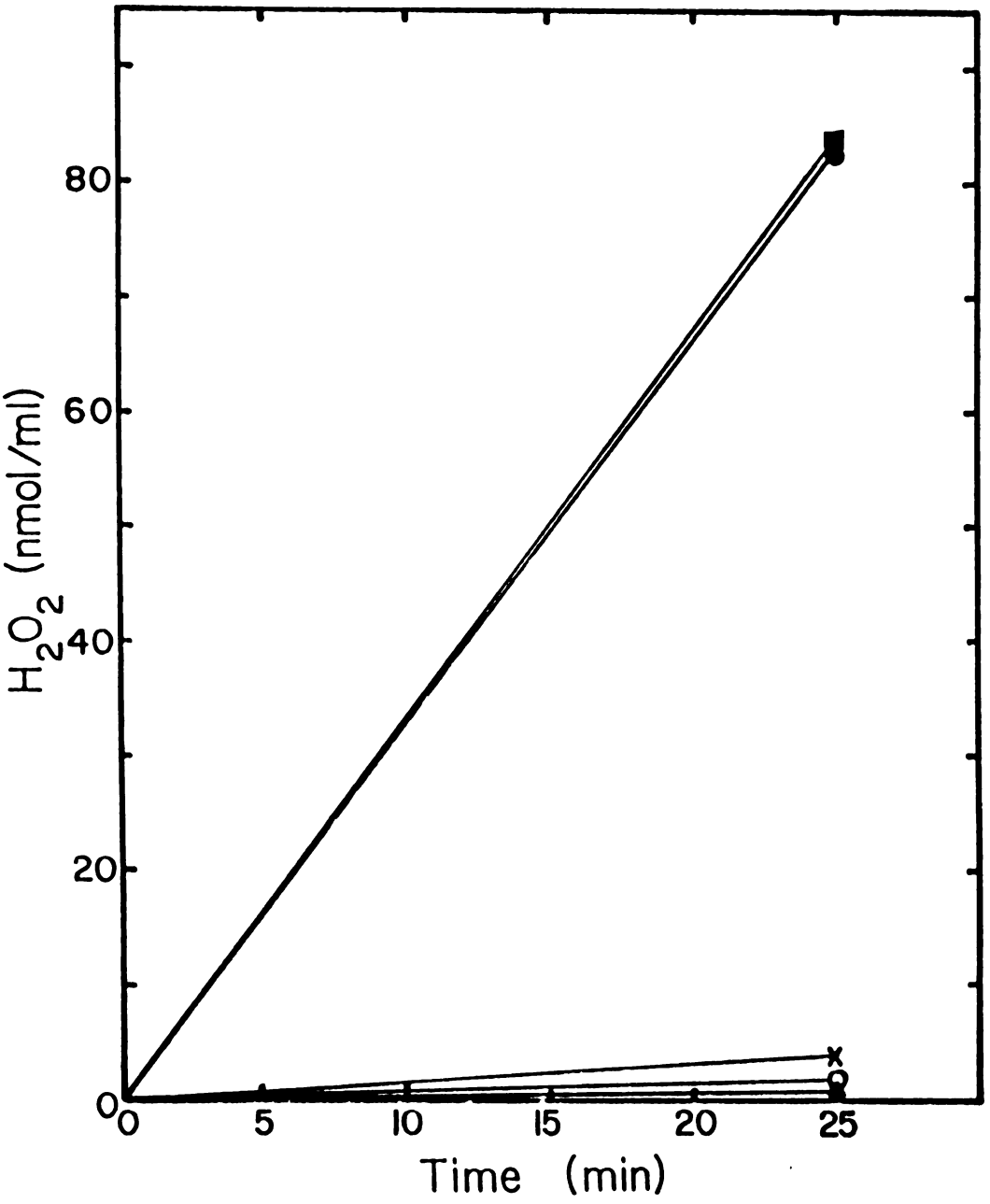


Figure 19: Effect of Ferric Chelates on Microsomal Hydrogen Peroxide Formation:
All incubations contained rat liver microsomes (0.25 mg/ml), and NADPH (0.5 mM) in 50 mM NaCl, pH 7.0 at 37°C. The following iron chelates were added (0.5 mM chelator: 0.1 mM $FeCl_3$) as indicated: none, (●); desferrioxamine-Fe^{3+}, (■); DTPA-Fe^{3+}, (X); EDTA-Fe^{3+}, (O); ADP-Fe^{3+}, (▲). Hydrogen peroxide was assayed as detailed in Materials and Methods.

Table 10. Stoichiometry of Oxygen Reduction by the Microsomal Mixed Function Oxidase System

Pretreatment	NADPH Oxidation	Superoxide Production	Hydrogen Peroxide Production
None	30	3.4	20
Phenobarbital	40	8.1	28
HBB	43	9.4	25

Microsomes were isolated from untreated rats or rats treated with phenobarbital or HBB (10 mg/kg in corn oil given by gavage). NADPH oxidation was monitored at 340 nm in incubations containing 0.1 mM NADPH in 0.05 M Tris pH 7.5. Hydrogen peroxide was measured as outlined in Material and Methods and $O_2^{\cdot}$ was quantitated as described in Table 8. Values are in nmol/min/mg protein.

Even though cytochrome P450 activates molecular oxygen and is postulated to produce $O_2^{\cdot-}$ and H_2O_2 via uncoupled reduction of oxygen, the possibility exists that the either in microsomes or the reconstituted system, the presence of cytochrome P450 isozymes may alter the electron transfer properties of the reductase such that generates $O_2^{\cdot-}$ and/or H_2O_2.[33] This hypothesis can be tested by examining the rates of NADPH oxidation and $O_2^{\cdot-}$ production from liver microsomes isolated from rats treated with isosafrole.

Isosafrole pretreatment results in the induction of cytochrome P450d, and its <u>in vivo</u> metabolism results in the formation of a metabolite which binds tightly to the heme iron of cytochrome P450d, preventing the binding of CO or oxygen and the metabolism of substrates.[40] Thus, these microsomes contain a specific isozyme previously shown to produce significant amounts of $O_2^{\cdot-}$, but that is specifically blocked by the isosafrole metabolite from activating oxygen. The metabolite can be removed by treating microsomes with butanol,[41] restoring its CO and oxygen binding capacities and its MFO activity. Comparison of the rates of NADPH oxidation and $O_2^{\cdot-}$ production by microsomes with or without the metabolite allows an assessment of the contribution of P450d to the rates of oxygen activation in these microsomes. The data are shown in Figure 11. Butanol treatment of the microsomes restored CO binding capacity and resulted in a near 6-fold increase in $O_2^{\cdot-}$ production. This was

Table 11. Effect of Butanol Pretreatment on NADPH Oxidation and Superoxide Production from Liver Microsomes Isolated from Isosafrole-Induced Rats

	NADPH Oxidation	Superoxide Production	Cytochrome P450	Total Cytochrome P450
Microsomes	36	5.2	1.45	2.75
Microsomes + Butanol	52	31	2.25	2.50

Microsomes were isolated from rats pretreated with isosafrole (150 mg/kg isosafrole in corn oil given by gavage daily for 4 days) by differential centrifugation and washed with 1.15% KCl, 10 mM EDTA pH 7.0 and then chromatographed on Sepharose 2B as in Figure 17. The isosafrole metabolite was displaced from microsomes by treating them with butanol (100 mM), for 1 hr. at room temperature prior to chromatography. Cytochrome P450 was determined by a carbon monoxide difference spectrum[42] and total cytochrome P450 was the sum of that determined with carbon monoxide and that of the isosafrole metabolite peak having $A_{455}-A_{490}$ of approximately 75 mM^{-1}.[43] Rates of NADPH oxidation and O_2 production are in nmol/min/mg protein and cytochrome P450 contents are in nmol/mg protein.

accompanied by a near stoichiometric increase in NADPH oxidation.

DISCUSSION

It was originally reported that the reductase produced $O_2^{\cdot}$, however, the epinephrine oxidation assay used to detect $O_2^{\cdot}$ formation has since been demonstrated to be rather poor for quantitating $O_2^{\cdot}$.[9,11] The reduction of cytochrome c is perhaps the assay of choice for the measurement of $O_2^{\cdot}$ under many circumstances, but microsomes and the reductase catalyze the direct reduction of cytochrome c. Acetylation (or succinoylation) of ferricytochrome c limits its potential for direct enzymatic reduction. Therefore, the reduction of acetylated cytochrome c is a more specific measure of $O_2^{\cdot}$ production than the reduction of unmodified cytochrome c. However, modification of cytochrome c does not totally eliminate the possibility of its direct reduction. Therefore, a highly modified cytochrome c preparation with a minimal tendency for direct enzymatic reduction has been used in these investigations.

The differences between xanthine oxidase- and reductase-dependent reduction of a highly acetylated cytochrome c preparation were readily apparent. Superoxide dismutase completely inhibited xanthine oxidase-dependent acetylated cytochrome c reduction, but only partially inhibited reductase-dependent reduction. Also, the xanthine oxidase-dependent reduction of acetylated cytochrome c was not linear; a net oxidation of acetylated ferrocytochrome c

became evident after several minutes of reaction. Minakami had previously reported that the susceptibility to oxidation of acetylated cytochrome c was enhanced as its percent modification was increased.[44] Because xanthine oxidase may generate H_2O_2 directly or by $O_2^{\cdot-}$ dismutation, the net oxidation of acetylated ferrocytochrome c that was observed was attributed to an accumulation of H_2O_2 in the incubation. The addition of catalase to the incubation prevented the oxidation of ferrocytochrome c, presumably by catalyzing the dismutation of H_2O_2 thus preventing its accumulation. Catalase was also added 3-4 min after the addition of xanthine oxidase (when a net oxidation of acetylated cytochrome c was occurring). In this case, oxidation of acetylated ferrocytochrome c was also prevented, and the rate of reduction of acetylated cytochrome c was equal to the rate of reduction in incubations to which catalase was added prior to xanthine oxidase.

In contrast, the rate of reductase-dependent reduction of acetylated cytochrome c was linear in the absence of catalase and not affected by the presence of catalase. Superoxide dismutase did not greatly inhibit the rate of reduction. Therefore, these results are consistent with the reductase generating only small amounts of $O_2^{\cdot-}$. Efficient scavenging of $O_2^{\cdot-}$ by acetylated cytochrome c prevents a significant accumulation of H_2O_2, and prevents the oxidation of acetylated cytochrome that was observed with xanthine oxidase. These results would also suggest that the

reductase does not produce H_2O_2 by direct 2 electron reduction of oxygen in agreement with previous studies of its electron transfer properties.[45]

The rate of reduction of acetylated cytochrome c which was inhibited by SOD was used as the measure of $O_2^{\cdot-}$ production. This technique capitalizes on the known specificity of SOD for $O_2^{\cdot-}$[46] and is, therefore, a more reliable method for quantitating $O_2^{\cdot-}$ production. In some instances the rate of $O_2^{\cdot-}$ production was expressed as a function of the unacetylated cytochrome c reductase activity of the purified reductase or intact microsomes. In this way the amount of $O_2^{\cdot-}$ relative to the total NADPH-dependent reduction capacity of the reductase (or microsomes) became apparent. The reductase generated 2.3 nmol $O_2^{\cdot-}$/unit cytochrome c reductase activity, a rate which was approximately 0.2% of its NADPH-dependent reduction capacity.

Because certain ferric chelates will react with $O_2^{\cdot-}$,[47-49] they may compete with acetylated cytochrome c and inhibit its rate of reduction. This was apparently the case with xanthine oxidase-dependent acetylated cytochrome c reduction, as added chelators or ferric chelates inhibited the reduction of acetylated cytochrome c by as much as 20%. However, SOD still completely inhibited the reduction.

In contrast, certain ferric chelates stimulated acetylated cytochrome c reduction by the purified reductase or intact microsomes. Both $O_2^{\cdot-}$-independent and $O_2^{\cdot-}$-

dependent rates of reduction were enhanced. The ferric chelates that markedly stimulated reductase-dependent acetylated cytochrome c reduction, and $O_2^{\cdot-}$ production (EDTA-Fe^{3+} and DTPA-Fe^{3+}) also stimulated NADPH oxidation in the absence of acetylated cytochrome c (Chapter I).

An explanation consistent with the data can be proposed. Oxygen must be considered as a poor substrate for the reductase relative to unmodified cytochrome c or cytochrome P450, its physiological electron acceptor. Therefore, $O_2^{\cdot-}$ production by the reductase in the absence of more suitable electron acceptors appears to be a slow process, accounting for only a minor percentage of its potential reduction capacity. The addition of EDTA-Fe^{3+} or DTPA-Fe^{3+} (better substrates than oxygen) results in an increased rate of NADPH oxidation with concomitant direct reduction of the ferric chelate (Chapter I). The resultant ferrous chelate could then reduce acetylated cytochrome c directly, accounting for the $O_2^{\cdot-}$-independent component of this reduction. The $O_2^{\cdot-}$-dependent component of reduction is probably a result of ferrous chelate oxidation. Ferrous-EDTA autoxidizes quite rapidly in contrast to DTPA-Fe^{2+}, and therefore, the $O_2^{\cdot-}$-dependent component of acetylated cytochrome c reduction in the presence of EDTA-Fe^{3+} was much greater than that observed in the presence of DTPA-Fe^{3+}. This was despite the fact that the total rate of acetylated cytochrome c reduction in the presence of DTPA-Fe^{3+} was greater. However, the mechanism(s) of autoxidation of

ferrous chelates has (have) not been elucidated, so it is not know whether the $O_2^{\cdot-}$-dependent fraction of acetylated cytochrome c reduction is due only to autoxidation. Conceivably, ferric chelates could also enhance $O_2^{\cdot-}$ production by the reductase by a different but unknown mechanism.

As with the purified reductase, microsomal $O_2^{\cdot-}$ production was low (15.6 nmol $O_2^{\cdot-}$/unit cytochrome c reductase activity), accounting for only a minor percentage of its total reduction potential (about 1.5%). Data in the literature as well as the results in this chapter undoubtedly indicate that the difference in $O_2^{\cdot-}$ production between the purified reductase and microsomes is due at least in part to cytochrome P450s. Ferric chelates, especially EDTA-Fe^{3+} and DTPA-Fe^{3+} stimulated microsomal $O_2^{\cdot-}$ production, although the degree of stimulation was less than with the purified reductase. This result brings into question the results of previous experiments in which EDTA was added to microsomal incubations in which NADPH oxidation, and $O_2^{\cdot-}$ and H_2O_2 production were measured. It is not clear exactly what effects trace EDTA-Fe^{3+} concentrations might have had on the observed stoichiometry of microsomal oxygen radical formation. Results in this Chapter and Chapter I would suggest a stimulation of NADPH oxidation and $O_2^{\cdot-}$ production, perhaps yielding a stoichiometry in which a greater percentage of NADPH oxidized resulted in $O_2^{\cdot-}$ production, a conclusion that

Kuthan et al. reached.[20] The stoichiometry obtained in this chapter agrees more closely with that of Zhukov and Archakov,[34] but is debatable whether the excess NADPH oxidation (over H_2O_2 formation) shown in this chapter is truly indicative of direct 4 electron reduction of oxygen to water as previously suggested.[33,34] Previous results obtained using a reconstituted system[33] would appear to be better evidence for a 4-electron oxidase activity than results obtained with microsomes, due to the problem of catalatic activity in microsomes.

The ability of cytochrome P450 isozymes to enhance $O_2^{\cdot-}$ production over that generated by the reductase alone was also demonstrated. Increased rates of $O_2^{\cdot-}$ production were observed as the molar ratio of cytochrome P450/reductase was increased, until the ratio reached approximately 4 or 5 to 1. This is consistent with previous studies that had shown that the rate of metabolism of MFO substrates in a reconstituted system increased until the cytochrome P450/reductase ratio approached 5.[50]

The production of $O_2^{\cdot-}$ by the reconstituted system followed previous observations of the requirements for obtaining drug metabolism in a reconstituted MFO system, i.e. the presence of DLPC, the detergent-solubilized reductase, and cytochrome P450 isozymes.[51] Use of the protease-solubilized enzyme or the exclusion of DLPC resulted in a marked inhibition in the rates of $O_2^{\cdot-}$ production.

Superoxide production by the reconstituted MFO system was confirmed using EPR spin trapping. However, because of the slow rate of reaction of $O_2^{\cdot-}$ with DMPO, the signal observed was apparently a combination of DMPO·OOH and DMPO·OH adducts. The direct production of ·OH as a source of DMPO·OH was prevented by the addition of desferrioxamine, which inhibits the iron-catalyzed Haber-Weiss reaction.[39] The addition of SOD to the incubation prevented both DMPO·OOH and DMPO·OH adduct signals, so the DMPO·OH most probably arose from DMPO·OOH decomposition.

A difference in the rates of $O_2^{\cdot-}$ production was observed depending upon the isozyme of cytochrome P450 that was reconstituted with the reductase. Cytochrome P450d, the isozyme isolated in the high spin conformation from rats treated with 3-MC type inducers produced slightly more $O_2^{\cdot-}$ than did cytochrome P450b, the major phenobarbital-inducible isozyme. Cytochrome P450c, the other major isozyme induced by 3-MC produced somewhat less $O_2^{\cdot-}$. A recent report has indicated that the reductase binds more tightly to high spin cytochrome P450.[52] This is consistent with the data illustrated in Figure 12; the slope of the curve with cytochrome P450d is considerably greater than those of the other two isozymes, indicating that high spin cytochrome P450d is able to saturate the reductase at lower concentrations than either cytochromes P450b or P450c.

Treatment of rats with isosafrole results in increased levels of hepatic microsomal cytochrome P450d. However, the

binding of an isosafrole metabolite to cytochrome P450d prevents the determination of cytochrome P450 using a CO difference spectra and inhibits $O_2^{\cdot-}$ production presumably by preventing the coordination of oxygen to the heme iron. Nevertheless, these microsomes still catalyze appreciable rates of NADPH oxidation and $O_2^{\cdot-}$ production. Removal of the isosafrole metabolite by preincubating with butanol resulted in microsomes having increased capacity for $O_2^{\cdot-}$ production as well as a near stoichiometric increase in the rate of NADPH oxidation. These results would suggest that $O_2^{\cdot-}$ production by cytochrome P450d is prevented when the heme iron of cytochrome P450d is occupied with other ligands. This would tend to discount the hypothesis of Gorsky et al.[33] who suggested that the presence of cytochrome P450 isozymes might stimulate the reductase to produce more $O_2^{\cdot-}$ or other partially reduced oxygen species.

However, from the data on the rates of NADPH oxidation and $O_2^{\cdot-}$ production from microsomes isolated from rats pretreated with MFO inducers it was apparent that the presence of particular isozymes of cytochrome P450 was not the only factor influencing oxygen radical production by microsomes. While induction with phenobarbital resulted in microsomes having greater basal rates of NADPH oxidation and $O_2^{\cdot-}$ and H_2O_2 production, induction of cytochrome P450d by HBB did not result in much of an increase in microsomal uncoupling (compared to microsomes from phenobarbital-treated rats) despite the fact that cytochrome P450d

stimulated $O_2^{\cdot-}$ production in the reconstituted system to a greater extent than cytochrome P450b. In fact, if rates of $O_2^{\cdot-}$ production were expressed per unit of reductase activity, $O_2^{\cdot-}$ production was comparable (11.0, 13.6 and 17.9 nmol/unit for microsomes from control, phenobarbital-, and HBB-treated rats, respectively). This result would suggest that under certain conditions, the level of reductase may limit $O_2^{\cdot-}$ production by microsomes in much the same fashion as it has been proposed to limit MFO activity.[30,53]

Finally, the potential of the MFO system to generate partially reduced oxygen species <u>in vivo</u> should be addressed. From one perspective, uncoupling represents a futile cycle with reducing equivalents from NADPH eventuating in the net reduction of oxygen to water without substrate oxidation. Futile cycles are certainly not the rule in biological systems and the cell usually prevents such unnecessary consumption of energy by inhibiting the competing enzyme or pathway. However, in at least one respect the microsomal electron transport system represents an atypical enzyme system. It appears to be ideally suited to handle a wide variety of structurally-diverse substrates. As such, a limited amount of uncoupling of MFO activity may be the biological price to be paid for the low degree of specificity exhibited by the MFO system relative to more conventional enzymes or enzyme systems.

On the other hand, oxygen radical production by microsomes may be an artifact of isolation and, therefore,

essentially a property of microsomes but not the intact endoplasmic reticulum. There is evidence for an asymmetric distribution of MFO components within the endoplasmic reticulum, and the normal homogenization procedure might disrupt the organization of the electron transport system and promote oxygen radical formation.

A limited amount of data would suggest that MFO-dependent oxygen radical production does indeed occur in vivo. However, that data had been obtained using indirect determinations, for example, monitoring the alteration of metabolism of methanol during the in vivo metabolism of ethylene glycol.[54] A recent report details the decrease of H_2O_2 production by the MFO system upon the treatment with allylisopropylacetamide,[55] a cytochrome P450 inhibitor, but this study used as a measure of H_2O_2 production the residual catalase activity remaining in liver following aminotriazole treatment. Thus, the evidence for in vivo formation of H_2O_2 can not be considered as particularly strong at the present time.

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CHAPTER III

The Roles of Superoxide and Hydrogen Peroxide in NADPH-Dependent Microsomal Lipid Peroxidation

ABSTRACT

The requirement for $O_2^{\cdot-}$ and H_2O_2 in the initiation of NADPH-dependent microsomal lipid peroxidation has been investigated. To assess the potential involvement of H_2O_2 in the generation of $\cdot OH$, the concentrations of microsomal H_2O_2 have been altered and resulting rates of lipid peroxidation have been measured. Hydrogen peroxide concentration in microsomes was altered by washing to remove endogenous catalatic activity, by adding exogenous catalase, adding exogenous H_2O_2 and/or by inhibiting endogenous catalase activity with azide. In only one instance was the rate of lipid peroxidation altered; exogenous H_2O_2 added to azide-treated microsomes inhibited lipid peroxidation, the opposite effect from that predicted if $\cdot OH$ generated from H_2O_2 is actually the major initiating species. These results do not support the role of free H_2O_2 in the initiation of microsomal lipid peroxidation. The participation of $O_2^{\cdot-}$ generated by the reductase in NADPH-dependent lipid peroxidation of microsomes was also investigated and compared with results obtained with a xanthine oxidase-dependent lipid peroxidation system. Peroxidation of phospholipid liposomes or rat liver microsomes in the presence of ADP-Fe^{3+} was demonstrated to be independent of $O_2^{\cdot-}$ generation by the reductase.

INTRODUCTION

Lai et al.,[1-3] Fong et al.,[4] and Koster and Slee[5] have all suggested that the ·OH is the initiating species in reductase-dependent peroxidation of microsomal lipids. They propose that, during oxidation of NADPH by microsomes, reduced iron and H_2O_2, formed by a $O_2^{\cdot-}$-dependent mechanism, react to form ·OH. This series of reactions is known as the iron-catalyzed Haber-Weiss reaction:

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

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

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

It has been questioned whether this mechanism represents the predominant means by which the initiator of microsomal lipid peroxidation is generated.[6] The endogenous catalase activity present in microsomes[7] should scavenge H_2O_2, thereby limiting the amount available to react with reduced iron to generate ·OH. Thurman et al.[8] have shown that, an inhibitor of catalase must be added to microsomes in order to quantitate H_2O_2 production, indicating that the steady-state concentration of H_2O_2 must be very low in microsomes incubated with NADPH. Furthermore, Cohen and Cederbaum[9] have shown that azide-treated microsomes produce more ·OH than do microsomes incubated in the absence of azide. Therefore, addition of exogenous H_2O_2 and/or the

inhibition of endogenous catalase activity should cause an increase both in ·OH production and in subsequent rates of microsomal lipid peroxidation if it is indeed initiated by the mechanism shown above.

However, there are no reports that thoroughly investigate the effects of H_2O_2, azide, and catalase on microsomal lipid peroxidation. There have been several reports claiming that catalase inhibited NADPH-dependent lipid peroxidation,[2,5] whereas other investigators have reported no effect.[10,11] Since catalase is available from several sources and because antioxidants are sometimes included in the preparations, the effect of several different catalase preparations on lipid peroxidation was examined.

Likewise, there is controversy as to whether in NADPH-dependent lipid peroxidation reduction of ferric is via $O_2^{\cdot-}$ (1) or not.[3-6,12] Previous studies presented in Chapter I indicated that microsomes possess a NADPH-dependent ferric reductase activity, that might eliminate a requirement for $O_2^{\cdot-}$ for example as is proposed in the Haber-Weiss reaction. Thus, relative to xanthine oxidase, microsomes or the purified reductase should promote greater rates of lipid peroxidation. Moreover, lipid peroxidation promoted by xanthine oxidase should be inhibited by SOD whereas NADPH-dependent peroxidation should not.

MATERIALS AND METHODS

Chemicals: Butylated hydroxytolulene, 2-thiobarbituric acid, glutathione reductase and sodium azide were all purchased from Sigma Chemical Company. Malondialdehyde bis (dimethyl acetal) was obtained from Aldrich Chemical. Catalase preparations were from Sigma, Boehringer-Mannheim and Millipore (3 preparations). Other chemicals were of reagent grade and used without further purification. Solutions used in lipid peroxidation assays were treated with Chelex 100 to remove contaminating transition metal ions.

Preparation of Phospholipid Liposomes: Microsomes were prepared as described in Chapter I and microsomal phospholipids were extracted by the procedure of Folch et al.,[13] and stored at -20°C in $CHCl_3$:CH_3OH (2:1). Phosphate was assayed by the procedure of Bartlett.[14] Liposomes were prepared by taking an aliquot of the $CHCl_3$:CH_3OH extract and drying it under a stream of Argon, diluting it with 50 mM NaCl and sonicating until all lipid was resuspended.

Lipid Peroxidation Assays: Stock solutions of microsomes or phospholipid liposomes, ADP-Fe^{3+}, NADPH, purified reductase, or xanthine oxidase prepared in 30 mM NaCl (pH 7.0) were used to constitute lipid peroxidation mixtures that were

incubated in a shaking water bath maintained at 37°C. Final concentrations of the solutions are given in the figure and table legends. Lipid peroxidation was initiated by the addition of NADPH or xanthine oxidase. When both azide and H_2O_2 were added to microsomes, azide was always added prior to H_2O_2 to inhibit the endogenous microsomal catalatic activity. When only H_2O_2 was added, it was added just before the initiation of peroxidation with NADPH. At specific times aliquots (0.5 ml or 1 ml) were removed and assayed for MDA by the thiobarbituric acid assay.[15] The procedure of Paglia and Valentine was used for the determination of H_2O_2-dependent glutathione oxidase activity.[16]

RESULTS

Role of Hydrogen Peroxide in Microsomal Lipid Peroxidation: Figure 20 is time course of microsomal NADPH-dependent lipid peroxidation and shows the effect of H_2O_2 added either without or with azide to inhibit endogenous catalase activity. The addition of either azide (0.4 mM) or H_2O_2 (0.1 mM) to microsomal incubation mixtures containing both NADPH and ADP-Fe^{3+} had no effect on the rate of lipid peroxidation. However, upon the addition of both H_2O_2 and azide, the initial rate of lipid peroxidation was decreased markedly. The simultaneous addition of H_2O_2 (0.1 mM), NADPH (0.1 mM) and azide (0.4 mM) to microsomes in the absence of ADP-Fe^{3+} did not result in lipid peroxidation (Figure 20), indicating that neither endogenous heme iron, such as cytochrome P-450 or cytochrome b_5, nor non-heme iron was able to decompose H_2O_2 at least not to an extent sufficient to observe lipid peroxidation.

The ability of azide to inhibit the endogenous catalase activity of microsomes was demonstrated by the results shown in Figure 21. In the absence of azide, the catalase activity of microsomes was approximately 140 units/mg protein. Fifty percent inhibition of this activity was obtained by the addition of 0.025 mM azide (Figure 21, inset), and 0.4 mM azide caused greater than 97 percent inhibition of the microsomal catalase activity. Although

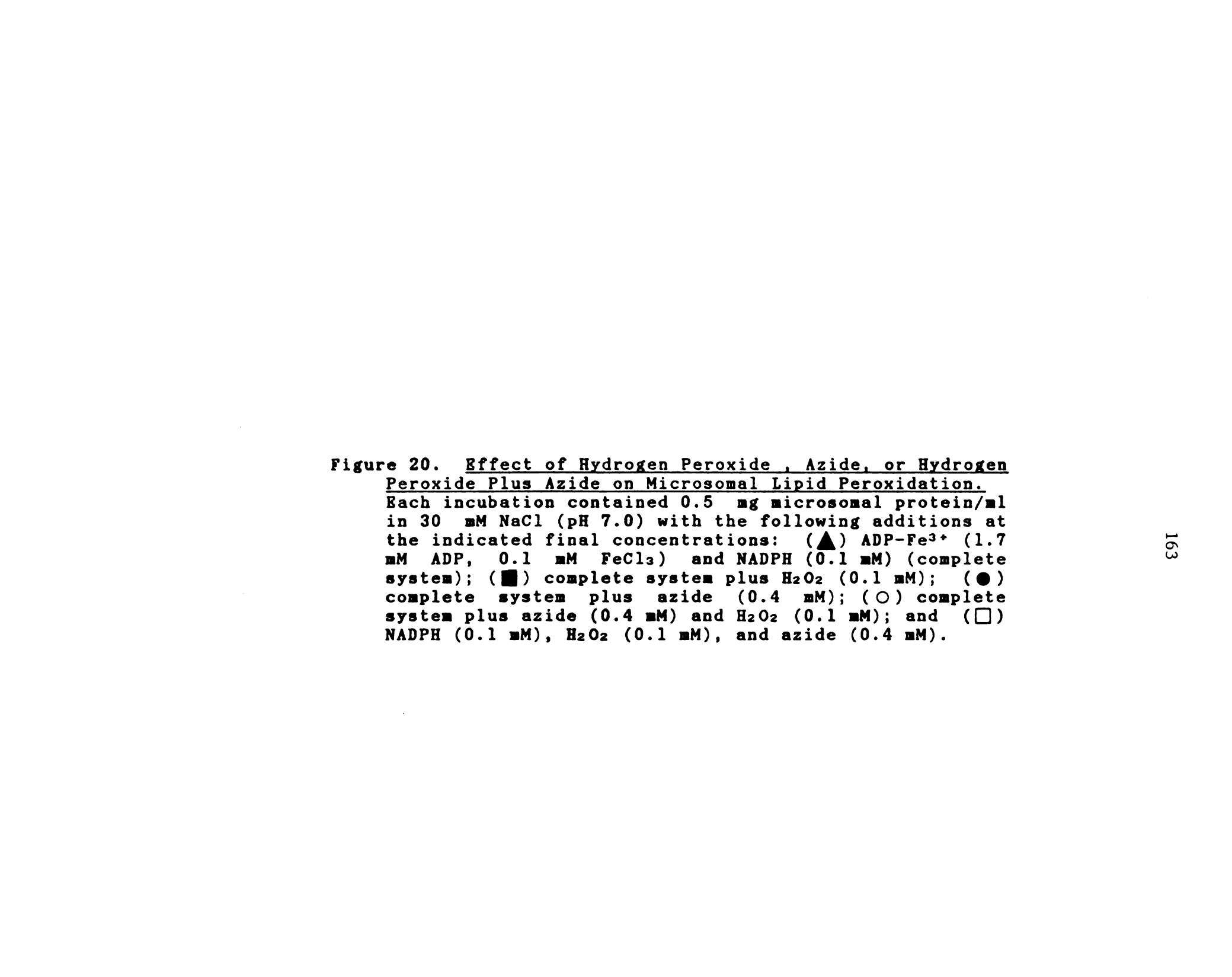


Figure 20. Effect of Hydrogen Peroxide , Azide, or Hydrogen Peroxide Plus Azide on Microsomal Lipid Peroxidation. Each incubation contained 0.5 mg microsomal protein/ml in 30 mM NaCl (pH 7.0) with the following additions at the indicated final concentrations: (▲) ADP-Fe^{3+} (1.7 mM ADP, 0.1 mM $FeCl_3$) and NADPH (0.1 mM) (complete system); (■) complete system plus H_2O_2 (0.1 mM); (●) complete system plus azide (0.4 mM); (○) complete system plus azide (0.4 mM) and H_2O_2 (0.1 mM); and (□) NADPH (0.1 mM), H_2O_2 (0.1 mM), and azide (0.4 mM).

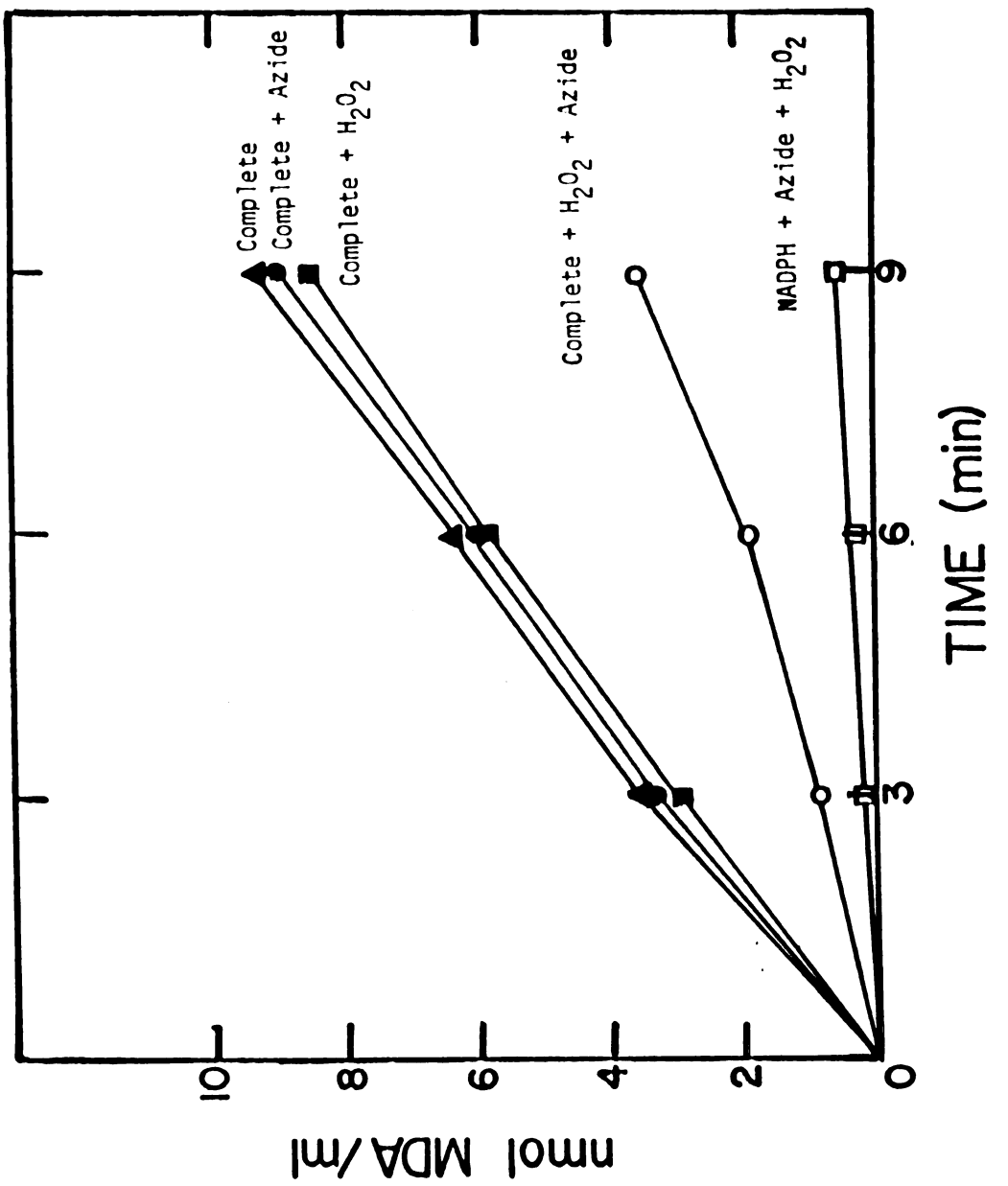
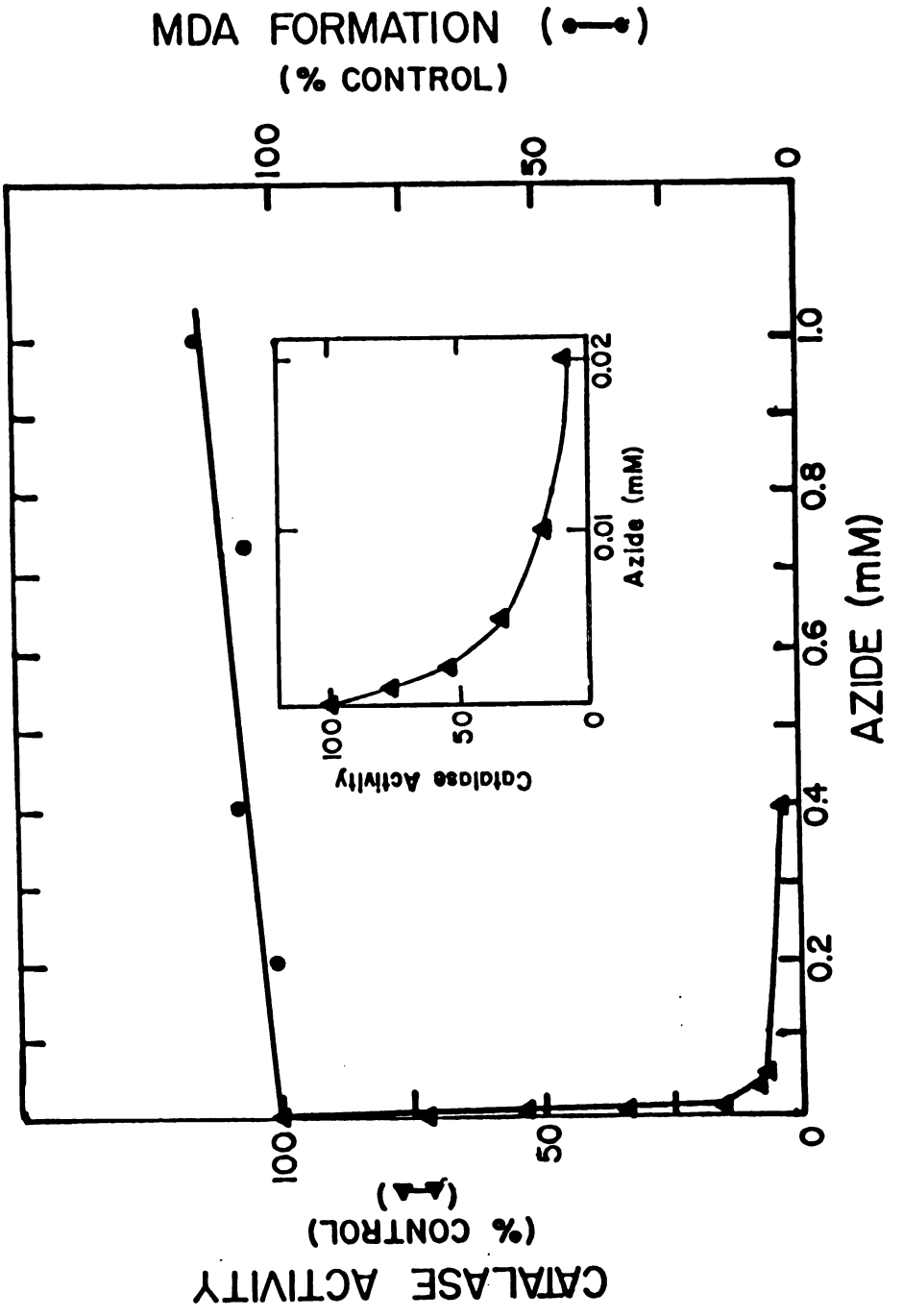

nmol MDA/ml
0
2
4
6
8
10
3
6
9
TIME (min)
Complete
Complete + Azide
Complete + H_2O_2
Complete + H_2O_2 + Azide
NADPH + Azide + H_2O_2

Figure 21. Effect of Azide on Endogenous Catalase Activity in Microsomes and Microsomal Lipid Peroxidation. Endogenous catalase activity in microsomes was measured in a 1 ml cuvette containing 0.1 mg microsomal protein/ml, and 20 mM H_2O_2 in 0.05 M KH_2PO_4 (pH7.5) with various concentrations of sodium azide. Microsomal lipid peroxidation incubations contained the complete system (described in Figure 20) to which were added various concentrations of sodium azide. Rates of MDA formation are expressed as percentages of that observed in the complete system.

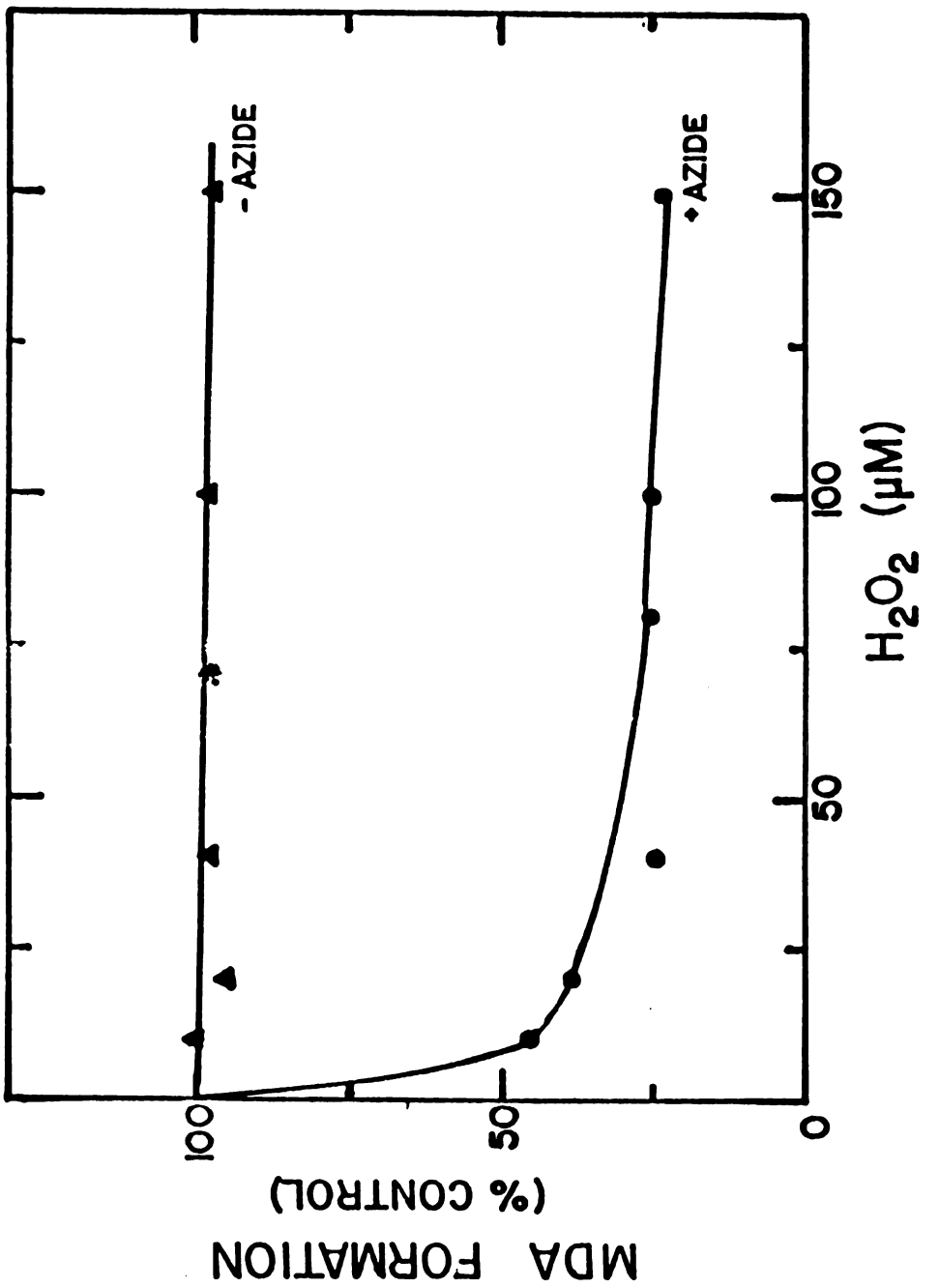

MDA FORMATION (•—•)
(% CONTROL)
100
50
0
1.0
0.8
0.6
0.4
0.2
0
AZIDE (mM)
0.02
0.01
Azide (mM)
Catalase Activity
100
50
0
CATALASE ACTIVITY
(% CONTROL)
(▲—▲)

inhibition of catalase activity by azide should increase the H_2O_2 concentration in microsomes, NADPH and ADP-Fe^{3+}-dependent lipid peroxidation was clearly not inhibited by azide concentrations up to 1 mM (Figure 21).

The effect of H_2O_2 on microsomal lipid peroxidation was also studied by adding exogenous H_2O_2 to microsomes (Figure 22). In the absence of azide, H_2O_2 concentrations up to 150 uM were without effect on the rate of MDA formation. However, in agreement with the results shown in Figure 20, the addition of H_2O_2 in the presence of azide (0.4 mM) caused a significant decrease in the rate of lipid peroxidation. Fifty percent inhibition occurred in the presence of approximately 20 μM H_2O_2. The results of control experiments indicated that neither H_2O_2 nor azide had an effect on the thiobarbituric acid assay for MDA.

Effect of Catalase on Lipid Peroxidation: The involvement of H_2O_2 in microsomal lipid peroxidation has been studied previously by adding exogenous catalase. However, the results of these studies are contradictory.[2,5,10,11] Accordingly, five different commercial catalase preparations were tested for their effects on this process. The results are shown in Figure 23. Three of the five catalase preparations had no effect on microsomal lipid peroxidation (Figure 23A). However, two catalase preparations significantly inhibited the rate of MDA formation (Figure 23B). Pederson and Aust suggested that the conflicting

Figure 22. Effect of Hydrogen Peroxide on Microsomal Lipid Peroxidation. All incubation mixtures contained the complete system (Figure 20) in the absence (▲) or presence (●) of azide to which were added various concentrations of H_2O_2. Rates of MDA formation are expressed as percentages of that observed in the complete system.


MDA FORMATION
(% CONTROL)
100
50
0
- AZIDE
+ AZIDE
50
100
150
H_2O_2 (μM)

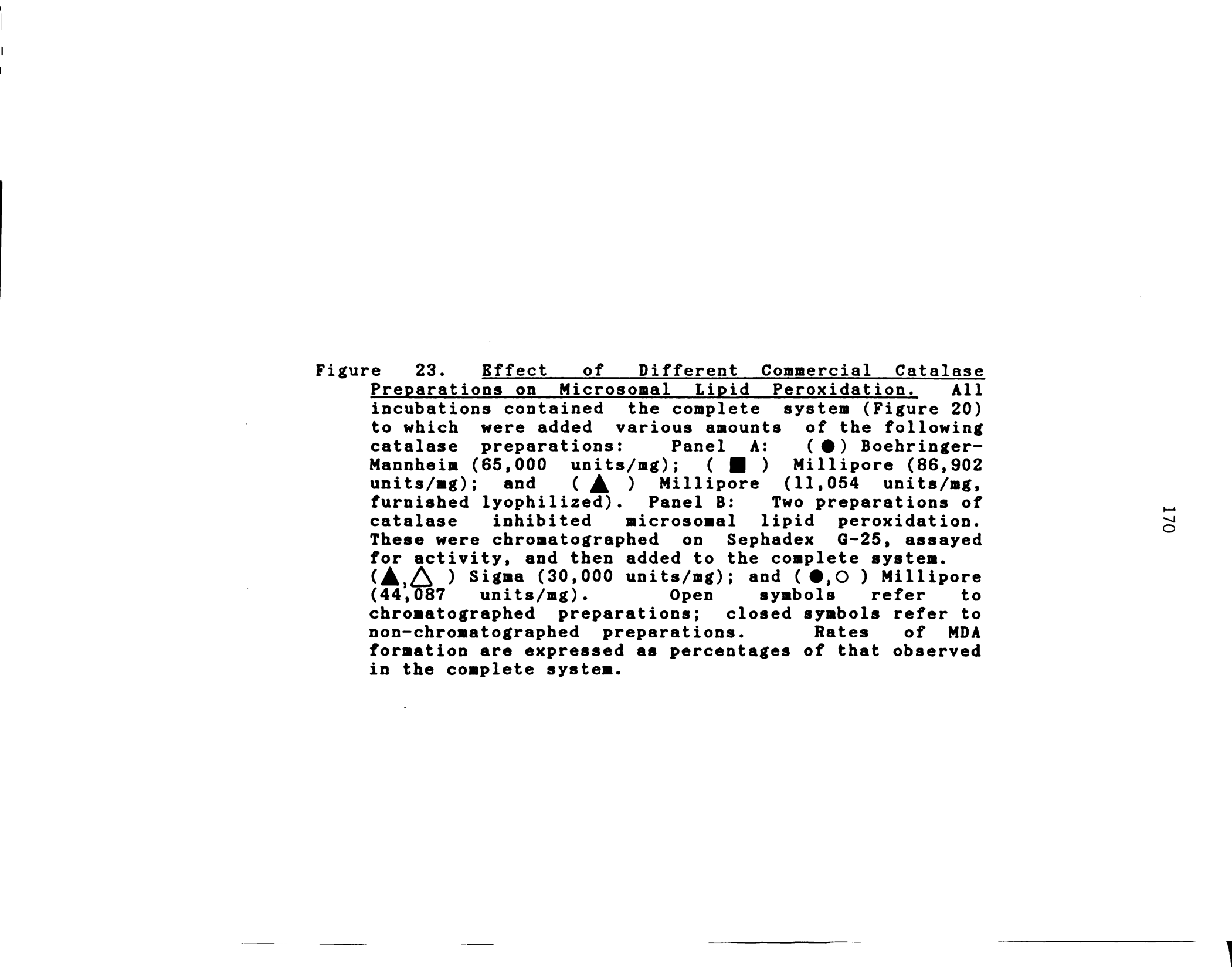
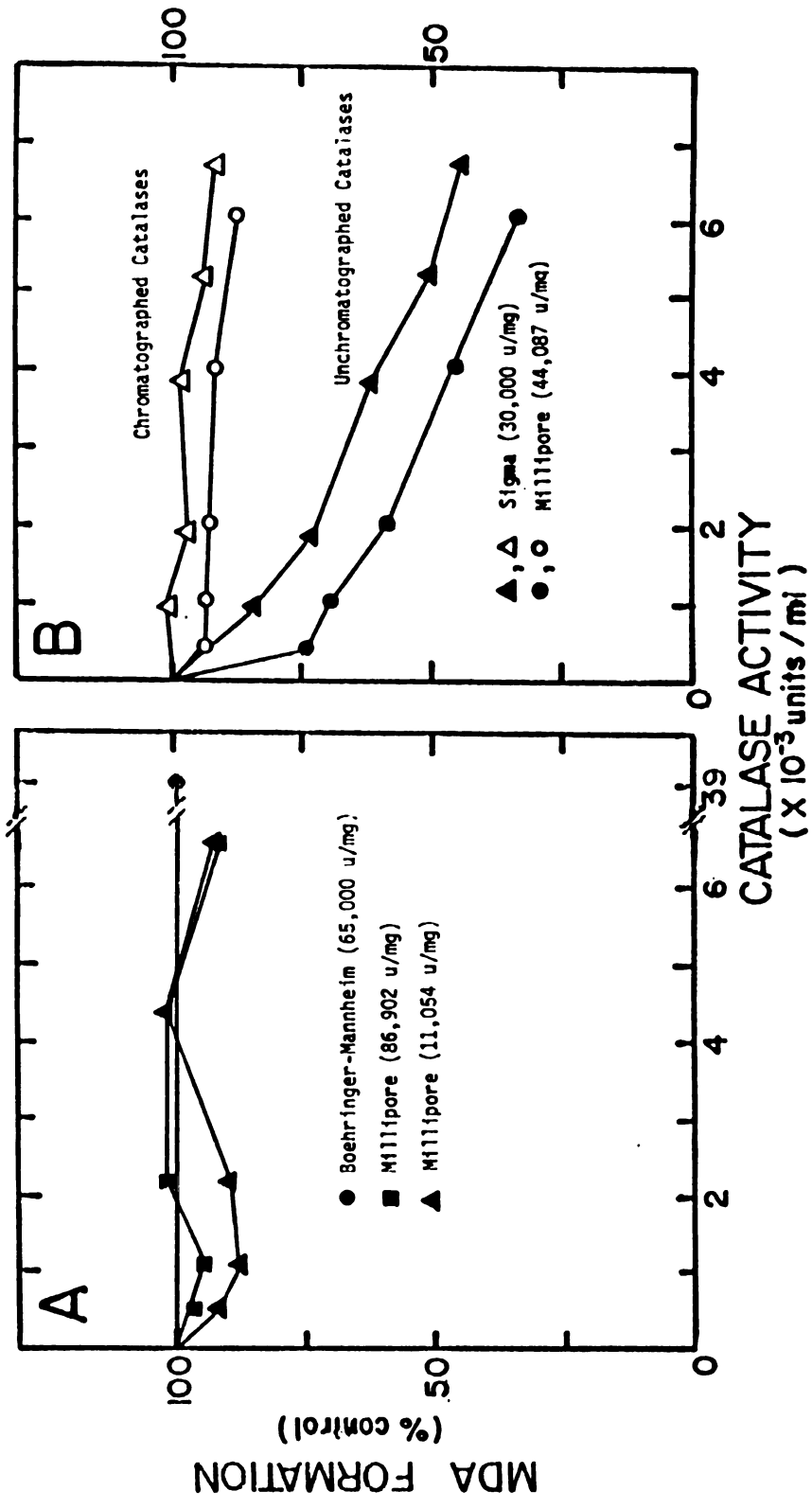


Figure 23. Effect of Different Commercial Catalase Preparations on Microsomal Lipid Peroxidation. All incubations contained the complete system (Figure 20) to which were added various amounts of the following catalase preparations: Panel A: (●) Boehringer-Mannheim (65,000 units/mg); (■) Millipore (86,902 units/mg); and (▲) Millipore (11,054 units/mg, furnished lyophilized). Panel B: Two preparations of catalase inhibited microsomal lipid peroxidation. These were chromatographed on Sephadex G-25, assayed for activity, and then added to the complete system. (▲,△) Sigma (30,000 units/mg); and (●,○) Millipore (44,087 units/mg). Open symbols refer to chromatographed preparations; closed symbols refer to non-chromatographed preparations. Rates of MDA formation are expressed as percentages of that observed in the complete system.


A
B
MDA FORMATION
(% control)
CATALASE ACTIVITY
(X 10⁻³ units / ml)
Boehringer-Mannheim (65,000 u/mg)
Millipore (86,902 u/mg)
Millipore (11,054 u/mg)
Chromatographed Catalases
Unchromatographed Catalases
▲,△ Sigma (30,000 u/mg)
●,○ Millipore (44,087 u/mg)
100
50
0
2
4
6
39

reports of the effect of exogenous catalase on microsomal lipid peroxidation could be due to antioxidants present in several commercial enzyme preparations. When the catalase preparations which inhibited lipid peroxidation [Sigma (30,000 units/mg) and Millipore (44,087 units/mg)] were chromatographed on Sephadex G-25 to remove low molecular weight contaminants, no inhibition of MDA formation could be observed upon their addition to the complete system (Figure 23B).

Previously, it was demonstrated that endogenous catalase activity could be significantly removed from microsomes by washing.[17] In this study, washed microsomes were found to contain both lower catalase activity and lower H_2O_2-dependent, glutathione oxidase activity than was found in unwashed microsomes, yet rates of microsomal lipid peroxidation were essentially unaffected (Table 12). Washed microsomes to which exogenous catalase was added to an activity equal to that of unwashed microsomes also showed rates of lipid peroxidation similar to those seen with unwashed control microsomes (Table 12). Chromatography of microsomes on Sepharose 2B reduced catalase activity to approximately 10 units/mg.

The ability of the ·OH trapping agents benzoate and mannitol to inhibit NADPH-dependent lipid peroxidation was investigated and compared to their effect on ·OH-dependent lipid peroxidation promoted by Fenton's reagent (Figures 24 and 25, respectively). Whereas either mannitol or benzoate

Table 12. Effect of Washing and the Addition of Catalase on Microsomal Lipid Peroxidation

Microsomes	Catalase activity (units/mg)	H_2O_2-dependent glutathione oxidation (μmoles NADPH·mg^{-1}·min^{-1})	MDA formation (% control)	
			No catalase added	Catalase added
Unwashed	143	0.119	100	---
Washed once	33	0.060	113	97
Washed twice	23	0.059	100	100

Microsomes were washed by homogenization in 1.15% KCl and centrifugation at 105,000 xg for 1 hour. Lipid peroxidation incubation mixtures contained microsomes (0.5 mg/ml) ADP-Fe^{3+} (1.7 mMADP-0.1 mM $FeCl_3$) and NADPH (0.1 mM) in 30 mM NaCl pH 7.0. In some incubations exogenous catalase was added back to washed microsomes to a specific activity equal to that of unwashed microsomes. Rates of MDA formation are expressed as percentages of that observed with unwashed microsomes.

Figure 24. Effect of Benzoate on Microsomal Lipid Peroxidation Dependent Upon Hydroxyl Radical or NADPH. NADPH-dependent incubations are described in Figure 20. Hydroxyl radical-dependent incubations contained rat liver microsomes (0.5 mg/ml) $FeCl_2$ (0.2 mM) and H_2O_2 (0.1 mM).

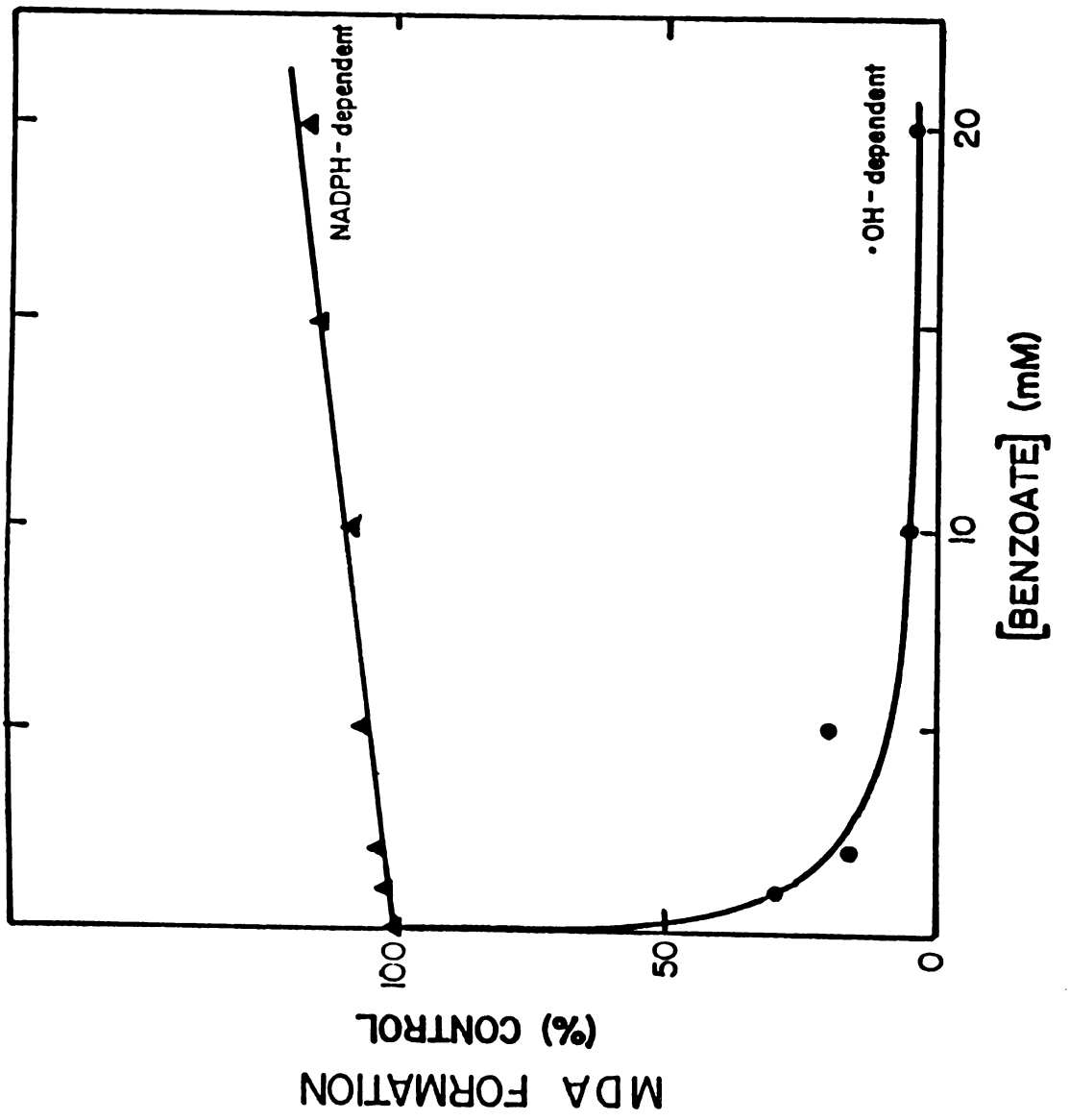
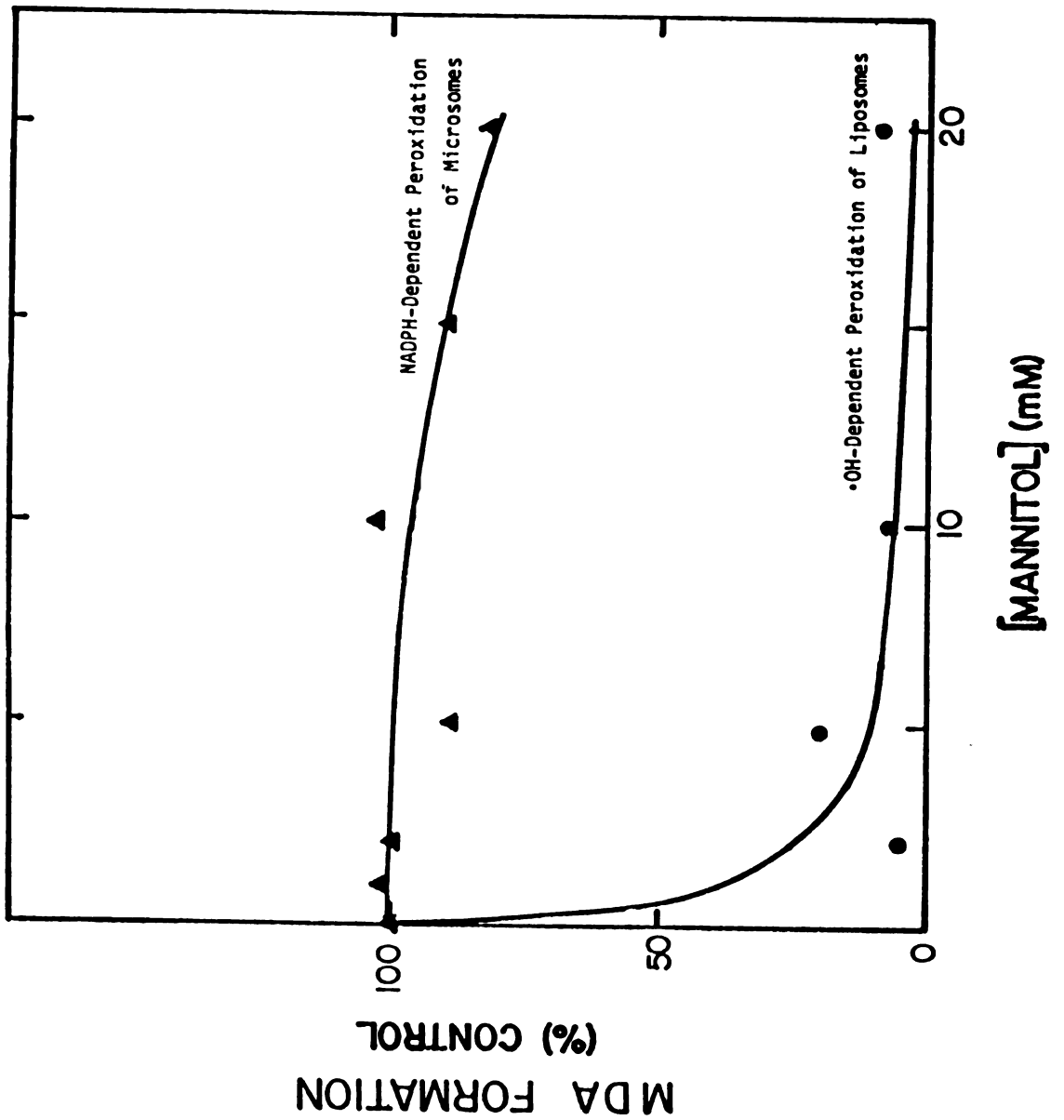

MDA FORMATION
(%) CONTROL
100
50
0
NADPH-dependent
•OH-dependent
10
20
[BENZOATE] (mM)

Figure 25. Effect of Mannitol on Microsomal Lipid Peroxidation Dependent Upon Hydroxyl Radical or NADPH. NADPH-dependent incubations are described in Figure 20. Hydroxyl radical-dependent incubations are described in Figure 24.


MDA FORMATION
(%) CONTROL
100
50
0
NADPH-Dependent Peroxidation of Microsomes
•OH-Dependent Peroxidation of Liposomes
10
20
[MANNITOL] (mM)

inhibited ·OH-dependent lipid peroxidation, neither inhibited the NADPH-dependent lipid peroxidation of microsomes.

Role of Superoxide in NADPH-Dependent Lipid Peroxidation: Comparison with Xanthine Oxidase-Dependent Peroxidation:

To determine the extent to which $O_2^{\cdot-}$ production was involved in microsomal lipid peroxidation, incubations containing ADP-Fe^{3+} and microsomes and either NADPH or xanthine and xanthine oxidase were constituted such that the activity of xanthine oxidase was sufficient to produce $O_2^{\cdot-}$ at a rate equal to that generated by microsomes. As evident in Figure 26, xanthine oxidase-dependent microsomal lipid peroxidation was completely inhibited by SOD, but SOD did not affect NADPH-dependent lipid peroxidation. The absolute rate of MDA formation was also greater in NADPH-dependent incubations than in those containing xanthine oxidase.

Essentially the same results were obtained with a reconstituted microsomal lipid peroxidation system in which liposomes were incubated with either NADPH and the reductase or xanthine and xanthine oxidase. Again, the concentrations of enzymes were adjusted so that equal rates of $O_2^{\cdot-}$ production were occurring in each incubation. As in the intact microsomal system, SOD completely inhibited xanthine oxidase-dependent peroxidation of liposomes but did not inhibit NADPH-dependent peroxidation (Figure 27).

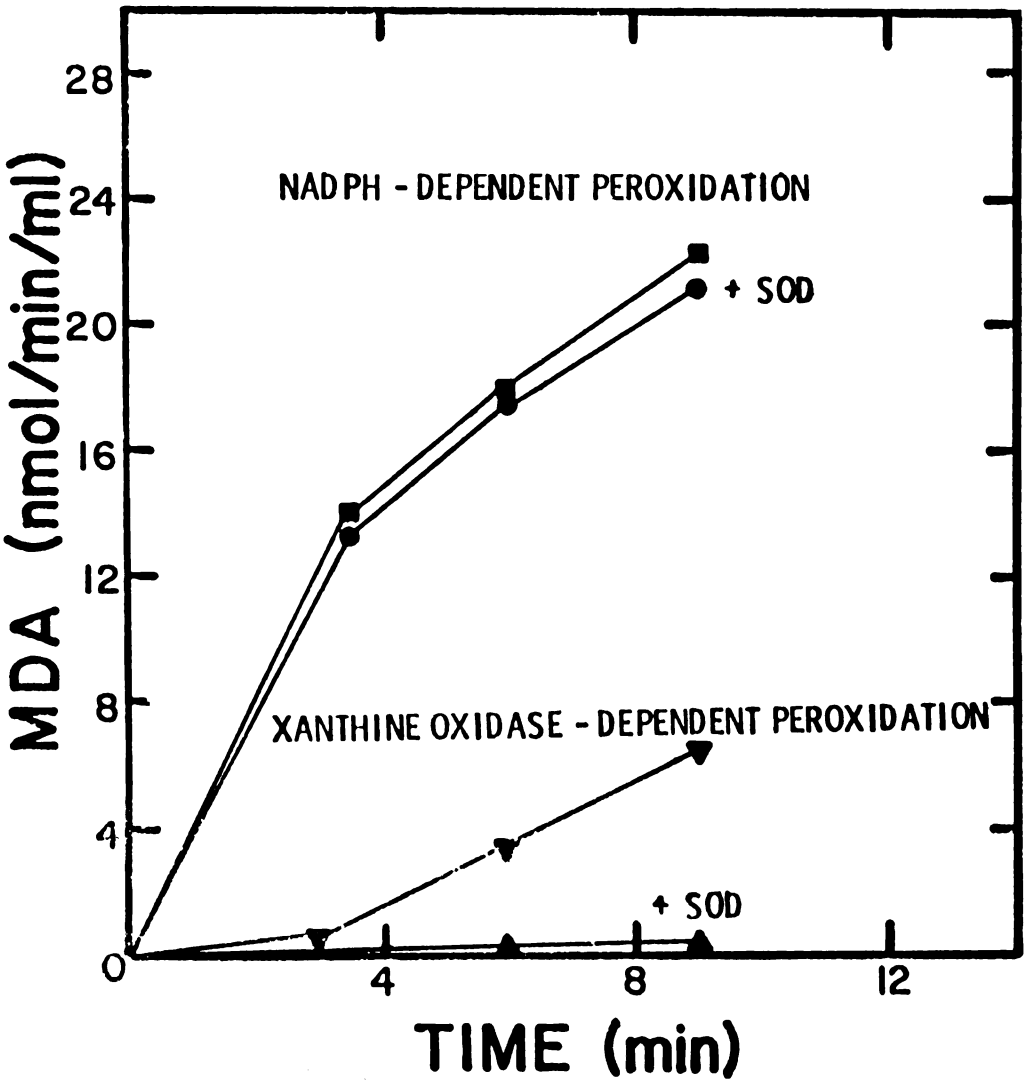


Figure 26. The Effect of Superoxide Dismutase on NADPH-Dependent Lipid Peroxidation of Microsomes: A Comparison with Xanthine Oxidase-Dependent Peroxidation. All incubations contained microsomes (0.5 mg/ml) and ADP-Fe^{3+} (1.7 mM ADP, 0.1 mM $FeCl_3$) in 0.3 M NaCl, pH 7.0, and either NADPH (0.1 mM) (■, ●) or xanthine (0.17 mM) and xanthine oxidase (0.004 u/ml) (▼, ▲). When added, the concentration of SOD was 100 u/ml.

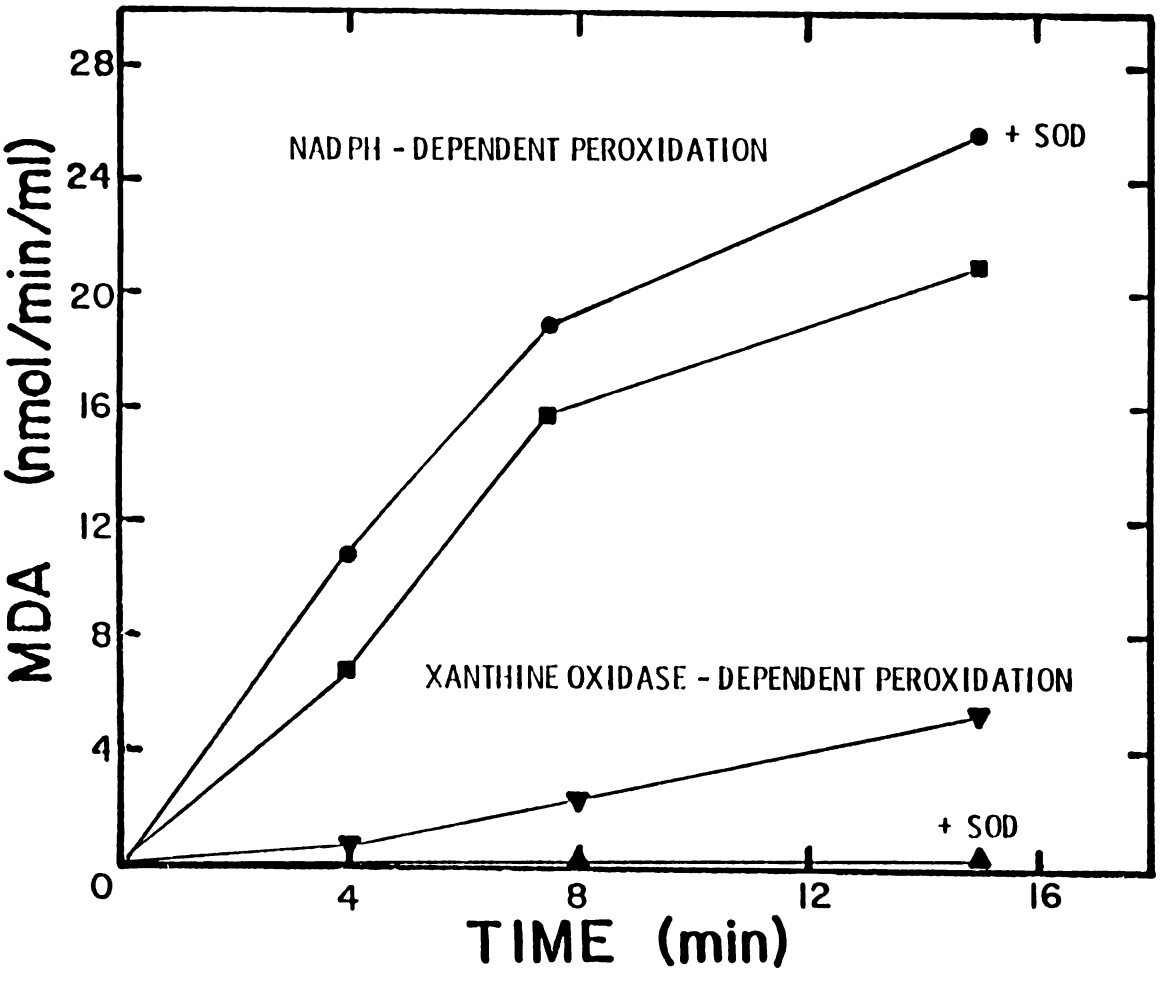


Figure 27. Effect of Superoxide Dismutase on NADPH-Dependent Lipid Peroxidation of Liposomes: A Comparison with Xanthine Oxidase-Dependent Peroxidation. All incubations contained liposomes (1 μmol lipid phosphate/ml), ADP-Fe^{3+} (1.7 mM ADP, 0.1 mM $FeCl_3$), and EDTA-Fe^{3+} (0.11 mM EDTA, 0.1 mM $FeCl_3$) in 0.3 M NaCl, pH 7.0, and either NADPH (0.1 mM) and purified reductase (0.5 u/ml) (■,●) or xanthine (0.17 mM) and xanthine oxidase (0.02 u/ml) (▼,▲). When added, the concentration of SOD was 100 u/ml.

DISCUSSION

In this paper, the potential role of microsomal H_2O_2 in the promotion of lipid peroxidation has been examined. It was postulated that by altering the concentration of H_2O_2 in microsomal lipid peroxidation incubations, corresponding changes in lipid peroxidation should be observed if it is initiated by an iron-catalyzed Haber-Weiss reaction.

The concentration of H_2O_2, generated *in vitro*, was altered by the addition of azide (0.4 mM) to microsomes. This treatment, which caused 97 percent inhibition of microsomal catalase activity, did not inhibit the rate of lipid peroxidation. Correspondingly, the rate of microsomal lipid peroxidation was also not affected by washing microsomes to remove both endogenous catalase activity and H_2O_2-dependent glutathione oxidase activity. Furthermore, the addition of exogenous catalase (previously chromatographed to remove contaminating antioxidants) to microsomes had no effect on lipid peroxidation. Using even extremely high concentrations of catalase (0.6 mg protein/ml), we could not observe the inhibition of MDA formation which was reported by Koster and Slee.[5] These results clearly indicate that the endogenous catalase activity in microsomes is neither critical for, nor inhibitory toward, the formation of the initiating species of microsomal lipid peroxidation.

The addition of organic hydroperoxides to microsomes has been shown to promote lipid peroxidation by a mechanism dependent upon the peroxidase activity of cytochrome P-450.[18] From these results, it appears that exogenous H_2O_2 does not interact with cytochrome P450 in a manner similar to that of organic hydroperoxides. The addition of only H_2O_2 to azide-treated microsomes did not initiate lipid peroxidation. However, when H_2O_2 was added to microsomes incubated with azide, ADP-Fe^{3+}, and NADPH, decreased rates of MDA formation were observed, in agreement with results reported by Kornbrust and Mavis.[10] This is the opposite effect from that which one would expect if the initiation of microsomal lipid peroxidation occurred via an iron-catalyzed Haber-Weiss reaction. In the absence of azide, exogenous H_2O_2 did not affect microsomal NADPH and ADP-Fe^{3+}-dependent lipid peroxidation, presumably because the H_2O_2 would be efficiently scavenged by endogenous catalase.

Small increases in the H_2O_2 concentration in azide-treated microsomes have been reported to cause increased $\cdot OH$ production and should cause corresponding increases in MDA formation. However, our results suggest that the formation of an initiator of microsomal lipid peroxidation is more complex than the exclusive generation of free $\cdot OH$ by an iron-catalyzed Haber-Weiss mechanism.

Other results would also indicate that H_2O_2 may actually inhibit lipid peroxidation,[11,19] because the addition of catalase was shown to stimulate lipid

peroxidation. This was postulated to be due to the oxidation of ADP-Fe^{2+} by H_2O_2 produced by xanthine oxidase.[19] Although rat liver microsomes generate H_2O_2, the endogenous levels of catalase activity in microsomes could prevent any accumulation of H_2O_2 and subsequent inhibition of lipid peroxidation. Therefore, such a mechanism of inhibition by H_2O_2 is entirely consistent with the data presented in this chapter.

Oxidation of ADP-Fe^{2+} by H_2O_2 should result in the production of ·OH and more lipid peroxidation. However, ·OH scavengers benzoate and mannitol had no effect on microsomal lipid peroxidation. When these results are integrated with the ability of H_2O_2 to inhibit lipid peroxidation, a strong argument against free ·OH in the initiation of microsomal lipid peroxidation can be made.

Having quantitated $O_2^{\cdot-}$ production by microsomes and the purified reductase in Chapter II, the participation of $O_2^{\cdot-}$ in NADPH-dependent lipid peroxidation can be more clearly established. Two types of microsomal lipid peroxidation incubations were constituted; one containing microsomes, NADPH, and ADP-Fe^{3+} and the other containing microsomes, xanthine oxidase, xanthine, and ADP-Fe^{3+}. The concentration of xanthine oxidase was adjusted to give a rate of $O_2^{\cdot-}$ generation equal to that produced by microsomes. Under these conditions the relative sensitivity of the rates of lipid peroxidation in both systems to SOD was examined. The rate of xanthine oxidase-dependent peroxidation was somewhat

slower than that of NADPH-dependent peroxidation, and was completely inhibited by SOD. Inhibition by SOD was expected as the reduction of ADP-Fe^{3+} in the xanthine oxidase system is via $O_2^{\cdot-}$. However, NADPH-dependent lipid peroxidation was not inhibited by SOD. If the analogous experiment is performed with a reconstituted lipid peroxidation system, similar results are obtained. Under conditions where equal rates of $O_2^{\cdot-}$ generation were occurring, the rate of xanthine oxidase-dependent lipid peroxidation was again lower than that of NADPH-dependent peroxidation and was completely inhibited by SOD.

The failure of SOD to inhibit NADPH-dependent lipid peroxidation has been previously reported, but some researchers still conclude that $O_2^{\cdot-}$ generation is required for iron reduction.[4] Failure of SOD to inhibit peroxidation has been rationalized by speculating that SOD was somehow inaccessible to $O_2^{\cdot-}$ generated at the membrane surface.[5,12] Certainly this explanation is less plausible in the reconstituted system where the protease-solubilized reductase was utilized, since it does not bind to membranes.[20]. It is apparent from the data that, despite stimulation by ferric chelates, $O_2^{\cdot-}$ production still accounts for only a minor percentage of the NADPH-dependent reduction capacity of microsomes or the purified reductase. It has also been clearly demonstrated that the reduction of certain ferric chelates can occur without $O_2^{\cdot-}$ generation (Chapter I). Certainly, NADPH-dependent microsomal lipid

peroxidation in these systems appears to occur without $O_2^{\cdot}$ generation, since SOD failed to inhibit peroxidation. Perhaps the apparent discrepancy over $O_2^{\cdot}$ participation in microsomal lipid peroxidation has evolved from the use of iron chelators other than ADP-Fe^{3+} which might be reduced and/or promote peroxidation by different mechanisms. It has been demonstrated that certain iron chelates are not suitable artificial electron acceptors for the reductase. Therefore, these chelates may have to be reduced by indirect means (perhaps via $O_2^{\cdot}$) in order to participate in lipid peroxidation.

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CHAPTER IV

Cytochrome P450-Dependent Lipid Peroxidation of Phospholipid Vesicles

ABSTRACT

A reconstituted lipid peroxidation system containing cytochrome P450 and the reductase incorporated into phospholipid vesicles was developed and characterized. The phospholipid vesicles underwent peroxidation when incubated with NADPH and ADP-Fe^{3+}. The extent of peroxidation depended upon the concentration of vesicles in the incubation, the concentration of ADP-Fe^{3+} and the cytochrome P450 content of the vesicles. No peroxidation of vesicles containing only the reductase occurred, unless EDTA-Fe^{3+} was also added. Peroxidation was inhibited by 30-40 percent by SOD. Thus NADPH-dependent reduction of ADP-Fe^{3+} is proposed to be both via $O_2^{\cdot-}$-dependent and -independent mechanisms, with the $O_2^{\cdot-}$-independent or direct reduction mechanism predominating.

INTRODUCTION

Microsomal lipid peroxidation was reported by Hochstein and Ernster, who demonstrated the requirement for NADPH, ADP, and the enzymatic nature of the process.[1] Their subsequent study of the peroxidative process also identified the necessity for iron[2] which had been a contaminant of their original ADP solutions. That the enzymatic nature of the process was responsible for the reduction of ADP-Fe^{3+} became evident in subsequent studies in which reductants such as ascorbate could substitute for NADPH,[2] or where ferrous iron addition to microsomes resulted in lipid peroxidation without the inclusion of reducing agents.[2,3]

Pederson and Aust further characterized the enzymic nature of the process, being the first to demonstrate that the reductase was the enzyme linking NADPH oxidation to the eventual ADP-Fe^{3+}-dependent peroxidation of microsomal membranes.[4] Using purified reductase, they developed a reconstituted NADPH-dependent lipid peroxidation system, observing that besides an ADP-Fe^{3+} requirement an additional ferric chelate, EDTA-Fe^{3+} was also necessary to initiate the peroxidation of microsomal phospholipid liposomes. Since no peroxidation occurred in the absence of EDTA-Fe^{3+}, they postulated that EDTA-Fe^{3+} was involved in electron transfer to ADP-Fe^{3+}. Reduction of ADP-Fe^{3+} was inferred to occur in intact microsomes since there was no apparent requirement

for EDTA-Fe^{3+}. Results presented in Chapter I demonstrated that rat liver microsomes but not the purified reductase could reduce ADP-Fe^{3+} under anaerobic conditions, thus confirming their hypothesis.

A question that remains is the identity of the microsomal component responsible for ADP-Fe^{3+} reduction and subsequent lipid peroxidation. Cytochrome P450 is the terminal electron acceptor of the MFO system and, therefore, is a logical choice. Moreover, cytochrome P450 is postulated to have a function in the peroxidative process, since cytochrome P450 inhibitors and substrates inhibit lipid peroxidation.[5,6] Cytochrome P450 also functions in the decomposition of lipid hydroperoxides generating alkoxyl radicals that can abstract methylene hydrogens from neighboring polyunsaturated fatty acids in a process termed lipid hydroperoxide-dependent lipid peroxidation.[7] Therefore, it can not be determined whether inhibition of lipid peroxidation by MFO inhibitors or substrates is due to inhibition of the initiation reaction, the lipid hydroperoxide-dependent or propagation phase, both, or neither.

Only one investigation has attempted to address in a definitive manner the potential of cytochrome P450 to participate in the initiation phases of lipid peroxidation. Elkstrom and Ingelman-Sundberg reported in a communication that phospholipid vesicles containing cytochrome P450 and reductase underwent NADPH-dependent peroxidation to a

greater extent than vesicles containing only the reductase.[8] Unfortunately, they did not demonstrate that the peroxidation process required exogenously added iron, despite the fact that the iron chelators desferrioxamine, DTPA and EDTA inhibited peroxidation. Therefore, due to absence of added iron it is conceivable that the cytochrome P450-dependent lipid peroxidation they observed was due to the cleavage of preformed hydroperoxides.

Previous studies in Chapter I had shown that a reconstituted MFO system could reduce ADP-Fe^{3+} anaerobically. The purpose of this investigation is to develop a model lipid peroxidation system dependent upon cytochrome P450 and characterize its requirements for iron chelates. In particular it is postulated that the requirement for EDTA-Fe^{3+} in the present reconstituted system can be fulfilled by cytochrome P450 isozymes.

MATERIALS AND METHODS

Chemicals: Asolectin was purchased from Associated Concentrates (Long Island, NY), and Bio Beads SM-2 was a product of Bio Rad (Richmond, CA).

Preparation of Enzymes: A partially purified preparation of cytochrome P450 isozymes isolated from the livers of male rats treated with phenobarbital were used for most of the phospholipid vesicle studies. It was prepared using the method of Dignam and Strobel[9] consisting of solubilization of microsomes with 1.5% Emulgen-911 and chromatography on DEAE Sephadex A25. The cytochrome P450 isozymes present in the flow-through of the column were precipitated with 18% polyethylene glycol (Sigma) and centrifuged at 105,000 x g for 1 hr. The precipitate was suspended in a small volume of 10 mM KH_2PO_4 pH 7.4, 20% glycerol, 0.2% Emulgen-911, 0.5% sodium cholate and 0.1 mM EDTA and the detergent was then removed by treating with Bio-Beads (0.3 gm/ml solution) for 3 hours at 4°C. The Bio-Beads were removed by decantation of the solution through nylon mesh and the resulting solution was dialyzed against 50 mM Tris pH 7.5 containing 20% glycerol. Preparations of the reductase prepared as outlined in Chapter I were dialyzed against this same solution and both preparations were stored at -70° C prior to use.

Preparation of Phospholipid Vesicles: Asolectin was dissolved (1.5 gm/15 ml) in Argon-purged chloroform: methanol (2:1 v/v) and the slightly turbid solution was allowed to stand in a capped test tube at -20° C overnight. Insoluble material collected on the bottom of the test tube and the clear organic phase was decanted and used for vesicle preparation. One ml aliquots of asolectin were placed in test tubes and dried under a stream of Argon. Chelexed 50 mM Tris pH 7.5 was added along with reductase and various concentrations of cytochrome P450 isozymes to give a final volume of 3 ml. The reductase was preincubated with cytochrome P450 prior to the addition to lipid. The head space of the test tube was purged with Argon and the tube was then capped with a rubber stopper. The tube was immediately placed into a 150 ml beaker containing ice cold H_2O and the water solution sonicated using a Branson Sonifier equipped with blunt-end probe at 60% power output until all dried lipid was completely resuspended (approximately 5 min.). The beaker containing the phospholipid vesicles and enzymes was then placed in a Branson bath sonicator and sonicated for 1 hr. with ice replenished periodically in order to maintain a temperature of 0-5° C. The sonicated solution was centrifuged at 105,000 xg for 1 hr. and the turbid supernatant was used for lipid peroxidation assays.

RESULTS

Requirements for NADPH-Dependent Peroxidation: In Table 13, the requirements for NADPH-dependent microsomal lipid peroxidation are shown. As has been previously observed, ADP-Fe^{3+} and NADPH are both required to obtain optimal rates of microsomal lipid peroxidation. The addition of EDTA-Fe^{3+}, although not a requirement for lipid peroxidation, did enhance the rate of MDA formation, in agreement with previous results in which EDTA-Fe^{3+} accelerated the decomposition of lipid hydroperoxides.[7]

Effect of Cytochrome P450 Induction on Microsomal Lipid Peroxidation: Results presented in Chapter II demonstrated that isozymes of cytochrome P450 produced differing amounts of $O_2^{\cdot-}$. Moreover, liver microsomes isolated from rats pretreated with the MFO inducers phenobarbital or HBB produced $O_2^{\cdot-}$ at greater rates than microsomes isolated from untreated rats. The effect of enzyme induction on the initial rates of NADPH-dependent peroxidation of microsomes is shown in Table 14. Liver microsomes isolated from uninduced, phenobarbital- or HBB-induced rats all peroxidized at nearly the same rate, although there appeared to be a tendency for microsomes from phenobarbital-induced animals to exhibit a slightly greater rate of MDA formation. The addition of SOD to the lipid peroxidation incubations

Table 13. Requirements for NADPH-Dependent Peroxidation of Rat Liver Microsomes

	MDA (nmol/min/mg)
Microsomes	0.06
+ADP-Fe^{3+}	0.04
+NADPH	0.14
Complete system	3.00
+EDTA (0.11 mM)	0.03
+EDTA-Fe^{3+} (0.11 mM EDTA, 0.1 mM $FeCl_3$)	5.3

The complete system contained rat liver microsomes (0.5 mg/ml), ADP-Fe^{3+} (1.7 mM ADP, 0.1 mM $FeCl_3$) and NADPH (0.1 mM) in 0.05 M NaCl pH 7.0.

Table 14. Effect of Cytochrome P450 Induction on the Rates of NADPH-Dependent Microsomes Lipid Peroxidation

	MDA Formation (nmol/min/mg protein)	
Inducer	-SOD	+SOD
None	2.0 ± 0.3	1.5 ± 0.2
Phenobarbital	2.7 ± 0.3	2.0 ± 0.2
HBB	2.2 ± 0.4	1.8 ± 0.2

Liver microsomes (0.5-0.8 mg/ml) were incubated with ADP-Fe^{3+} (1.7 mM ADP, 0.1 mM $FeCl_3$) and NADPH (0.1 mM) in 50 mM NaCl pH 7.0. Some incubations also contained SOD (100 u/ml). Values are averages of 3 preparations of microsomes (minimum of 3 animals/preparation).

mixtures resulted in a 20-25% inhibition of the rates of MDA formation, regardless of the microsomal preparation used.

Effect of Cytochrome P450 on NADPH-Cytochrome P450 Reductase-Dependent Peroxidation of Liposomes: The addition of cytochrome P450 isozymes to the reductase in DLPC micelles resulted in the reduction of ADP-Fe^{3+} under anaerobic conditions (Chapter I) and the production of $O_2^{\cdot-}$ under aerobic conditions (Chapter II). Therefore, cytochrome P450 isozymes were reconstituted with the reductase in DLPC micelles and incubated with microsomal phospholipid liposomes to investigate the ferric chelate requirements for lipid peroxidation. The results are shown in Table 15. No peroxidation occurred in the presence of ADP-Fe^{3+} and NADPH; both ADP-Fe^{3+} and EDTA-Fe^{3+} where necessary for MDA formation, just as they were in the reconstituted lipid peroxidation system of Pederson and Aust.[4] Thus, cytochrome P450 present in phospholipid micelles appears to be unable to support the ADP-Fe^{3+}-promoted peroxidation of liposomes.

Incorporation of Cytochrome P450 and NADPH-Cytochrome P450 Reductase into Phospholipid Vesicles: Cytochrome P450 and reductase were incorporated into phospholipid vesicles using an indirect sonication procedure in the absence of detergents that has been previously used for the incorporation of several membrane proteins.[10,11] Several

Table 15. Requirements for the NADPH-Dependent Peroxidation of Phospholipid Liposomes Promoted by the Reconstituted Mixed Function Oxidase System

	MDA (nmol/min/ml)
Complete System	
+P450b	0.05
+EDTA-Fe^{3+}	0.00
+ADP-Fe^{3+}	0.04
+EDTA-Fe^{3+} + ADP-Fe^{3+}	1.32
+P450c	0.00
+EDTA-Fe^{3+}	0.00
+ADP-Fe^{3+}	0.07
+EDTA-Fe^{3+} + ADP-Fe^{3+}	0.92
+P450d	0.00
+EDTA-Fe^{3+}	0.05
+ADP-Fe^{3+}	0.00
+EDTA-Fe^{3+} + ADP-Fe^{3+}	1.50

The complete system contained reductase (0.1 u/ml), DLPC (20 µg/ml), phospholipid liposomes (1 µmol lipid phosphate/ml), and NADPH (0.1 mM) in 50 mM NaCl pH 7.0. The cytochrome P450 isozymes (0.02 nmol/ml final concentration) were preincubated with the reductase and DLPC for 5 min at room temperature, then 1 hr on ice before use. Ferric chelate concentrations were: EDTA-Fe^{3+} (0.11 mM EDTA, 0.1 mM $FeCl_3$) and ADP-Fe^{3+} (0.5 mM ADP, 0.1 mM $FeCl_3$).

preparations of vesicles containing cytochrome P450 and the reductase were subjected to gel permeation chromatography on Sepharose CL-4B. The resulting fractions were analyzed for phosphate, reductase activity, and cytochrome P450 content. A column profile is shown in Figure 28. The majority of phosphate, reductase, and cytochrome P450 eluted at the void volume of the column, indicating that the proteins were incorporated into phospholipid vesicles.

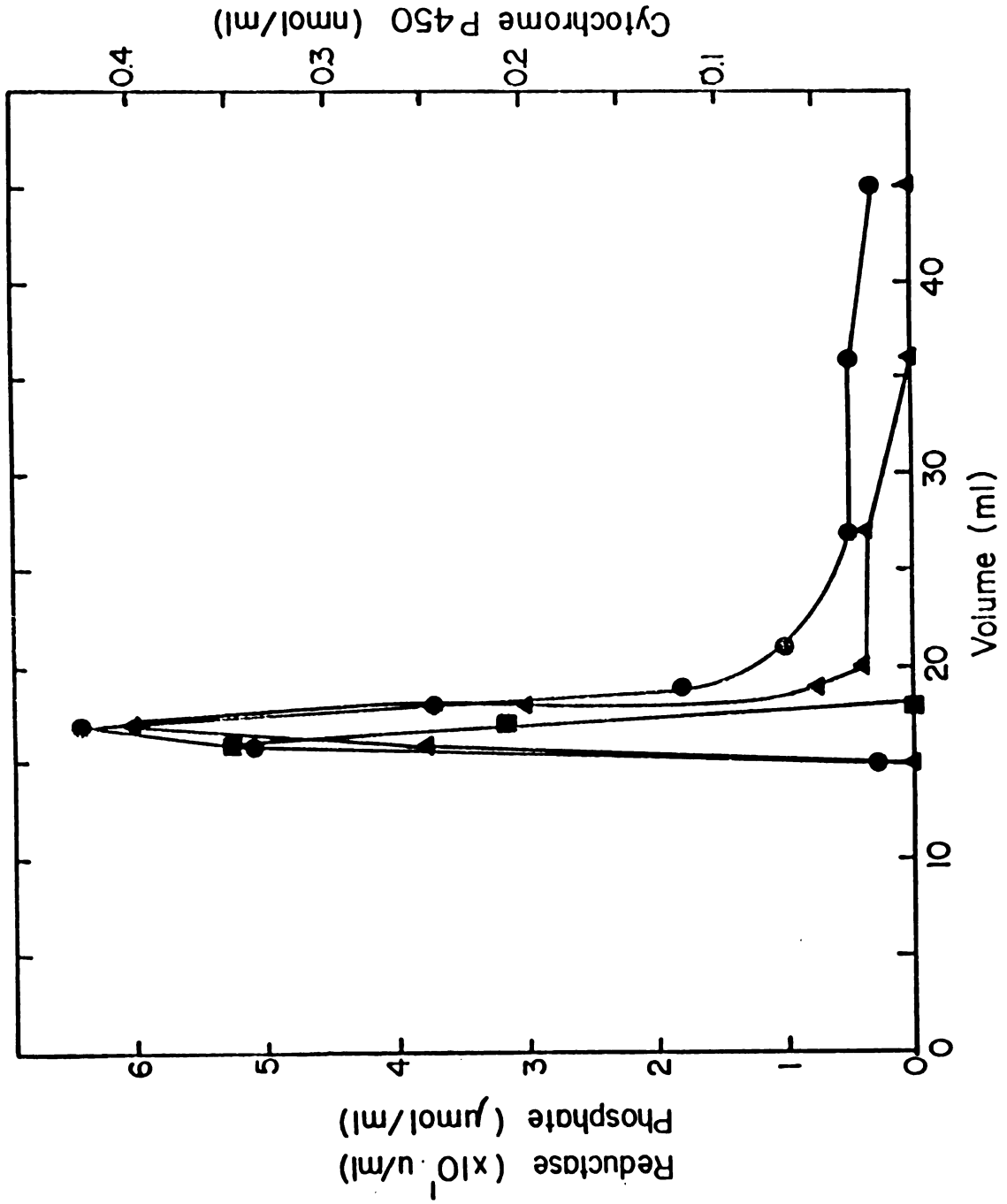
NADPH-Dependent Peroxidation of Phospholipid Vesicles: Incubation of phospholipid vesicles containing reductase and cytochrome P450 isozymes with ADP-Fe^{3+} and NADPH resulted in the peroxidation of the phospholipids as evidenced by MDA formation. Low rates of peroxidation were observed in the absence of either NADPH or ADP-Fe^{3+} or when EDTA-Fe^{3+} was substituted for ADP-Fe^{3+} (Table 16). The addition of SOD resulted in a 42% inhibition of the rates of MDA formation. Catalase also inhibited somewhat, but to a lesser degree than SOD. As the concentration of vesicles incubated with ADP-Fe^{3+} and NADPH was increased, the corresponding rates of MDA formation also increased (Figure 29). Likewise, greater MDA formation was observed in incubations containing increased concentrations of ADP-Fe^{3+} (Figure 30). Vesicle preparations containing equal amounts of reductase but different cytochrome P450 contents were also prepared. Vesicles having higher cytochrome P450 contents peroxidized more rapidly (Figure 31). The addition of SOD to these

Table 16. Requirements for the NADPH-Dependent Peroxidation of Cytochrome P450-Containing Phospholipid Vesicles

	MDA (nmol/15 min/ml)
Vesicles	0.2
+ADP-Fe^{3+}	0.5
+NADPH	0.4
+EDTA-Fe^{3+} + NADPH	0.2
Complete System	7.4
+SOD (250 u/ml)	4.3
+Catalase (250 u/ml)	6.2
+EDTA-Fe^{3+} (0.11 mM EDTA, 0.1 mM $FeCl_3$)	31.1

The complete system contained vesicles (0.43 u/ml reductase, 0.33 nmol/ml cytochrome P450, 3.7 mol lipid phosphate/ml), ADP-Fe^{3+} (0.75 mM ADP, 0.15 mM $FeCl_3$) and NADPH (0.2 mM) in 50 mM Tris pH 7.5.

Figure 28. Elution Profile of NADPH-Cytochrome P450 Reductase and Cytochrome P450-Containing Phospholipid Vesicles Subjected to Gel Filtration. A 1 ml aliquot of vesicles was chromatographed on a 1x30 cm Sepharose CL-4B column. Fractions (1 ml) were collected and assayed for reductase activity (●), cytochrome P450 content (■) and phosphate (▲).


Reductase ($x10^1$ u/ml)
Phosphate (μmol/ml)
Cytochrome P450 (nmol/ml)
Volume (ml)
0
1
2
3
4
5
6
10
20
30
40
0.1
0.2
0.3
0.4

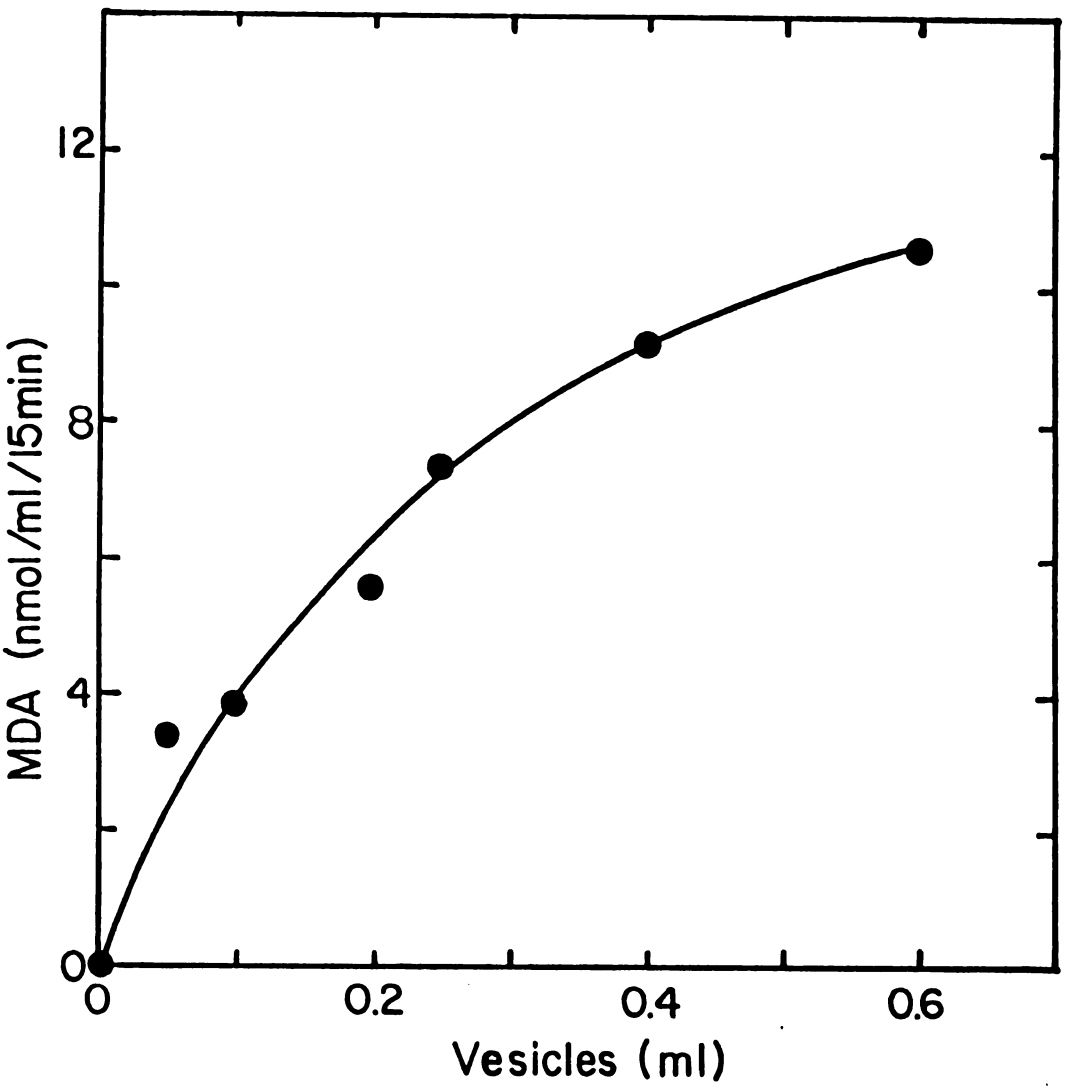


Figure 29. Effect of Vesicle Concentration on the Peroxidation of Phospholipid Vesicles. Various concentrations of phospholipid vesicles (4.2 units reductase: 3.3 nmol cytochrome P450: 37 μmol lipid phosphate) were incubated with ADP-Fe^{3+} (0.75 mM ADP, 0.15 mM $FeCl_3$) and NADPH (0.2 mM) in 50 mM Tris pH 7.5 at 37° C. The final incubation volumes were 2.5 ml.

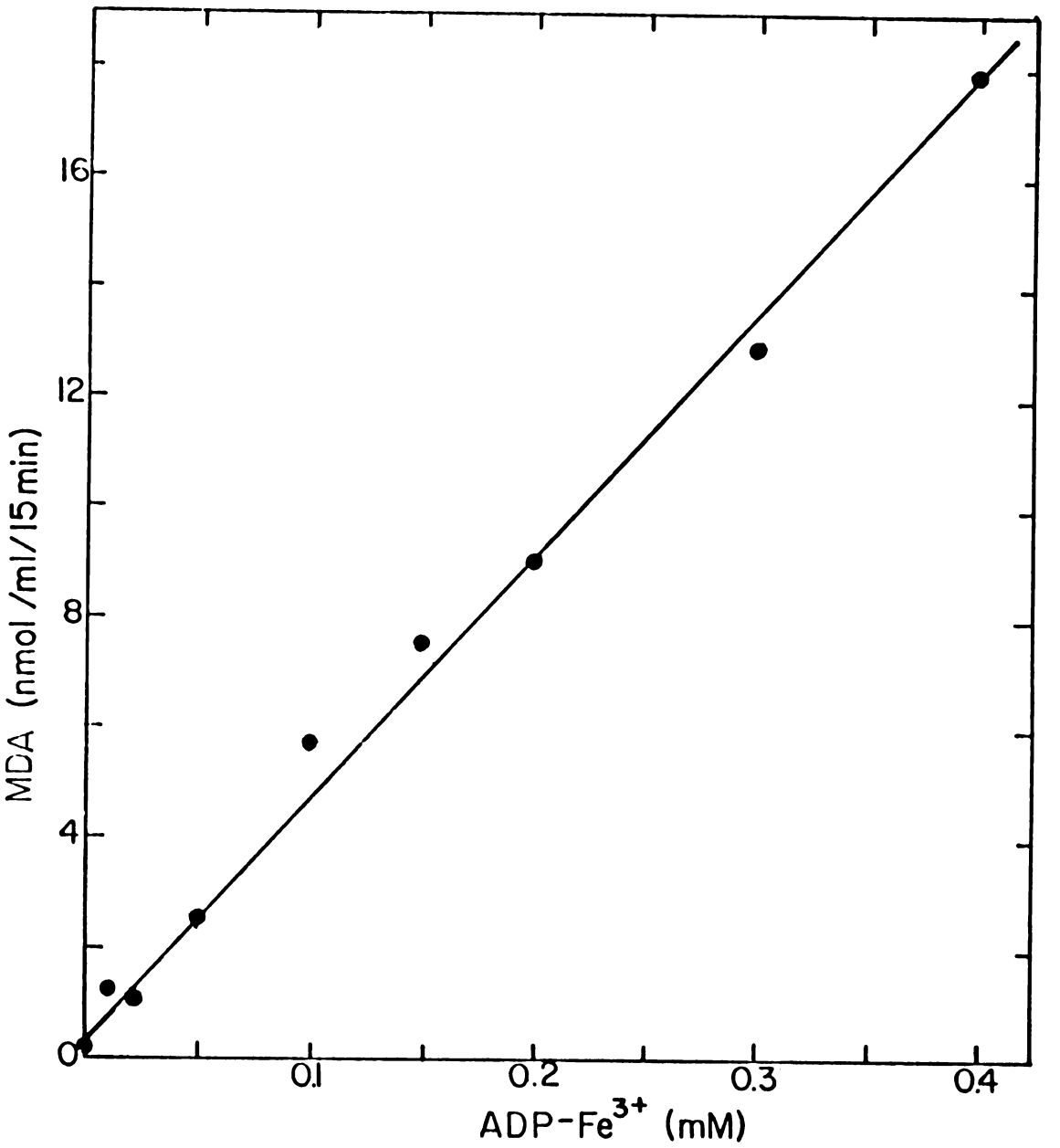
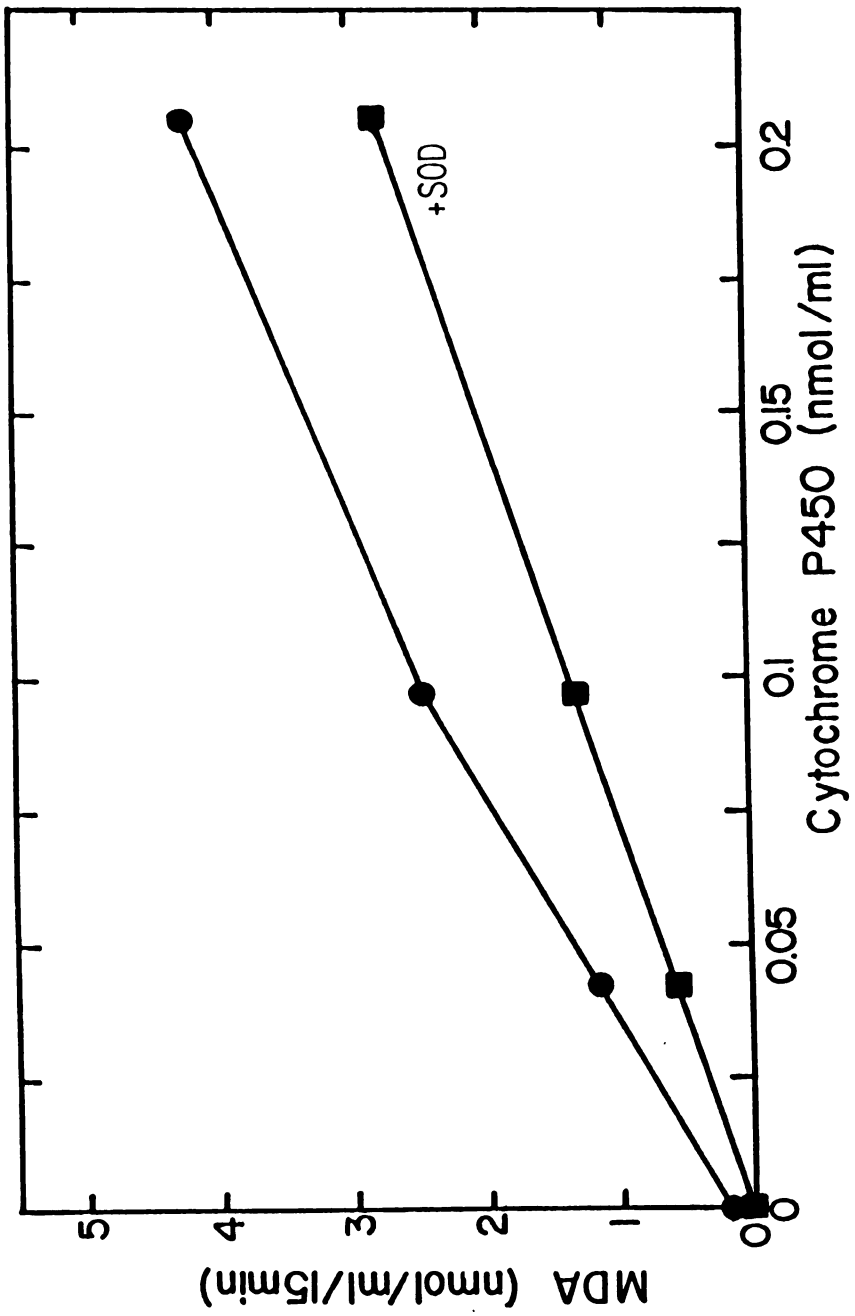


Figure 30. Effect of ADP-Fe^{3+} Concentration on the Peroxidation of Phospholipid Vesicles. Phospholipid vesicles (0.4 units reductase activity/ml, 0.25 nmol cytochrome P450/ml; 3.5 μmol lipid phosphate/ml) were incubated with NADPH (0.2 mM) and various concentrations of ADP-Fe^{3+} (prepared as a 5:1 chelate) in 50 mM Tris pH 7.5.

Figure 31. Effect of Increasing Cytochrome P450 Content on the Peroxidation of Phospholipid Vesicles. Vesicle preparations having different cytochrome P450 contents were incubated with ADP-Fe^{3+} (0.5 mM ADP, 0.1 mM $FeCl_3$) and NADPH (0.2 mM) in 50 mM Tris pH 7.5. The reductase activity in the incubations was 0.33 + 0.02 units/ml and the phospholipid content was 1.93 + 0.4 µmol lipid phosphate/ml. When added, SOD was 240 u/ml.


MDA (nmol/ml/15min)
0
1
2
3
4
5
0
0.05
0.1
0.15
0.2
Cytochrome P450 (nmol/ml)
+SOD

incubations resulted in 30-50% inhibition, with higher degrees of inhibition occurring at lower cytochrome P450 contents.

DISCUSSION

The reactions leading to the formation of an initiating species of lipid peroxidation are thought to involve ferrous iron and oxygen (or perhaps partially-reduced forms of oxygen). The function of the microsomal MFO is usually considered to the reduction of ferric chelates. Microsomal lipid peroxidation is promoted by ADP-Fe^{3+} and NADPH, but a reconstituted lipid peroxidation system that included the purified reductase could not support peroxidation unless EDTA-Fe^{3+} was also included.[4] Thus it was postulated that EDTA-Fe^{3+} replaced an endogenous component of microsomes permitting the reduction of ADP-Fe^{3+} and the subsequent initiation of peroxidation.

If this hypothesis is indeed correct, cytochrome P450 isozymes are probably the most likely microsomal components necessary for ADP-Fe^{3+} reduction. In Chapter I, cytochrome P450 was shown to reduce ADP-Fe^{3+} under anaerobic conditions when incubated with NADPH and the reductase. Thus, what remained to be shown was that the addition of cytochrome P450 to a reconstituted lipid peroxidation system already containing the reductase, ADP-Fe^{3+}, and NADPH could result in peroxidation, without the addition of EDTA-Fe^{3+}.

However, the incubation of cytochrome P450 with the reductase in DLPC micelles did not result in ADP-Fe^{3+}-dependent peroxidation, unless EDTA-Fe^{3+} was also added.

This is somewhat surprising in that this reconstitution yields an active MFO system that is capable of reducing ADP-Fe^{3+} (Chapter I), but it is essentially no different from reconstituted system of Pederson and Aust with respect to its ferric chelate requirements.

The reductase and cytochrome P450 were incorporated in asolectin vesicles using a sonication procedure. Incubation of these vesicles with NADPH and ADP-Fe^{3+} resulted in peroxidation as evidenced by the formation of MDA. Essentially no peroxidation was observed in the absence of NADPH or ADP-Fe^{3+}, or when EDTA-Fe^{3+} was substituted for ADP-Fe^{3+}. Increasing the concentration of vesicles in the incubation resulted in increased MDA formation, as did increasing the concentration of ADP-Fe^{3+}. Moreover, vesicles containing increasing cytochrome P450 contents exhibited increased rates of lipid peroxidation. Thus, it appears that the incorporation of cytochrome P450 and the reductase in phospholipid vesicles results in the peroxidation of the vesicles incubated with NADPH and ADP-Fe^{3+}.

The addition of SOD to these incubations resulted in a moderate inhibition of the rates of MDA formation, just as was the case with intact microsomes. However, as the cytochrome P450 content of the vesicles increased, MDA formation was inhibited by SOD to lesser extents. It has been a subject of debate as to whether or not NADPH-dependent microsomal lipid peroxidation is dependent upon

$O_2^{\cdot-}$.[12-16] Whereas the addition of cytochrome P450 to the reductase resulted in increased rates of $O_2^{\cdot-}$ production (Chapter II) it also resulted in the direct anaerobic reduction of ADP-Fe^{3+} (Chapter I). Thus, regardless of the mechanism of iron reduction, the data in this thesis would support the role of cytochrome P450 in the initiation of microsomal lipid peroxidation. Inhibition by SOD was less than 50%, so the major pathway for ADP-Fe^{3+} reduction in this reconstituted vesicular system would appear to be direct.

In a previous communication, Elkstrom and Ingelman-Sundberg had reached a similar conclusion.[8] However, they did not demonstrate a requirement for low molecular weight chelates, despite that they reported an inhibition by iron chelators desferrioxamine, EDTA, and DTPA. Thus, it could not be ascertained whether cytochrome P450 was promoting lipid peroxidation via enhancing initiation reactions by reducing low molecular weight iron chelates or via participating in the decomposition of lipid hydroperoxides as had previously been demonstrated.[7] The data in this chapter supports a role for cytochrome P450 in the reduction of iron in the promotion of lipid peroxidation. It should be indicated that if the asolectin used as a phospholipid source in these studies had contained a significant amount of lipid hydroperoxides, peroxidation of the vesicles would have been promoted by EDTA-Fe^{3+} and reductase alone, since the reductase readily reduces EDTA-Fe^{3+} and EDTA-Fe^{3+}

readily decomposes lipid hydroperoxides.[7] In this case, no requirement for ADP-Fe^{3+} or for cytochrome P450 would have been evident.

It is also interesting to speculate on the apparent necessity for cytochrome P450 to be incorporated into the phospholipid vesicles in order to promote lipid peroxidation. The MFO reconstituted system customarily used in drug metabolism investigations was not able to promote lipid peroxidation despite being capable of reducing ADP-Fe^{3+} (Chapter I). This might indicate a different type of "site specific" mechanism, that is the reduction of ADP-Fe^{3+} must occur close to or at the membrane surface in order to efficiently promote lipid peroxidation. This differs from other "site specific" hypotheses in that it has been postulated that the iron that acts as a catalyst for potentially deleterious redox reactions is bound at critical cellular sites or chelated by chemicals having selective cellular binding sites.[17,18]

Relatedly, in Chapter I it was shown that microsomes or the reconstituted MFO system reduced ADP-Fe^{3+} at a slow rate relative to EDTA-Fe^{3+}. This would indicate that in order to efficiently promote lipid peroxidation the low level of ADP-Fe^{3+} formed must be able to successfully interact with the lipid bilayer, a more likely prospect when cytochrome P450 is incorporated into vesicles.

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SUMMARY

Several mechanisms for the formation of an initiating species of microsomal lipid peroxidation have been proposed, but one feature common to all of these proposals is the reduction of ferric iron. Iron reduction has been proposed to be either $O_2^{\cdot-}$-dependent or direct ($O_2^{\cdot-}$-independent). In this dissertation, two activities of the microsomal MFO system ($O_2^{\cdot-}$ production and direct iron reduction) have been examined and their relative significance in the initiation of lipid peroxidation of phospholipid membranes has been assessed.

A summary of the data is presented in Table 17. Rat liver microsomes incubated with NADPH produced $O_2^{\cdot-}$ and reduced the ferric chelates ADP-Fe^{3+} and EDTA-Fe^{3+}. The NADPH-dependent peroxidation of microsomes required only the presence of ADP-Fe^{3+}, in agreement with earlier work. Despite the greater rates of EDTA-Fe^{3+} reduction exhibited by microsomes, no lipid peroxidation promoted by EDTA-Fe^{3+} was observed. This emphasizes that the reduction of any ferric chelate is not necessarily sufficient to result in the initiation of lipid peroxidation. However, the reduction of ADP-Fe^{3+} does result in the peroxidation of membrane phospholipids. Peroxidation was not inhibited by SOD, indicating that the direct iron reduction mechanism has

Table 17. Summary of Results

	Superoxide Production	Direct Iron Reduction	Lipid Peroxidation
Reductase	+/-	EDTA-Fe^{3+}	EDTA-Fe^{3+} ADP-Fe^{3+}
Microsomes	+	EDTA-Fe^{3+} ADP-Fe^{3+}	ADP-Fe^{3+}
Reconstituted MFO	+	EDTA-Fe^{3+} ADP-Fe^{3+}	ADP-Fe^{3+}

greater significance in the initiation of lipid peroxidation than does $O_2^{\cdot-}$ production.

Purified reductase generated very low rates of $O_2^{\cdot-}$ production under aerobic conditions, but catalyzed the reduction of EDTA-Fe^{3+} under anaerobic conditions. Lipid peroxidation of phospholipid liposomes promoted by the reductase could not be initiated in the presence of ADP-Fe^{3+} alone, consistent with the inability of the enzyme to reduce ADP-Fe^{3+}. However, the reductase did reduce EDTA-Fe^{3+} and the incubation of EDTA-Fe^{3+} and ADP-Fe^{3+} with liposomes resulted in NADPH-dependent peroxidation. The small amount of $O_2^{\cdot-}$ produced by the reductase could not promote ADP-Fe^{3+} reduction or subsequent lipid peroxidation. Peroxidation promoted by EDTA-Fe^{3+} and ADP-Fe^{3+} was not inhibited by SOD, indicating that the direct reduction of EDTA-Fe^{3+} was of utmost importance in the initiation of lipid peroxidation.

The addition of cytochrome P450 isozymes to the purified reductase resulted in increased rates of $O_2^{\cdot-}$ production, indicating that cytochrome P450 is the microsomal component mainly responsible for $O_2^{\cdot-}$ generation. Furthermore, this reconstituted MFO system exhibited ADP-Fe^{3+} and EDTA-Fe^{3+} reduction activities. When incorporated into phospholipid vesicles, the reconstituted MFO system promoted NADPH-dependent peroxidation of the vesicles incubated with ADP-Fe^{3+}. Peroxidation of vesicles containing increased cytochrome P450 contents was inhibited to decreasing extents by SOD.

In conclusion, these data indicate that direct iron reduction by the microsomal MFO system or its purified components is of greater importance in the initiation of lipid peroxidation than reduction of iron dependent upon $O_2^{\cdot -}$.

APPENDIX A

Appendix A

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