MECHANISMS FOR THE RELEASE OF IRON FROM FERRITIN AND THEIR RELATIONSHIP TO LIPID PEROXIDATION AND TOXICITY

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ABSTRACT

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This work was undertaken in an effort to determine what physiological iron chelates may provide iron for redox reactions leading to the formation of oxidants which initiate membrane lipid peroxidation (LP). Initial studies were designed to separate and characterize low molecular weight (MW) iron complexes from rat liver cytosol. Gel filtration experiments revealed that essentially 100% of the total cytosolic iron was recovered in fractions ranging from 100-500,000 MW, with 70% of the iron present in ferritin, the major iron storage protein.

Subsequent work demonstrated that superoxide ($O_2^{\cdot-}$), generated by xanthine oxidase, released iron from ferritin and initiated the peroxidation of phospholipid liposomes. Iron release and LP were inhibited by superoxide dismutase (SOD) but stimulated by catalase. ESR spin trapping studies supported an inverse relationship between hydroxyl radical formation and LP. Catalase stimulated LP by preventing rapid H_2O_2-dependent oxidation of Fe^{2+}, allowing more efficient formation of an initiator of LP.

Superoxide, generated by the redox cycling of paraquat and catalyzed by NADPH-cytochrome P450 reductase, also released iron from ferritin and promoted LP. SOD inhibited paraquat-dependent Fe^{2+}

release only 50-60%. Anaerobically, the paraquat cation radical rapidly released all of the iron from ferritin, accounting for the SOD-insensitive Fe^{2+} release.

The antitumor anthracycline antibiotics, adriamycin and daunomycin, whose clinical use is limited by cardiotoxicity, also underwent redox cycling to generate $O_2^{\cdot-}$, releasing Fe^{2+} from ferritin. ESR studies demonstrated that, anaerobically, their semiquinone free radicals transferred electrons to ferritin to release Fe^{2+}. Accordingly, aerobic iron release was inhibited only 50% by SOD. The semiquinone radical of diaziquone, a relatively non-toxic chemotherapeutic drug, was incapable of electron transfer and Fe^{2+} release.

Another study characterized the endogenous iron in rat liver microsomes. Development of an ELISA for ferritin revealed that 83% of the iron in microsomes is in ferritin. A chromatographic procedure was devised which removed ferritin, as well as contaminating SOD and catalase, from microsomes. The iron in ferritin was released by $O_2^{\cdot-}$, whereas the remaining 17% of the iron was directly reduced by the reductase. Both iron pools were capable of supporting microsomal LP.

To My Grandmother and Grandfather (late)
Ma-Mu and Pap-Pap

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Much of the credit for this work must go to my family, particularly my parents. It is they who subtilely impressed upon me the value of education and the virtues of hard work.

Finally, I cannot thank enough my wife, Jean, who saw me through the difficult times and shared in the laughter of the good times,and whom had the pleasure (?) of typing this dissertation. Her continued support made it all worthwhile.

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ABBREVIATIONS

ADP	adenosine-5'-diphosphate
BCA	bicinchoninic acid
DDC	diethyldithiocarbamate
DMPO	5,5'-dimethyl-1-pyrroline-N-oxide
DMPO-OH	DMPO spin trap adduct with ·OH
DMPO-OOH	DMPO spin trap adduct with $O_2^{-\cdot}$
DTPA	diethylenetriamine penta-acetic acid
EDTA	ethylenediaminetetracetate
ELISA	enzyme linked immunosorbent assay
ESR	electron spin resonance
GSH	glutathione, reduced form
Hepes	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
hfs	hyperfine splitting
MDA	malondialdehyde
MFO	mixed function oxygenase
NADPH	reduced nicotinamide adenine dinucleotide phosphate
PBS	phosphate buffered saline
PUFA	polyunsaturated fatty acid
SDS	sodium dodecyl sulfate
SOD	superoxide dismutase
TBA	2-thiobarbituric acid

INTRODUCTION

A free radical can be defined simply as any species possessing an unpaired electron. The need to pair electrons imparts upon free radicals their characteristic reactivity towards a variety of organic and inorganic molecules. Since the demonstration by McCord and Fridovich in 1969 that radical species of molecular oxygen exist in biological systems, a plethora of literature reports on the generation and consequences of oxygen radicals has appeared.

This topic is of great interest as it indicates that survival in an oxygen environment presents a perplexing dilemma to aerobic organisms. While molecular oxygen is required for normal metabolic function, the potential for cells to produce reactive, partially reduced species of dioxygen necessitates careful control over the generation of such species. Consequently, cells have evolved a series of concerted mechanisms which control the production of, or limit the accumulation of oxygen radicals. For example, electron transfer pathways such as that of the mitochondria appear to be tightly coupled such that the ultimate product of dioxygen reduction is water, with little escape from the organelle of any of the partially reduced oxygen species. In addition, all oxygen utilizing organisms possess

enzyme systems which serve to remove or "detoxify" potentially deleterious oxygen radicals.

Perhaps one of the most important means by which dioxygen activation can be controlled is by preventing its interaction with transition metals such as iron. Dioxygen exists as a diradical in the ground state, containing two unpaired electrons of parallel spin in the $2p\pi^*$ antibonding orbital. Fortunately, it is this property which limits its direct reaction with organic molecules as this would require compounds to possess two electrons of parallel spin, in contradiction to the Pauli Exclusion Principle. However, transition metals contain unpaired electrons and can readily form complexes with dioxygen resulting in the production of partially reduced oxygen species. Therefore, one would expect that a critical control point for limiting the generation of active oxygen would be to prevent interactions between oxygen and transition metals. Accordingly, the transport and storage of these metals by specialized proteins is a highly regulated process which serves to minimize inadvertent oxygen reduction.

While cellular production of oxygen radicals has been intensively studied, there is an increasing awareness of a lack of knowledge concerning the potential for biological iron-containing proteins to participate in oxygen radical reactions. However, it is becoming more apparent that iron-oxygen interactions do occur *in vivo*. In particular, iron-dependent peroxidation of membrane polyunsaturated fatty acids is implicated in a variety of toxicological and pathological states. In this dissertation, evidence is presented that ferritin, the major iron storage protein, can provide iron for the

formation of radical species capable of initiating membrane lipid peroxidation. Chapter I describes studies which demonstrate that $O_2^{\cdot-}$, generated by xanthine oxidase, can reductively release iron from ferritin. Once released from ferritin this iron can promote the peroxidation of phospholipid liposomes. ESR spin trapping studies demonstrate an inverse correlation between ·OH formation and lipid peroxidation.

In Chapter II the cyclic reduction and autoxidation of paraquat, catalyzed by NADPH-cytochrome P450 reductase, is shown to generate $O_2^{\cdot-}$ and release iron from ferritin. The inability of ·OH scavengers to inhibit lipid peroxidation again provides evidence that lipid peroxidation may be initiated by a Fe^{2+}-O_2-Fe^{3+} complex, rather than ·OH.

A study of iron release from ferritin by paraquat (Chapter III) revealed that the reduced paraquat cation radical can mediate a very rapid, complete release of iron from ferritin. ESR and visible spectrophotometric studies demonstrated a rapid transfer of electrons from the radical species of paraquat to ferritin, thereby reducing and releasing the iron. The ferritin protein structure was not altered by the radical and retained its ability to take up additional iron. The semiquinone free radicals of the cardiotoxic anthracycline antibiotics, adriamycin and daunomycin, are also capable of reductively releasing iron from ferritin (Chapter IV). Importantly, a relatively non-toxic autitumor drug, diaziquone, is also reduced to a semiquinone free radical but is unable to mediate iron release from ferritin.

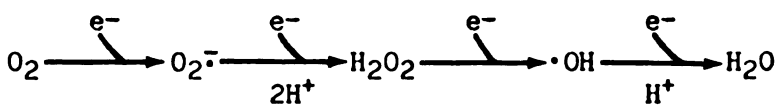
The last Chapter (V) summarizes results obtained from a careful study of the role of endogenous iron in microsomal lipid peroxidation. Nearly 90% of microsomal iron was found to be associated with ferritin. A procedure for ferritin removal was devised and the ability of ferritin to promote microsomal lipid peroxidation was investigated.

Each of the aforementioned chapters is written in a format similar to that of many scientific journals, thus each chapter contains an Abstract, Introduction, Materials and Methods, and Discussion as well as its own List of References. As the subject matter of this dissertation encumbers a variety of topics including oxygen radicals, lipid peroxidation, and iron metabolism a Literature Review detailing each of these topics, and containing its own List of References, precedes Chapter I.

LITERATURE REVIEW

Univalent Reduction of Molecular Oxygen

1. Superoxide and Hydrogen Peroxide. The sequential one electron reduction of molecular oxygen produces superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$) and, ultimately, water.



(1)

Superoxide is known to be produced by many enzyme systems such as xanthine oxidase and flavin dehydrogenases. Many enzyme systems known, or proposed to generate $O_2^{\cdot-}$ and H_2O_2 are dependent upon iron as a means of reducing dioxygen. Xanthine oxidase, which oxidizes xanthine and hypoxanthine to uric acid, contains iron sulfur centers as well as molybdenum and FAD. This electron transport system is capable of storing either 6 (1), or more recently, 8 electrons (2). Its ability to produce both $O_2^{\cdot-}$ and H_2O_2 has led to its widespread use in oxygen radical-related research. Stimulated polymorphonuclear leukocytes also produce large fluxes of $O_2^{\cdot-}$ as a desired product of their electron transport system, which utilizes a b type cytochrome (3). This generation of $O_2^{\cdot-}$ is thought to be required for the bactericidal properties exhibited by these leukocytes.

The inadvertent reduction of dioxygen by transition metal-dependent electron transport systems, evolved for other metabolic functions in the cell, has been proposed. The iron and copper containing terminal oxidase of the mitochondrial electron transport chain, cytochrome c oxidase, catalyzes the 4 electron reduction of molecular oxygen to water. However, H_2O_2 production by mitochondria has been demonstrated and is thought to arise from autoxidation of reduced intermediates of the system, particularly ubisemiquinone (4,5). Likewise, generation of $O_2^{\cdot-}$ and H_2O_2 by the MFO system of the endoplasmic reticulum has also been reported (6,7). This electron transport system, which consists of two cytochromes and their respective flavoproteins, functions to insert one atom of molecular oxygen into the substrate (a host of endogenous compounds and xenobiotics) with the other oxygen atom reduced to water. The ability of inducers or uncouplers of MFO activity to enhance $O_2^{\cdot-}$ and/or H_2O_2 production and conversely, of MFO inhibitors to decrease their production, suggests that dioxygen reduction may result from inefficient electron transfer (8). The significance of these findings under normal cellular conditions remains to be determined.

The autoxidation of reduced, organic compounds such as ubiquinone (4), epinephrine (9), and reduced flavins (10) has been suggested to produce $O_2^{\cdot-}$ and H_2O_2. Similarly, the simple autoxidation of Fe^{2+} will also produce $O_2^{\cdot-}$ (11):

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

As the reaction of dioxygen with organic molecules is kinetically unfavorable, the proposed autoxidation of many compounds may also be related to the presence of adventitous transition metal ions.

While simplistic in theory, Fe^{2+} autoxidation is subject to variation, particularly in the presence of other ions or chelators. The influence of chelation on Fe^{2+} autoxidation is usually manifested as alterations in 1) the rate of autoxidation, 2) which reduced oxygen species is produced, and 3) the redox potential of the metal. The ability of numerous anions including chloride (12), sulfate (13), and phosphate (14) to affect Fe^{2+} autoxidation has generally indicated that autoxidation increases as the affinity of the anion for Fe^{3+} increases. The complexity of these effects is demonstrated by the ability of certain anoins to destabilize the Fe^{2+}-O_2 complex resulting in $O_2^{-\cdot}$ formation while others stabilize the complex, allowing reaction with a second Fe^{2+} to generate H_2O_2.

While H_2O_2 can be produced directly by Fe^{2+} autoxidation without $O_2^{-\cdot}$ as an intermediate product, the majority of non-enzymatic H_2O_2 production likely involves formation of $O_2^{-\cdot}$ initially. Once $O_2^{-\cdot}$ is generated, H_2O_2 can be formed via several pathways, for example, by reaction with Fe^{2+}:

$$Fe^{2+} + O_2^{-\cdot} \xrightarrow{2H^+} Fe^{3+} + H_2O_2 \qquad (3)$$

Hydrogen peroxide is also readily formed by non-enzymatic dismutation of $O_2^{-\cdot}$ or the action of SOD as shown in reaction (4):

$$O_2^{-\cdot} + O_2^{-\cdot} \xrightarrow{2H^+} H_2O_2 + O_2 \qquad (4)$$

At physiological pH the second order rate constant for the non-enzymatic disproportionation of $O_2^{\cdot-}$ is 8 x 10^4 $M^{-1}s^{-1}$ while the SOD-catalyzed reaction proceeds at 2 x 10^9 $M^{-1}s^{-1}$ (15). Superoxide dismutase actually refers to three related, yet distinct enzymes which differ primarily with respect to the redox active metal utilized to catalyze dismutation. The cytosolic SOD contains Cu^{2+} and Zn^{2+}, a mitochondrial SOD contains Mn^{2+}, while an Fe^{3+}-containing variant has been found in bacteria (15). These enzymes exemplify the use of metals in controlled oxygen radical reactions and it is often proposed that their existence in nearly all aerobic organisms is testimony to the importance of controlling the cellular flux of $O_2^{\cdot-}$.

Hydrogen peroxide is also known to be generated by many enzymes such as D-amino acid oxidase and urate oxidase which are localized within peroxisomes. Not surprisingly, this organelle contains high levels of catalase, a hemoprotein which serves to disproportionate H_2O_2 to water and dioxygen. However, calculations show 40% of the generated H_2O_2 can diffuse out of the peroxisome (16). Many peroxisomal proliferating or hyperlipidemic drugs are proposed to result in the excessive production of H_2O_2 which may then "leak" out of the peroxisome (17). While this process is thought to damage cells, whether it actually occurs is questionable and furthermore, cells also contain the cytosolic, selenium-containing enzyme glutathione peroxidase which can remove intracellular H_2O_2 (18).

2. Formation of the Hydroxyl Radical. As the formation of H_2O_2 involves the addition and pairing of two electrons in the π^* antibonding orbital of dioxygen, H_2O_2 is not considered a radical

although it is a strong oxidizing agent and is generally included in the category of "active oxygen". However, H_2O_2 readily reacts with transition metals to generate oxidants capable of reacting with numerous organic molecules. One of the most well known reactions is that of H_2O_2 with Fe^{2+}, known as the Fenton's reaction (19):

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + \cdot OH + {}^{-}OH \qquad (5)$$

In fact, Fenton's reagent has been used for many years as a proficient hydroxylating reagent as ·OH is a very reactive species capable of reacting with virtually all organic molecules in aqueous media, at nearly diffusion controlled rates. Reaction types of ·OH can be hydrogen abstraction, addition, or electron transfer and its indiscriminate reactivity has led to proposals of its reaction with numerous biomolecules including phospholipids, proteins, and DNA (20).

Numerous in vitro studies have demonstrated that, as with autoxidation, chelation can markedly affect the reaction of metals with H_2O_2. Unchelated Fe^{2+} readily promotes ·OH formation while chelates which lack a free coordination site such as DTPA and desferrioxamine apparently prevent ·OH generation (21). On the other hand, chelation of Fe^{2+} by EDTA may increase the yield of ·OH by 1) producing additional H_2O_2 by rapid autoxidation or 2) maintaining the resulting Fe^{3+} in a soluble form (22). However, in the presence of adequate amounts of H_2O_2, EDTA may also decrease ·OH production by facilitating the autoxidation of Fe^{2+}, in which case Fe^{2+} availability becomes rate limiting.

Lipid Peroxidation

1. An Overview of Initiation, Propagation and Termination. A dramatic example of the concerted interaction of dioxygen and transition metals in promoting oxidative stress is the peroxidation of PUFA of membrane lipids. The lowered bond dissociation energy of the allylic hydrogens of their methylene carbons renders PUFA more susceptible to oxidative damage. The overall process of lipid peroxidation can be divided into three main phases: initiation, propagation, and termination (23). Initiation refers to the abstraction of an allylic hydrogen from the PUFA of the phospholipid (LH) by an oxidant, perhaps one of the partially reduced species of oxygen. This results in the production of a carbon centered lipid radical (L•):

$$LH \xrightarrow[-H\bullet]{} L\bullet \qquad (6)$$

Subsequently, L• undergoes rearrangement (diene conjugation) and reacts rapidly with molecular oxygen to produce a lipid peroxyl radical (LOO•):

$$L\bullet + O_2 \longrightarrow LOO\bullet \qquad (7)$$

The propagative phase of lipid peroxidation begins when LOO• reacts with another divinyl methane of the same molecule or of a neighboring PUFA, generating a lipid hydroperoxide (LOOH) and another L•:

$$LOO\bullet + LH \longrightarrow LOOH + L\bullet \qquad (8)$$

Propagation of lipid peroxidation is also promoted by transition metal ions which can cleave LOOH to reactive LOO· or alkoxy lipid radicals (LO·) which can also react with LH:

$$M^{n+} + LOOH \longrightarrow LO\cdot + OH^- + M^{(n+1)} \quad (9)$$

$$M^{(n+1)+} + LOOH \longrightarrow LOO\cdot + H^+ + M^{n+} \quad (10)$$

Certain hemoproteins may also promote the peroxidative process by cleaving LOOH (24). The generation of reactive intermediates during the propagative phase of lipid peroxidation, which themselves can abstract susceptible hydrogens, imparts an auto-catalytic nature to the process. It has been estimated that 8 to 14 propagation cycles occur for each free radical generated (25). Hydroperoxides are often formed during isolation and preparation of phospholipids and, in the presence of transition metals, these PUFA readily undergo LOOH-dependent peroxidation. It is likely that discrepancies among laboratories with regard to initiation of lipid peroxidation via hydrogen abstraction is due to contaminating LOOH.

Lipid peroxyl radicals are also known to undergo internal cyclization to form a PUFA endoperoxide shifting the unpaired electron to a carbon center. On to this, dioxygen again rapidly adds to form a PUFA endoperoxide peroxyl radical which can then propagate the peroxidative process in the same manner as LOO·. Additionally, this radical can undergo a series of cleavage reactions to yield a diverse array of products including aldehydes, ketones, alcohols, and ethers. One of the predominant products from PUFA containing at least 3 double bonds is MDA (26). Malondialdehyde forms a Schiff base product with

TBA (1 MDA: 2 TBA) which absorbs maximally at 532 nm (27). While not totally specific for MDA, this assay is the most widely used index of in vitro lipid peroxidation.

Termination reactions or defense mechanisms are either primary or secondary. Primary defense mechanisms are those that prevent the formation of initiators of lipid peroxidation. These can involve limiting the generation or accumulation of $O_2^{\cdot-}$ and H_2O_2 by 1) efficient electron transport systems, or 2) the action of SOD, catalase, and glutathione peroxidase. Alternatively, the binding of iron to proteins such as transferrin and ferritin, to minimize its potential reaction with dioxygen and its reduction products, may represent an important defense mechanism (28).

Secondary mechanisms for controlling the peroxidative process exert their effect on the propagative phase. One of the most important defense systems is vitamin E (α-tocopherol) which is strongly lipophilic and integrates intimately with membrane phospholipids. Its phenolic group donates hydrogen atoms to L· or LOO·, thereby interrupting the free radical cascade of lipid peroxidation (29). The resultant tocopherol semiquinone chromanoxyl radical is fairly stable and may be reduced by ascorbate (30) or GSH (31), regenerating α-tocopherol. Also considered a secondary defense is glutathione peroxidase, which by reducing LOOH to the less reactive, corresponding alcohol minimizes the probability of further propagation.

The importance of initiation in lipid peroxidation is highlighted by the tremendous amount of research effort directed towards identifying oxidants capable of mediating methylene hydrogen

abstraction. Excessive intracellular generation of $O_2^{\cdot-}$ and H_2O_2 by numerous toxic chemicals capable of undergoing cyclic reduction and autoxidation has been proposed to result in lipid peroxidation (32). However, it is known that neither $O_2^{\cdot-}$ nor H_2O_2, in aqueous media at physiologic pH, is of sufficient reactivity to initiate the peroxidative process (33). The undissociated perhydroxyl radical ($HO_2^{\cdot}$), with a pKa of 4.8, is capable of initiating peroxidation of hydroperoxide-free PUFA but only 0.25% of any $O_2^{\cdot-}$ will be present in this form at pH 7.4 (34). It may be important in the acidic, phagocytic vacuole or in the vicinity of the hydrophobic membrane matrix where the pH is appreciably lower.

The limited reactivity of $O_2^{\cdot-}$ and H_2O_2 suggests that lipid peroxidation requires the formation of another, more potent oxidant within cells. At present, there are at least two widely proposed mechanisms for the formation of an initiator of lipid peroxidation, both of which are highly dependent upon the redox chemistry of transition metal ions. The two most intensively researched metals have been iron and copper, which may be expected considering their biological abundance. Some of the first evidence for the critical involvement of iron in lipid peroxidation arose from the fortuitous discovery by Hochstein et al. (35) that MDA formation occurred in rat liver microsomes during NADPH oxidation. It was subsequently determined that this process was attributable to endogenous iron present in the ADP being used (36). Rates of microsomal lipid peroxidation were subsequently shown to be markedly affected by washing microsomes (37,38) or by the addition of iron chelators to the system (39). The source, or the nature of, this endogenous iron which is found associated with microsomes is yet to be determined, however.

2. Initiation by the Hydroxyl Radical. Initiation of lipid peroxidation by ·OH has received considerable support. Its generation is most often proposed to occur via the iron-catalyzed Haber-Weiss reaction (19,40):

$$O_2^{\cdot-} + Fe^{3+} \longrightarrow Fe^{2+} + O_2 \qquad (11)$$

$$O_2^{\cdot-} + O_2^{\cdot-} \xrightarrow{2H^+} H_2O_2 + O_2 \qquad (4)$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {\cdot}OH + {}^-OH \qquad (5)$$

Unfortunately, ·OH involvement is generally invoked as a result of the ability of SOD, catalase, and ·OH scavengers such as mannitol and benzoate to inhibit peroxidation. In this manner xanthine oxidase dependent peroxidation of arachidonic acid was proposed to be initiated by ·OH (41) as peroxidation was inhibited by SOD (inhibits reaction 11), catalase (inhibits reaction 5), and mannitol. Similarly, inhibition of microsomal NADPH-dependent lipid peroxidation by these agents was taken as evidence for ·OH-dependent initiation (42).

Fenton's reagent (reaction 5) was capable of initiating peroxidation when the iron was chelated with EDTA (22). Conversely, unchelated Fe^{2+} appeared to initiate peroxidation solely by cleavage of pre-existing LOOH (43) although other work (44) suggests that at lower, more physiologically relevant levels of iron, ·OH formation occurs readily with free Fe^{2+}. Continuing studies (45) have provided evidence that at a strict EDTA:Fe^{2+} ratio of one, ·OH formation is favored and accordingly, lipid peroxidation is observed. At EDTA:Fe^{2+} ratios greater than one, lipid peroxidation is inhibited while at

ratios of less than one, EDTA-Fe^{2+} functions to decompose LOOH, generating reactive LO· which are then capable of rapidly peroxidizing other PUFA. Similar results were obtained by Tien et al. (46) however, they suggested that the optimal rates of lipid peroxidation, noted at a ratio of less than one, were due to optimum ·OH generation resulting from a rapidly autoxidizing pool (EDTA-Fe^{2+}) and the slower oxidizing free Fe^{2+}.

The aforementioned studies are indicative of the many factors which can affect ·OH production, even in what appears to be a relatively simple system. They also demonstrate that several interpretations of similar data are possible. In spite of these shortcomings, the purported formation of ·OH in a variety of even more complex systems, subject to many more variables, is touted as indirect evidence for ·OH-dependent initiation of lipid peroxidation. McCord and Day (47) have demonstrated, using a xanthine oxidase system to generate $O_2^{-\cdot}$, that EDTA-Fe^{3+} effectively catalyzes ·OH formation which has since been confirmed by others (43,44,48). Hydroxyl radical formation with di- and triphosphate nucleotides, in the presence of Fe^{2+}, was demonstrated by Floyd (49,50) with ESR spin trapping. This was postulated to be a function of decreased autoxidation of chelated Fe^{2+}, thereby facilitating interaction with H_2O_2. Even more speculative is the report which suggests that H_2O_2-dependent ·OH formation, catalyzed by trace amounts of cellular iron, was responsible for killing of mouse 3T3 cells (51).

3. Alternatives to the Hydroxyl Radical in Initiation. While support for the involvement of ·OH in lipid peroxidation continues,

there is a growing body of evidence which suggests that other oxidants, particulary an iron-bound oxygen complex, may in many instances be the actual initiator. Some of the first evidence refuting ·OH involvement was provided by Pederson and Aust (52) who demonstrated that NADPH-dependent microsomal lipid peroxidation was not inhibited by SOD or catalase and similarly, Hochstein and Ernster (35) found catalase to be without effect. It is known that microsomes contain endogenous catalase and significant accumulation of the H_2O_2 required to promote the Haber-Weiss reaction is doubtful. The addition of exogenous catalase or azide, a catalase inhibitor, to microsomes also had no effect on the rate of lipid peroxidation (53). Recently, direct quantitation of $O_2^{-\cdot}$ generation by microsomes has shown it to account for only a minor percentage of their total reduction potential, suggesting that ·OH formation may not adequately account for the initiation of microsomal lipid peroxidation (54).

In lieu of the above findings it has been proposed that initiation may be mediated by an oxidant other than ·OH. In a reconstituted peroxidation system employing liposomes prepared from extracted microsomal lipid, and xanthine oxidase to generate $O_2^{-\cdot}$, nucleotide-chelated Fe^{3+} was capable of initiating peroxidation (55). Inhibition of peroxidation by SOD, but no effect of catalase or mannitol, suggests initiation to be via a chelated, reduced iron-oxygen complex such as the perferryl ion (or ferrous dioxygen, Fe^{2+}-O_2) originally proposed by Hochstein and Ernster (35).

The identification of NADPH-cytochrome P450 reductase as the microsomal enzyme catalyzing the transfer of reducing equivalents to Fe^{3+} chelates, necessary for lipid peroxidation, made it possible to

study NADPH-dependent lipid peroxidation in a liposomal system (56). In contrast to the xanthine oxidase system, the addition of ADP-Fe^{3+} resulted in no peroxidation. Activity was dependent upon the inclusion of EDTA-Fe^{3+} to this system. This dependence upon EDTA-Fe^{3+} is due to an inability of NADPH-cytochrome P450 reductase to generate significant amounts of $O_2^{\cdot-}$, or to directly reduce ADP-Fe^{3+} (54). However, the enzyme is capable of directly reducing EDTA-Fe^{3+} and electron transfer from EDTA-Fe^{2+} to ADP-Fe^{3+} results in the formation of an ADP-Fe^{2+}-O_2 complex capable of initiating peroxidation. Accordingly, SOD, catalase and mannitol had no effect on rates of MDA formation (52).

In an effort to further elucidate the nature of this postulated Fe^{2+}-oxygen complex, peroxidation initiated directly by Fe^{2+}-chelates was evaluated in hopes that elimination of a reducing agent or system would simplify the situation. As discussed, Fe^{2+} autoxidation can be quite complex and markedly affected by the nature of the chelating ligands, however, it is reasonable to assume that all autoxidations will produce some of the partially reduced forms of oxygen or their iron-bound equivalent forms. The ability of EDTA- or DTPA-chelated Fe^{2+} to generate $\cdot OH$ and to decompose peroxides has been reported. Accordingly, peroxidation initiated by these Fe^{2+}-chelates was sensitive to SOD or catalase (57,58). Ferrous chelates of possible physiological significance, such as phosphate, ADP, oxalate, and citrate, initiated peroxidation but were largely unaffected by SOD or catalase, suggesting formation of an initiator other than $\cdot OH$

Lipid peroxidation initiated by nucleotide-chelated Fe^{2+} consistently exhibited a significant, initial lag phase before MDA

formation was observed (59). The addition of chelated-Fe^{3+}, under conditions that minimized Fe^{2+} autoxidation, abolished the lag period in a concentration-dependent fashion. Recent work in our laboratory has demonstrated similar results with other Fe^{2+}-chelates, and that the rate and extent of lipid peroxidation is maximal at a Fe^{3+}:Fe^{2+} ratio of one (60). Of great interest was the finding that mannitol and benzoate, which are thought to inhibit lipid peroxidation by scavenging ·OH, significantly affected Fe^{2+} autoxidation and therefore affected lipid peroxidation by altering the Fe^{3+}:Fe^{2+} ratio. These data suggest that the initiator of lipid peroxidation may not be a Fe^{2+}-O_2 complex, but rather a Fe^{2+}-O_2-Fe^{3+} complex of undetermined structure or reactivity.

The inability of ·OH scavenging agents to inhibit peroxidation in systems employing nucleotide-chelated iron does not exclude ·OH formation in the system but suggests initiation to be via another species. Indeed, ·OH formation by nucleotide-Fe^{2+} chelates has been described. One of the arguments against ·OH as an initiator of lipid peroxidation capitalizes on its extreme reactivity. While clearly capable of hydrogen abstraction, it is likely to react at, or close to, its site of generation. For example, Tien et al. (22) were unable to demonstrate lipid peroxidation in a liposomal system in the presence of EDTA-Fe^{3+} and xanthine oxidase. However, when the phospholipid was dispersed with detergent or replaced with detergent-dispersed linoleate, peroxidation occurred (46). Catalase, mannitol and SOD significantly inhibited activity implying the involvement of ·OH. The inability of this system to initiate

The propensity of ·OH to initiate peroxidation only in highly artificial systems (e.g. synthetic iron chelators and detergent-dispersed lipid) leads one to question the role of ·OH *in vivo*. As an alternative to ·OH, several investigators have proposed the existence of a similar species known as crypto ·OH (61,62). Its generation involves the homolytic cleavage of H_2O_2 in which the radical species formed remains constricted within its region of generation; such as in an enzyme crevice. Others envision the ·OH to be "surrounded" by solvent forming a cage-like structure. These type of restrictions may impart a degree of selectivity towards potential substrates such as the allylic hydrogens of PUFA. Indirect evidence for damage by such complexes, or by a "site-directed" Fenton's reaction, where the metal is bound to a biological macromolecule and cleaves H_2O_2 in close proximity to susceptible moieties such as phospholipids, has been presented (63,64).

It is readily apparent from all of the above studies that iron is intimately involved in the initiation and propagation of lipid peroxidation. While the true identity of an initiator of lipid peroxidation remains elusive, a critical role of iron is implicit in virtually all proposals. Most *in vitro* studies employ low molecular weight iron chelates, particularly ADP and EDTA, and much of this work has demonstrated the dramatic effects which chelation can impart to the peroxidative process. This knowledge has led to an increasing awareness of the need to isolate and identify physiologic iron chelates, and to assess their potential for providing iron for participation in the formation of an initiator of lipid peroxidation.

Iron Metabolism

1. Absorption and its Regulation. Although iron metabolism has been well studied, the potential for known physiological iron sources to provide iron for participation in redox activities is not well known. Highly regulated cellular control of iron is necessitated not only by its potential toxicity, but also by the very limited solubility of free Fe^{3+} in aqueous solutions. These principles are illustrated by the ability of cells to rapidly synthesize ferritin in response to iron loading (65) and the elaborate siderophore-dependent, iron storing capacity of many microorganisms (66). Extensive studies examining iron metabolism indicate that the oxidation state of the iron significantly affects its incorporation or release from biological iron-containing molecules. Thus, the ability of organisms to reduce cellular iron suggests the potential for initiation of lipid peroxidation.

Mechanisms for the excretion of body iron are quite limited, therefore regulation of iron absorption is an important control point for maintaining acceptable iron levels. Accordingly, variations in iron absorption appear to reflect changes in total body iron stores. Means of controlling absorption are not fully understood but are thought to be related to levels of ferritin in the mucosa or to the total iron content of the mucosal cell (67).

Intestinal absorption of iron occurs predominately in the proximal portion of the small intestine, particularly the duodenum (68). Uptake of iron requires iron as Fe^{2+}, therefore reduction of dietary iron is necessary (69). Recent evidence indicates that

xanthine oxidase in brush border cells promotes the incorporation of iron into mucosal transferrin for transport to the portal blood stream (70). Others have suggested that iron enters mucosal cells when complexed with a low molecular weight membrane carrier (71-74). In most studies, however, it is accepted that a mucosal transferrin ultimately transports dietary iron to the serosal cell surface.

2. Transport of Iron. Iron circulates in the blood bound to transferrin, a single-chain glycoprotein capable of binding two Fe^{3+} (75). The uptake and release of iron from transferrin is not fully understood, although several hypothesis have been proposed. Iron binding by transferrin requires two bicarbonate anions which have been shown to bind to the metal but their structural role in assembling the metal site is unknown (76,77). The anion appears to bind weakly to the protein prior to the iron, perhaps resulting in a conformational change which facilitates iron binding. Interestingly, the binding constant of apotransferrin for Fe^{2+} is 17 times less that of Fe^{3+} (78), again emphasizing the importance of the oxidation state of the iron. Ceruloplasmin, which serves as the major transport protein of copper has significant ferroxidase activity and has been suggested to facilitate oxidation of Fe^{2+} for binding to transferrin (79).

Iron appears to enter cells with transferrin following receptor mediated endocytosis (86,87). The iron is subsequently released from transferrin in an acidic, endocytic vesicle with the apoprotein recycled to the cell surface. The mechanism of iron release from the ternary complex is unknown but the weak affinity of transferrin for Fe^{2+} suggests that reduction of the complexed iron would facilitate its release and most investigators also envision protonation of the

anion in the vesicle as a means of freeing bound iron. Studies with various reductants suggest that iron release is dependent upon not only the redox potential of the agent, but also upon its effect on protein conformation (88). It is speculated that at low pH, decreased stability of the Fe^{3+}-transferrin complex makes the metal more susceptible to reduction.

An alternative hypothesis proposes that cells contain low molecular weight iron chelates which can remove the iron from the protein and also function in its intracellular transport. It has been demonstrated that phosphate containing compounds such as pyrophosphate and ATP are effective in iron removal from transferrin (89) while in rabbit reticulocytes rates of iron uptake from transferrin directly correlate with ATP concentrations (90). Iron release from transferrin which is dependent solely on chelation occurs quite slowly but is enhanced significantly at lowered pH. Thus, irrespective of mechanism, iron release from transferrin is likely to occur within the acidic millieu of the non-lysosomal endocytic vesicle.

In spite of intensive study, mediators of intracellular iron transport have not been positively identified although most investigators suggest that a low molecular weight intermediate may function in this respect (91). For example, phosphate compounds involved in the removal of iron from transferrin may also shuttle iron throughout the cell cytosol. It is this elusive "pool" that is generally proposed to be the most likely candidate for participating in oxidative processes. While some evidence for its existence in reticulocytes has been presented (92,93) definitive identification in other cells is lacking although chelators such as ADP (42) and citrate (94) have been proposed.

3. Iron Storage in Ferritin: Uptake and Release. Ultimately, the vast majority of intracellular iron is stored in ferritin which is predominately a cytosolic protein although very recent work indicates an association with the endoplasmic reticulum. It is a large (Mr=450,000) protein comprised of 24 subunits arranged symmetrically about a hollow central core (95). Iron is stored in this core with an average compositon of $(FeOOH)_8 \cdot FeO \cdot OPO_3H_2$. Each ferritin molecule is capable of binding up to 4500 atoms of iron although ferritin is normally only 20% loaded with iron (96). Access to the core is via 6 narrow hydrophobic channels and 8 smaller, hydrophilic channels which average 0.9 nm to 1.2 nm in width (97).

The mechanisms of deposition and mobilization of iron from ferritin remain enigmatic but appear to involve oxidation and reduction, respectively (98). It is generally thought that deposition of iron occurs after the protein subunits are assembled, because apoferritin accelerates the rate of oxidation of Fe^{2+} (99). The electron acceptor in vivo is likely to be dioxygen although whether $O_2^{\cdot-}$, H_2O_2 or H_2O is the product remains to be resolved (100,101). Elucidation of mechanism(s) and identification of products is complicated by evidence that oxidation occurs at two sites; one on the protein and the other on the iron core, and that oxidation may occur via different mechanisms at each site (102).

While it is accepted that mobilization of iron from ferritin requires reduction, physiological reductant(s) remain unknown. The "crystal growth theory" (103) proposes that reductants traverse the length of the channels to directly reduce the ferric iron core while others (104) envision electron transfer along the channels, initiated

at oxido-reduction sites on the interior of the channels. Reduced flavins, which are among the most effective mobilizers of iron from ferritin, appear to at least partially enter the channels (105). Therefore, size constraints may limit the number of potential biological reductants.

Physiological reductants such as GSH, ascorbate and cysteine release iron from ferritin at rates too slow to be considered of significance (106) while non-physiological reducing agents such as dithionite and thioglycolate readily release ferritin iron in vitro (107,108). Certain chelating agents have been shown to facilitate iron release from ferritin, but only in the presence of a reductant (109). Enzymatic means for releasing iron from ferritin have been suggested. Evidence has been presented for the existence of a NADH-FMN oxido-reductase which provides reduced flavins to mediate iron release but it is highly unstable and is not well characterized (110,111). Several studies have also indicated that hepatic xanthine oxidase, in its NAD-requiring dehydrogenase form, may play a role in ferritin iron release (112,113). Another factor to be considered is the redox potential of the reductant as recent work reports the reduction potential for iron in ferritin to be approximately -230 mV at pH 7.0 (114). Accordingly, the efficacy of a series of reduced flavins to mediate iron release from ferritin closely paralleled their respective redox potentials (105).

4. Biological Iron and Lipid Peroxidation. The similarities in the conditions under which the release of iron from ferritin and lipid peroxidation are favored, i.e. the presence of reducing agent(s) and chelator(s) is rather striking. Surprisingly, however, relatively few

studies have investigated the ability of ferritin to supply iron for the promotion of lipid peroxidation. This is likely a result of the general concensus that ferritin provides a secure means of storing iron in an inert form. However, it is becoming increasingly evident that ferritin may play a more dynamic role *in vivo*. For example, mitochondria contain distinct binding sites for ferritin from which iron is mobilized (115,116). Gutteridge (117) reported that ferritin promoted *in vitro* ascorbate-dependent lipid peroxidation while others demonstrated ·OH formation using ferritin and ascorbate (118). Similarly, Wills (119) demonstrated that ferritin may promote non-enzymatic peroxidation, however, it was subsequently suggested (38) that ferritin was not responsible for the increase in lipid peroxidation observed in liver microsomes isolated from iron-loaded rats. Thus, in lieu of the rather cursory examination of the ability of ferritin to promote lipid peroxidation, and the large amount of intracellular iron stored within this protein, a more thorough evaluation of this topic is warranted.

ESR spin trapping has demonstrated the ability of transferrin to inhibit (80,81) or catalyze (82,83) ·OH formation in the presence of a $O_2^{\cdot-}$ generating system. This is related to the degree of iron saturation as only fully loaded transferrin stimulates ·OH production and promotes lipid peroxidation. Partially loaded transferrin inhibits both ·OH generation and lipid peroxidation (84,85). Transferrin is normally only 30% saturated on the average, therefore it may serve in a protective capacity.

In addition to ferritin and transferrin, much of the total body iron is found in hemoglobin and myoglobin. While it is generally

concluded that these proteins would be unlikely to donate iron to participate in oxygen radical formation, several recent studies have addressed this topic. Hemoglobin can promote $O_2^{\cdot-}$-dependent, $\cdot OH$ formation and the peroxidation of red cell membranes (120). Other studies have shown that methemoglobin and metmyoglobin can form complexes with H_2O_2 which are apparently capable of initiating lipid peroxidation (121)

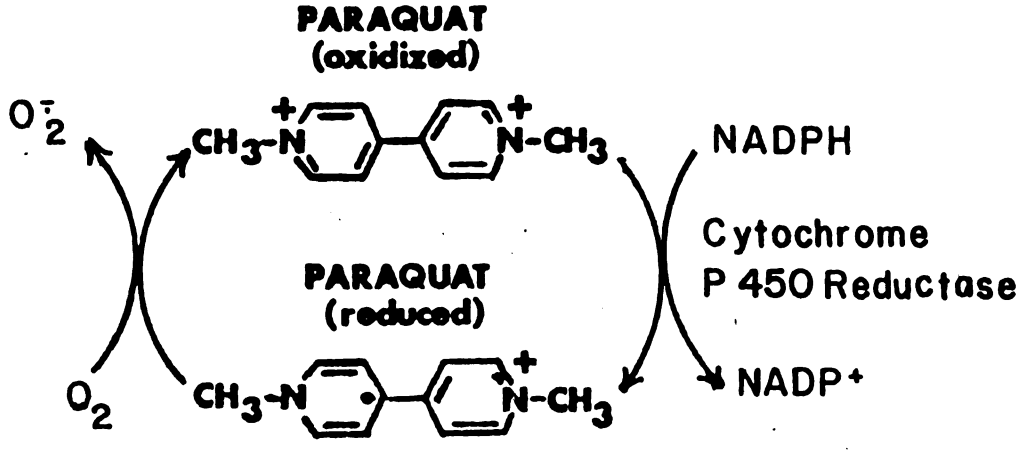
Traces of iron are also present in extracellular fluids although the nature of these complexes is unknown and their ability to catalyze oxygen radical formation is controversial. Winterbourn (122) was unable to detect $\cdot OH$ formation from plasma, lymph or synovial fluid upon exposure to H_2O_2. In contrast, Gutteridge et al. (123) demonstrated the ability of synovial fluid to catalyze $\cdot OH$ formation and promote lipid peroxidation. The physiological significance of these findings is unknown, however, it has been shown that in certain disease states, such as rheumatoid arthritis, the amounts of these iron salts are elevated (124,125). Correspondingly, a correlation between iron levels and the formation of TBA-reactive material has been demonstrated in the synovial fluid of these patients (126).

Iron in Proposed Oxygen Radical-Dependent Toxicities.

1. Paraquat. The toxicities associated with numerous drugs and chemicals is often attributed to their ability to be metabolized to free radical species, resulting ultimately in oxidative damage to critical cellular constituents (32). For many structurally diverse chemicals their metabolism is a virtue of their ability to serve as alternate electron acceptors for the microsomal enzyme,

NADPH-cytochrome P450 reductase. The addition of a single electron to these compounds produces an unstable, free radical intermediate that, in most instances, reacts rapidly with dioxygen to produce $O_2^{\cdot-}$ and regenerate the parent compound, a process referred to as redox cycling (32). As discussed, it is unlikely that the simple generation of $O_2^{\cdot-}$ and H_2O_2 will initiate oxidative damage to membrane PUFA, proteins or DNA. Thus, the toxicity of these chemicals is likely to require, or be greatly potentiated by, the presence of transition metal ions.

Paraquat (methyl viologen, 1-1-dimethyl-4,4'-bipyridylium dichloride) is a widely used herbicide which is highly toxic to mammals. Its herbicidal properties are attributed to the reduction within plant chloroplasts of its bipyridyl, dication structure to a mono-cation radical that readily autoxidizes to produce $O_2^{\cdot-}$. Its mammalian toxicity is presumably a result of reduction by NADPH-cytochrome P450 reductase and subsequent redox cycling to generate $O_2^{\cdot-}$ (127):



Numerous investigations have attempted to correlate paraquat toxicity with enhanced lipid peroxidation. While results are varied it does appear that, in general, iron is involved in the toxicity of paraquat. Many of the discrepancies between laboratories appears to concern the type and amount of iron present in the various *in vitro* systems. Trush *et al.* (128) demonstrated that paraquat stimulated rat lung microsomal peroxidation in the absence of added exogenous iron. However, peroxidation was completely inhibited by EDTA, thus trace amounts of endogenous iron probably were responsible for promoting the peroxidative activity. In a reconstituted liposomal peroxidation system utilizing isolated NADPH-cytochrome P450 reductase, paraquat also stimulated peroxidation when supplied with ADP-Fe^{3+} (127).

Others studies have not supported lipid peroxidation as a mechanism of toxicity for paraquat. One investigation reported no effect of paraquat on iron-catalyzed microsomal lipid peroxidation (129). Conversely, it has been suggested that paraquat inhibits lung microsomal lipid peroxidation dependent upon endogenous iron by interrupting microsomal electron transport which normally produces a reduced iron-oxygen intermediate capable of initiating peroxidation (130). These conflicts have not been totally resolved but may reflect NADPH depletion by continuous redox cycling of paraquat in the studies which report an inhibition of peroxidation. In fact, reduction of NADPH levels *in vivo* has also been suggested as a mechanism of toxicity for paraquat (131).

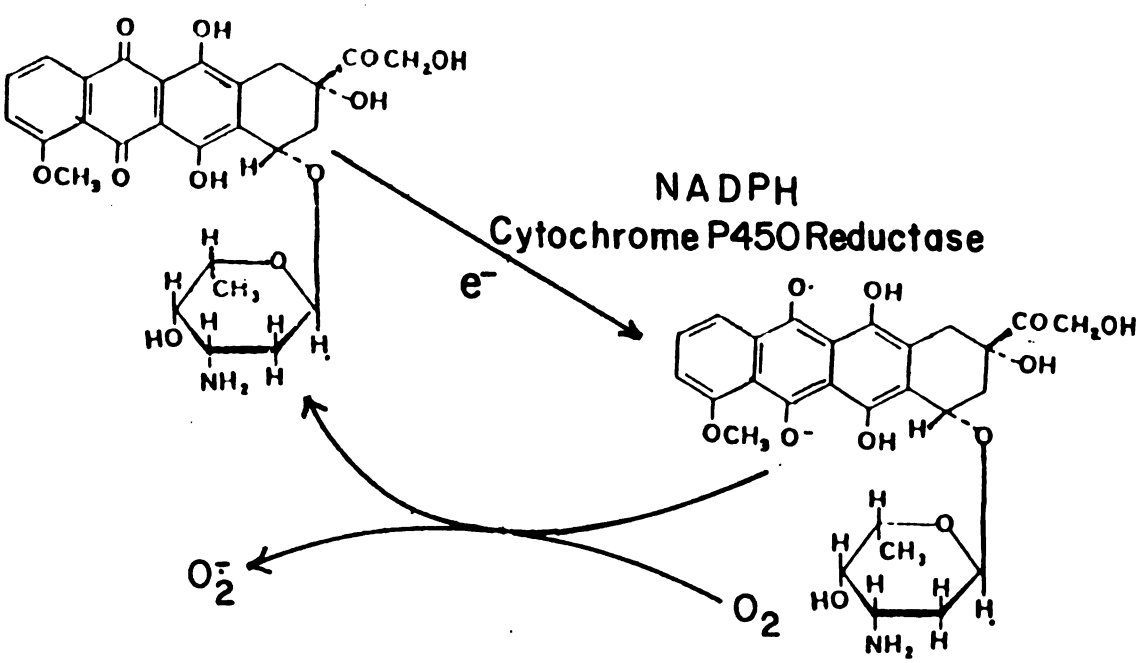
Recently, several *in vivo* studies have provided evidence that paraquat toxicity is mediated, in part, by redox active metals. In paraquat-treated mice, the co-administration of Fe_2SO_4 greatly

potentiated toxicity (132). On the other hand, treatment of the animals with the iron chelator desferrioxamine partially ameliorated toxicity (132). With E. Coli, cell inactivation by paraquat was significantly increased by small amounts of copper ions which enhanced degradation of the cytoplasmic membrane (133).

If it is assumed that paraquat toxicity does occur via iron catalyzed lipid peroxidation, the topic still to be addressed concerns the nature of the initiating species. It has been demonstrated with paraquat that, in vitro, trace amounts of EDTA- and DTPA-Fe^{3+} catalyzed ·OH formation of which 97% was free to diffuse into solution (134). Kohen and Chevion have proposed "site-specific" ·OH generation to rationalize paraquat-dependent E. Coli cell killing (133). Treatment of paraquat intoxicated animals with ·OH scavengers such as dimethylthiourea has generally not proven beneficial (135). Very recent work with Chinese hamster ovary cells reported that SOD provided protection against paraquat damage but catalase was without effect (136). Many other studies have provided very conflicting evidence on the ability of SOD to prevent or lessen paraquat-induced toxicity (137,138).

It can be seen that the only concensus that can be derived from the literature is that paraquat toxicity, if it indeed does involve lipid peroxidation, must require a source of iron. Thus, if lipid peroxidation is to remain a viable alternative to account for paraquat-dependent toxicity, it is imperative to identify physiological sources of iron which may be released or made available by paraquat, allowing the formation of an initiator of lipid peroxidation.

2. Adriamycin. There are a number of other toxicities in which iron is thought to play a vital role. One of the most important, and intensively studied, is the toxicity of adriamycin. This anthracycline antibiotic is widely used as a chemotherapeutic agent. However, its clinical use is limited by a dose-dependent cardiomyopathy leading to congestive heart failure. There are several proposed mechanisms for this selective cardiomyopathy, of which lipid peroxidation appears to be currently favored. The quinone moiety of adriamycin can be reduced by one electron to a semiquinone free radical by several flavoproteins including NADPH-cytochrome P450 reductase (139):



Subsequent redox cycling to generate $O_2^{\cdot-}$ and H_2O_2 is thought to lead to oxidative stress and eventually, cell death. The heart appears to be particularly susceptible to oxidative damage due to low levels of SOD and catalase relative to other oxygenated tissues (140).

Microsomal lipid peroxidation induced by adriamycin can be inhibited by SOD, catalase, ·OH scavengers, and iron chelators, indicative of initiation via the iron-catalyzed Haber-Weiss reaction (141). Recently it was shown that the concentration of ferrous ions markedly affects adriamycin-induced peroxidation (142) while others have suggested that in the absence of iron, adriamycin does not potentiate microsomal lipid peroxidation (143). Evidence for similar processes occurring in vivo was provided by Mimnaugh et al. (144) who demonstrated that in vivo administration of adriamycin resulted in biochemical changes which were manifested as an 18-fold increase in NADPH-dependent cardiac microsomal lipid peroxidation. Also implicating iron in anthracycline toxicity was the demonstration that co-administration of an EDTA derivative provided some protection against adriamycin-induced cardiac damage (145).

While a requirement for iron in adriamycin toxicity is generally accepted, again the mechanism(s) by which an initiating species is formed remains controversial. Elegant work by Nakano and co-workers (146) has demonstrated that while adriamycin may potentiate lipid peroxidation, the process is quite complex and could involve an oxidant other than ·OH. An absolute requirement of iron for adriamycin-stimulated peroxidation was originally demonstrated by these authors (146) who subsequently reported that adriamycin formed a complex with ferric iron and ADP (147). This complex was capable of undergoing "self-reduction" with the reduced complex capable of initiating peroxidation (148) in a ·OH-independent fashion. The necessity for reduction of the iron in this complex is demonstrated by the ability of the ferroxidase activity of ceruloplasmin to inhibit

complex-initiated peroxidation (149). Adriamycin is also capable of mediating oxidative damage to DNA. No concensus has been reached with regard to the nature of the damaging species with evidence presented for both ·OH (150) and an iron-oxygen complex (151).

In spite of these intensive research efforts which implicate iron as a causative factor in adriamycin-induced cardiomyopathy, the development of effective preventative or therapeutic regimens, or new drugs, is limited by a lack of knowledge concerning the *in vivo* source of iron which is accessible to adriamycin. Recent work by Demant (152) has shown that the chelative properties of adriamycin allow it to slowly mobilize iron from ferritin. Similarly, iron released from transferrin at acidic pH could be chelated by adriamycin (153). However, in both instances rates of iron accumulation by adriamycin were very slow. These mechanisms also preclude reduction of the anthracycline to its semiquinone free radical, which is regarded as a prerequisite for its toxicity. Thus, a definitive correlation or interaction between the redox cycling of adriamycin and biological iron stores has yet to be established.

LIST OF REFERENCES

LIST OF REFERENCES

1. J.S. Olson, D.P. Ballou, G. Palmer and V. Massey. The mechanism of action of xanthine oxidase. J. Biol. Chem. 249: 4363-4382 (1974).

2. R. Hille and V. Massey. The presence of a reducible disulfide bond in milk xanthine oxidase. J. Biol. Chem. 257: 8898-8901 (1982).

3. D.R. Light, C. Walsh, M.O. O'Callaghan, E.J. Goetzl, and A.I. Tucker. Characteristics of the cofactor requirements for the superoxide-generating NADPH oxidase of human polymorphonuclear leukocytes. Biochem. 20: 1468-1476 (1981).

4. A. Boveris, E. Cadenas, and A.O.M. Stoppani. Role of ubiquinone in the mitochondrial generation of hydrogen peroxide. Biochem. 156: 435-444 (1976).

5. E. Cadenas, A. Boveris, I. Ragan and A.O.M. Stoppani. Production of superoxide radicals and hydrogen peroxide by NADH-ubiquinone reductase and ubiquinol-cytochrome c reductase from beef heart mitochondria. Arch. Biochem. Biophys. 180: 248-257 (1977).

6. H. Kuthan, H. Tsuji, H. Graf, V. Ullrich, J. Werringloer and R.W. Estabrook. Generation of superoxide as a source of hydrogen peroxide in a reconstituted monooxygenase system. FEBS Lett. 91: 343-345 (1978).

7. J. Werringloer. The formation of hydrogen peroxide during hepatic microsomal electron transport reactions. In: Microsomes and Drug Oxidations (V. Ullrich, I. Roots, A.G. Hildebrandt, R.W. Estabrook, and A.H. Conney, eds.), pp. 261-268, Pergamon Press, Oxford (1977).

8. R.G. Thurman, H.G. Ley and R. Scholz. Hepatic microsomal ethanol oxidations: Hydrogen peroxide formation and the role of catalase. Eur. J. Biochem. 25: 420-430 (1972).

9. H.P. Misra and I. Fridovich. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J. Biol. Chem. 247: 3170-3180 (1972).

10. V. Massey, G. Palmer and D. Ballou. On the reaction of reduced flavins and flavoproteins with molecular oxygen. In: Flavins and Flavoproteins (H. Kamin, ed.), pp. 349-361, University Park Press, Baltimore, MD (1971).

11. G. Just. Kinetische untersuchung der autoxydation des in wasser gelosten ferrobicardonats. Che. Ber. 40: 3695-3701 (1907).

12. A.M. Posner. The kinetics of autoxidation of ferrous ions in concentrated HCl solutions. Trans. Faraday Soc. 49: 382-388 (1953).

13. A.B. Lamb and L.W. Elder. The electromotive activation of oxygen. J. Am. Chem. Soc. 53: 137-163 (1931).

14. M. Cher and N. Davidson. The kinetics of the oxygenation of ferrous iron in phosphoric acid solution. J. Am. Chem. Soc. 77: 793-798 (1954).

15. I. Fridovich. Superoxide radical: An endogenous toxicant. Ann. Rev. Pharmacol. Toxicol. 23: 239-257 (1983).

16. A. Boveris, N. Oshino and B. Chance. The cellular production of hydrogen peroxide. Biochem. J. 128: 617-630 (1972).

17. J.K. Reddy, J.R. Warren, M.K. Reddy and N.D. Lalwani. Hepatic and renal effects of peroxisome proliferators: Biological implications. Ann. N. Y. Acad. Sci. 386: 81-110 (1982).

18. R.A. Condell and A.L. Tappel. Evidence for the suitability of glutathione peroxidase as a protective enzyme: Studies of oxidative damage, renaturation, and proteolysis. Arch. Biochem. Biophys. 223: 407-416 (1983).

19. F. Haber and J.J. Weiss. The catalytic decomposition of hydrogen peroxide by iron salts. Proc. Roy. Soc. London Ser. A. 147: 332-351 (1934).

20. R.L. Willson. Free radicals and tissue damage: Mechanistic evidence from radiation studies. In: Biochemical Mechanisms of Liver Injury (T.F. Slater, ed.), pp. 123-224, Academic Press, New York (1978).

21. E. Graf, J.R. Mahoney, R.G. Bryant and J.W. Eaton. Iron-catalyzed hydroxyl radical formation: Stringent requirement for free iron coordination site. J. Biol. Chem. 259: 3620-3624 (1984).

22. M. Tien, B.A. Svingen and S.D. Aust. An investigation into the role of hydroxyl radical in xanthine oxidase-dependent lipid peroxidation. Arch. Biochem. Biophys. 216: 142-151 (1982).

23. J.F. Mead. Free radical mechanisms of lipid damage and consequences for cellular membranes. In: Free Radicals in Biology, Volume I (W.A. Pryor, ed.), pp. 51-68, Academic Press, New York (1976).

24. P.J. O'Brien. Intracellular mechanisms for the decomposition of a lipid peroxide. I. Decomposition of a lipid peroxide by metal ions, heme compounds, and nucleophiles. Canadian J. Biochemistry 47: 485-492 (1969).

25. G.S. Wu, R.A. Stein and J.F. Mead. Autoxidation of fatty acid mono-layers absorbed on silica gel: II. Rates and products. Lipids 12: 971-978 (1978).

26. N.A. Porter, J. Nixon and R. Isaac. Cyclic peroxides and the thiobarbituric assay. Biochim. Biophys. Acta 441: 506-512 (1976).

27. J.A. Buege and S.D. Aust. Microsomal lipid peroxidation. In: Methods in Enzymology, Volume 52 (S. Fleischer and L. Packer, eds.), pp. 302-310, Academic Press, New York (1978).

28. R.R. Crichton. Interactions between iron metabolism and oxygen activation. In: Oxygen Free Radicals and Tissue Damage, pp. 57-76, Excerpta Medica, Amsterdam (1979).

29. A.L. Tappel. Vitamin E and free radical peroxidation of lipids. Annal. New York Acad. Sci. 203: 12-28 (1972).

30. E. Niki, T. Saito, A. Kawakami and Y. Kamiya. Inhibition of oxidation of methyl linoleate in solution by vitamin E and vitamin C. J. Biol. Chem. 259: 4177-4182 (1984).

31. C.C. Reddy, R.W. Scholz, C.E. Thomas and E.J. Massaro. Vitamin E dependent reduced glutathione inhibition of rat liver microsomal lipid peroxidation. Life Sci. 31: 571-576 (1982).

32. H. Kappus and H. Sies. Toxic drug effects associated with oxygen metabolism: Redox cycling and lipid peroxidation. Experentia 37: 1233-1241 (1981).

33. J.A. Fee and J.S. Valentine. Chemical and physical properties of superoxide. In: Superoxide and Superoxide Dismutases (A.M. Michelson, J.M. McCord and I. Fridovich, eds.), pp. 19-60, Academic Press, New York (1977).

34. B.H.J. Bielski, R.L. Arudi and M.W. Sutherland. A study of the reactivity of HO_2/O_2^- with unsaturated fatty acids. J. Biol. Chem. 258: 4759-4761 (1983).

35. P. Hochstein and L. Ernster. ADP-activated lipid peroxidation coupled to the TPNH oxidase system of microsomes. Biochem. Biophys. Res. Comm. 12: 388-394 (1963).

36. P. Hochstein, K. Nordenbrand and L. Ernster. Evidence for the involvement of iron in the ADP-activated peroxidation of lipids in microsomes and membranes. Biochem. Biophys. Res. Comm. 14: 323-328 (1964).

37. J.L. Poyer and P.B. McCay. Reduced triphosphopyridine nucleotide oxidase-catalyzed alterations of membrane phospholipids. J. Biol. Chem. 246: 263-269 (1971).

38. E.D. Wills. Lipid peroxide formation in microsomes. The role of non-haem iron. Biochem. J. 113: 325-332 (1969).

39. E.D. Wills. Mechanisms of lipid peroxide formation in tissues. Role of metals and hematin proteins in the catalysis of the oxidation of unsaturated fatty acids. Biochem. Biophys. Acta 98: 238-251 (1965).

40. J.A. Fee. On the question of superoxide toxicity and the biological function of superoxide dismutases. In: Oxidases and Related Redox Systems (T.E. King, H.S. Mason and M. Morrison, eds.), pp. 101-149, Pergamon Press, New York (1982).

41. S.E. Fridovich and N.A. Porter. Oxidation of arachidonic acid in micelles by superoxide and hydrogen peroxide. J. Biol. Chem. 256: 260-265 (1981).

42. K.L. Fong, P.B. McCay, J.L. Poyer, H.P. Misra, and B.B. Keele. Evidence for superoxide-dependent reduction of Fe^{3+} and its role in enzyme-generated hydroxyl radical formation. Chem. Biol. Inter. 15: 77-89 (1976).

43. J.M.C. Gutteridge. Lipid peroxidation initiated by superoxide-dependent hydroxyl radicals using complexed iron and hydrogen peroxide. FEBS Lett. 172: 245-249 (1984).

44. W. Flitter, D.A. Rowley and B. Halliwell. Superoxide-dependent formation of hydroxyl radicals in the presence of iron salts. What is the physiological iron chelator? FEBS Lett. 158: 310-312 (1983).

45. J.M.C. Gutteridge. Ferrous ion-EDTA-stimulated phospholipid peroxidation. A reaction changing from alkoxyl-radical to hydroxyl-radical-dependent initiation. Biochem. J. 224: 697-701 (1984).

46. M. Tien, L.A. Morehouse, J.R. Bucher and S.D. Aust. The multiple effects of ethylenediaminetetracetate in several model lipid peroxidation systems. Arch. Biochem. Biophys. 218: 450-458 (1983).

47. J.M. McCord and E.D. Day, Jr. Superoxide-dependent production of hydroxyl radical catalyzed by the iron-EDTA-complex. FEBS Lett. 86: 139-142 (1978).

48. B. Halliwell. Superoxide-dependent formation of hydroxyl radicals in the presence of iron chelates: Is it a mechanism

for ·OH radical in biological systems? FEBS Lett. 92: 321-326 (1978).

49. R.A. Floyd. Direct demonstration that ferrous ion complexes of di- and triphosphate nucleotides catalyze hydroxyl free radical formation from hydrogen peroxide. Arch. Biochem. Biophys. 225: 263-270 (1983).

50. R.A. Floyd and C.A. Lewis. Hydroxyl free radical formation from hydrogen peroxide by ferrous iron-nucleotide complexes. Biochem. 22: 2645-2649 (1983).

51. A.C.M. Filho, M.E. Hoffman and R. Meneghini. Cell killing and DNA damage by hydrogen peroxide are mediated by intracellular iron. Biochem. J. 218: 273-275 (1984).

52. T.C. Pederson and S.D. Aust. The mechanism of liver microsomal lipid peroxidation. Biochim. Biophys. Acta 385: 232-241 (1975).

53. L.A. Morehouse, M. Tien, J.R. Bucher and S.D. Aust. Effect of hydrogen peroxide on the initiation of microsomal lipid peroxidation. Biochem. Pharmacol. 32: 123-127 (1983).

54. L.A. Morehouse, C.E. Thomas and S.D. Aust. Superoxide generation by NADPH-cytochrome P450 reductase: The effect of iron chelators and the role of superoxide in microsomal lipid peroxidation. Arch. Biochem. Biophys. 232: 366-377 (1984).

55. J.R. Bucher, M. Tien, L.A. Morehouse and S.D. Aust. Three mechanisms for the formation of an initiator of lipid peroxidation by xanthine oxidase. In: Oxy Radicals and Their Scavenger Systems, Volume I: Molecular Aspects (G. Cohen and R.A. Greenwald, eds.), pp. 296-299, Elsevier Science Publishing Co., New York (1983).

56. T.C. Pederson, J.A. Buege and S.D. Aust. Microsomal electron transport. The role of reduced nicotinamide adenine dinucleotide phosphate-cytochrome c reductase in liver microsomal lipid peroxidation. J. Biol. Chem. 248: 7134-7141 (1973).

57. J.R. Bucher, M. Tien, L.A. Morehouse and S.D. Aust. Redox cycling and lipid peroxidation: The central role of iron chelates. Fund. Appl. Toxicol. 3: 222-226 (1983).

58. J.R. Bucher, M. Tien, L.A. Morehouse and S.D. Aust. Influence of superoxide dismutase and catalase on strong oxidant formation drug autoxidation of ferrous chelates. In: Oxy Radicals and their Scavenger Systems. Volume I: Molecular Aspects (G. Cohen and R.A. Greenwald, eds.), pp. 292-295, Elsevier Science Publishing Co., New York (1983).

59. J.R. Bucher, M. Tien and S.D. Aust. The requirement for ferric in the initiation of lipid peroxidation by chelated ferrous iron. Biochem. Biophys. Res. Comm. 111: 777-784 (1983).

60. G. Minotti and S.D. Aust. The requirement for ferric in the initiation of lipid peroxidation by ferrous and hydrogen peroxide (Submitted).

61. R.J. Youngman. Oxygen activation: is the hydroxyl radical always biologically relevant? Trends in Biochem. Sci. 9: 280-283 (1984).

62. R.J. Youngman and E.F. Elstner. Oxygen species in paraquat toxicity: The crypto-OH radical. FEBS Lett. 129: 265-268 (1981).

63. A. Samuni, M. Chevion and G. Czapski. Unusual copper-induced sensitization of the biological damages due to superoxide radicals. J. Biol. Chem. 256: 12632-12635 (1981).

64. E.K. Hodgson and I. Fridovich. The interaction of bovine erythrocyte superoxide dismutase with hydrogen peroxide: Inactivation of the enzyme. Biochem. 14: 5294-5299 (1975).

65. G.E. Shull and E.C. Theil. Regulation of ferritin mRNA: A possible gene-sparing phenomenon. J. Biol. Chem. 258: 7921-7923 (1983).

66. J.B. Nielands. Microbial iron compounds. In: Annual Review of Biochemistry, Volume 50 (E.E. Snell, P.D. Boyer, A. Meister and C.C. Richardson, eds.), pp. 715-731, Annual Reviews Inc., Palo Alto, CA (1981).

67. R.G. Sheehan and E.P. Frenkel. The control of iron absorption by the gastrointestinal mucosal cell. J. Clin. Invest. 51: 224-231 (1972).

68. W.H. Crosby. The control of iron balance by the intestinal mucosa. Blood 22: 441-446 (1963).

69. W.A. Muir, U. Hopfer and M. King. Iron transport across brush-border membranes from normal and iron-deficient mouse upper small intestine. J. Biol. Chem. 259: 4896-4903 (1984).

70. R.W. Topham, M.C. Walker and M.P. Calisch. Liver xanthine dehydrogenase and iron mobilization. Biochem. Biophys. Res. Comm. 109: 1240-1246 (1982).

71. M.N. Pearson and M.B Reich. Studies of ferritin and a new iron-binding protein found in the intestinal mucosa of the rat. J. Nutr. 99: 137-140 (1969).

72. M. Boulard, M. Delin and Y.C. Najean. Identification and purification of a new non-heme, non-ferritin iron protein. Proc. Soc. Exptl. Biol. Med. 139: 1379-1384 (1972).

73. M.C. Linder and H.N. Munro. The mechanism of iron absorption and its regulation. Fed. Proc. 36: 2017-2023 (1977).

74. J.W. Halliday, L.W. Powell and V. Mack. Iron absorption in the rat: The search for possible intestinal mucosol carriers. Br. J. Haematol. 34: 237-250 (1976).

75. P. Aisen. Chemistry and physiology of the transferrins. In: The Biological Chemistry of Iron (H.B. Dunford, D. Dolphin, K.N. Raymond and L. Sieter, eds.), pp. 63-83, D. Reidel Publishing Co., Dordrecht, The Netherlands (1982).

76. G.W. Bates and M.R. Schlabach. The nonspecific binding of Fe^{3+} to transferrin in the absence of synergistic anions. J. Biol. Chem. 250: 2177-2181 (1975).

77. M.R. Schlabach and G.W. Bates. The synergistic binding of anions and Fe^{3+} by transferrin. J. Biol. Chem. 250: 2182-2188 (1975).

78. N. Kojima and G.W. Bates. The formation of Fe^{3+}-transferrin-CO_3^{2-} via the binding and oxidation of Fe^{2+}. J. Biol. Chem. 256: 12034-12039 (1981).

79. S. Osaki, D.A. Johnson and E. Frieden. The possible significance of the ferrous oxidase activity of ceruloplasmin in normal human serum. J. Biol. Chem. 241: 2746-2751 (1966).

80. D.A. Baldwin, E.R. Jenny and P. Aisen. The effect of human serum transferrin and milk lactoferrin on hydroxyl radical formation from superoxide and hydrogen peroxide. J. Biol. Chem. 259: 13391-13394 (1984).

81. J.M.C. Gutteridge, S.K. Paterson, A.W. Segal and B. Halliwell. Inhibition of lipid peroxidation by the iron-binding protein lactoferrin. Biochem. J. 199: 259-261 (1981).

82. J.V. Bannister, P. Bellavite, A. Davioli, P.J. Thornally and F. Rossi. The generation of hydroxyl radicals following superoxide production by neutrophil NADPH oxidase. FEBS Lett. 150: 300-302 (1982).

83. N. Motohashi and J. Mori. Superoxide-dependent formation of hydroxyl radical catalyzed by transferrin. FEBS Lett. 157: 197-199 (1983).

84. J.M.C. Gutteridge. Antioxidant properties of the proteins caeruloplasmin, albumin and transferrin. A study of their activity in serum and synovial fluid from patients with

rheumatoid arthritis. Biochim. Biophys. Acta. 869: 119-127 (1986).

85. J.M.C. Gutteridge, S.K. Paterson, A.W. Segal and B. Halliwell. Inhibition of lipid peroxidation by the iron-binding protein lactoferrin. Biochem. J. 199: 259-261 (1981).

86. S. Young and A. Bomford. Transferrin and cellular iron exchange. Clin. Sci. 67: 273-278 (1984).

87. R.D. Klausner, J.V. Renswonde, G. Ashwell, C. Kempf, A.N. Schechter, A. Dean and R.R. Bridges. Receptor-mediated endocytosis of transferrin in K562 cells. J. Biol. Chem. 258: 4715-4724 (1983).

88. N. Kojima and G.W. Bates. The reduction and release of iron from Fe^{3+}-transferrin-CO^{2-}_3. J. Biol. Chem. 254: 8847-8854 (1979).

89. E.H. Morgan. Studies on the mechanism of iron release from transferrin. Biochim. Biophys. Acta 580: 312-326 (1979).

90. S.G. Kailis and E.H. Morgan. Iron uptake by immature erythroid cells. Mechanism of dependence on metabolic energy. Biochim. Biophys. Acta 464: 389-398 (1977).

91. A. Jacobs. Low molecular weight intracellular iron transport compounds. Blood 50: 433-439 (1977).

92. G.R. Bartlett. Phosphate compounds in rat erythrocytes and reticulocytes. Biochem. Biophys. Res. Comm. 70: 1055-1062 (1976).

93. S. Pollack and T. Campana. Low molecular weight non-heme iron and a highly labeled heme pool in the reticulocyte. Blood 56: 564-566 (1980).

94. C.G.D. Morley and A. Bezkorovainy. Identification of the iron chelate in hepatocyte cytosol. IRCS Med. Sci. 11: 1106-1107 (1983).

95. P.M. Harrison. Ferritin: An iron-storage molecule. Sem. Hematol. 14: 55-70 (1977).

96. P. Aisen and I. Listowsky. Iron transport and storage proteins. In: Annual Review of Biochemistry, Volume 49 (E.E. Snell, P.D. Boyer, A. Meister and C.C. Richardson, eds.), pp. 357-393, Annual Reviews, Inc., Palo Alto, CA (1980).

97. D.W. Rice, G.C. Ford, J.L. White, J.M.A. Smith and P.M. Harrison. The spatial structure of horse spleen apoferritin. In: Iron Binding Proteins Without Cofactors or Sulfur

Clusters (E.C. Theil, G.L. Eichhorn and L.G. Marzilli, eds.), pp. 39-50, Elsevier, New York (1983).

98. E.C. Theil. Ferritin: Structure, function, and regulation. In: Iron Binding Proteins Without Cofactors or Sulfur Clusters (E.C. Theil, G.L. Eichhorn and L.G. Marzilli, eds.), pp. 1-38, Elsevier, New York (1983).

99. M. Wauters, M. Michelson and R.R. Crichton. Studies on the mechanism of ferritin formation. FEBS Lett. 91: 276-280 (1978).

100. G. Melino, S. Stefanini, E. Chiancone and E. Antonini. Stoichiometry of iron oxidation by apoferritin. FEBS Lett. 86: 136-138 (1978).

101. D.E. Mayer, J.S. Rohrer, D.A. Schoeller and D.C. Harris. Fate of oxygen during ferritin iron incorporation. Biochemistry 22: 876-880 (1983).

102. J.G. Wardeska, B. Viglione and N.D. Chasteen. Metal ion complexes of apoferritin. Evidence for the initial binding in the hydrophilic channels. J. Biol. Chem. 261: 6677-6683 (1986).

103. P.M. Harrison, T.G. Hoy, I.G. Macara and R.J. Hoare. Ferritin iron uptake and release. Structure-function relationships. Biochem. J. 143: 445-451 (1974).

104. C.F.A. Bryce and R.R. Crichton. The catalytic activity of horse spleen apoferritin. Preliminary kinetic studies and the effect of chemical modification. Biochem. J. 133: 301-309.

105. T. Jones, R. Spencer and C. Walsh. Mechanism and kinetics of iron release from ferritin by dihydroflavins and dihydroflavin analogues. Biochemistry 17: 4011-4017 (1978).

106. A. Mazur, S. Baez and E. Shorr. The mechanism of iron release from ferritin as related to its biological properties. J. Biol. Chem. 213: 147-160 (1955).

107. S. Granick and L. Michaelis. Ferritin II. Apoferritin of horse spleen. J. Biol. Chem. 147: 91-97 (1943).

108. R.R. Crichton. Ferritin. Struct. Bond 17: 67-134 (1973).

109. R.R. Crichton, F. Roman and F. Roland. Iron mobilization from ferritin by chelating agents. J. Inorg. Biochem. 13: 305-316 (1980).

110. S. Sirivech, J. Driskell and E. Frieden. NADH-FMN oxidoreductase activity and iron content of organs from riboflavin- and iron-deficient rats. J. Nutr. 107: 739-745 (1977).

111. Z. Zaman and R.L. Verwilghen. Effect of riboflavin deficiency on activity of NADH-FMN oxidoreductase (ferriductase) and iron content of rat liver. Biochem. Soc. Trans. 5: 306-308 (1977).

112. A. Mazur, S. Green, A. Saha and A. Carleton. Mechanism of release of ferritin iron in vivo by xanthine oxidase. J. Clin. Invest. 37: 1809-1817 (1958).

113. A. Mazur and A. Carleton. Hepatic xanthine oxidase and ferritin iron in the developing rat. Blood 26: 317-322 (1965).

114. G.D. Watt, R.B. Frankel and G.C. Papaefthymiou. Reduction of mammalian ferritin. Proceed. Natl. Acad. Sci. 82: 3640-3643 (1985).

115. R.J. Ulvik. Relevance of ferritin-binding sites on isolated mitochondria to the mobilization of iron from ferritin. Biochim. Biophys. Acta 715: 42-51 (1982).

116. R.J. Ulvik and I. Romslo. Reduction of exogenous FMN by isolated rat liver mitochondria. Significance to the mobilization of iron from ferritin. Biochim. Biophys. Acta 635: 457-469 (1981).

117. J.M.C. Gutteridge, B. Halliwell, A. Treffry, P.M. Harrison and D. Blake. Effect of ferritin-containing fractions with different iron loading on lipid peroxidation. Biochem. J. 209: 557-560 (1983).

118. M. O'Connell, B. Halliwell, C.P. Moorhouse, O.I. Aruoma, H. Baum and T.J. Peters. Formation of hydroxyl radicals in the presence of ferritin and haemosiderin. Biochem. J. 234: 727-731 (1986).

119. E.D. Wills. Mechanisms of lipid peroxide formation in animal tissues. Biochem. J. 99: 667-676 (1966).

120. S.M.H. Sadrzadeh, E. Graf, S.S. Panter, P.E. Hallaway and J.W. Eaton. Hemoglobin. A biologic Fenton reagent. J. Biol. Chem. 259: 14354-14356 (1984).

121. J. Kanner and S. Harel. Initiation of membranal lipid peroxidation by activated metmyoglobin and methemoglobin. Arch. Biochem. Biophys. 237: 314-321 (1985).

122. C.C. Winterbourn. Hydroxyl radical production in body fluids. Roles of metal ions, ascorbate, and superoxide. Biochem. J. 198: 125-131 (1981).

123. J.M.C. Gutteridge, D.A. Rowley and B. Halliwell. Superoxide-dependent formation of hydroxyl radicals and lipid

peroxidation in the presence of iron salts. Biochem. J. 206: 605-609 (1982).

124. J.M. McCord. Free radicals and inflammation: Protection of synovial fluid by superoxide dismutase. Science 185: 529-531 (1974).

125. B. Halliwell. Superoxide-dependent formation of hydroxyl radicals in the presence of iron salts. Its role in degradation of hyaluronic acid by a superoxide-generating system. FEBS Lett. 96: 238-242 (1978).

126. D. Rowley, J.M.C. Gutteridge, D. Blake, M. Farr and B. Halliwell. Lipid peroxidation in rheumatoid arthritis: thiobarbituric acid-reactive material and catalytic iron salts in synovial fluid from rheumatoid patients. Clin. Sci. 66: 691-695 (1984).

127. J.S. Bus, S.D. Aust and J.E. Gibson. Superoxide- and singlet oxygen-catalyzed lipid peroxidation as a possible mechanism for paraquat (methyl viologen) toxicity. Biochem. Biophys. Res. Comm. 58: 749-755 (1974).

128. M.A. Trush, E.G. Mimnaugh, E. Ginsburg and T.E. Gram. In vitro stimulation by paraquat of reactive oxygen-mediated lipid peroxidation in rat lung microsomes. Toxicol Appl. Pharmacol. 60: 279-286 (1981).

129. D.J. Kornbrust and R.D. Mavis. The effect of paraquat on microsomal lipid peroxidation in vitro and in vivo. Toxicol. Appl. Pharmacol. 53: 323-332 (1980).

130. H.P. Misra and L.D. Gorsky. Paraquat and NADPH-dependent lipid peroxidation in lung microsomes. J. Biol. Chem. 256: 9994-9998 (1981).

131. L.L. Smith, M.S. Rose and I. Wyatt. The pathology and biochemistry of paraquat. In: Oxygen Free Radicals in Tissue Damage (Ciba Foundation Symposuim 65) pp. 321-341, Excerpta Medica, New York (1979).

132. R. Kohen and M. Chevion. Paraquat toxicity is enhanced by iron and reduced by desferrioxamine in laboratory mice. Biochem. Pharmacol. 34: 1841-1843 (1985).

133. R. Kohen and M. Chevion. Transition metals potentiate paraquat toxicity. Free Rad. Res. Commun. 1: 79-88 (1985).

134. H.C. Sutton and C.C. Winterbourn. Chelated iron-catalyzed ·OH formation from paraquat radicals and H_2O_2: Mechanism of formate oxidation. Arch. Biochem. Biophys. 235: 106-115 (1984).

135. R.D. Fairshter, N.D. Vaziri, L.C. Dearden, K. Malott and M. Caserio. Effect of dimethylthiourea on paraquat toxicity in rats. Toxicol. Appl. Pharmacol. 74: 150-154 (1984).

136. A.C. Bagley, J. Krall and R.E. Lynch. Superoxide mediates the toxicity of paraquat for Chinese hamster ovary cells. Proceed. Natl. Acad. Sci. 83: 3189-3193 (1986).

137. A.P. Autor. Reduction of paraquat toxicity by superoxide dismutase. Life Sci. 14: 1309-1319 (1974).

138. C.E. Patterson and M.L. Rhodes. The effect of superoxide dismutase on paraquat mortality in mice and rats. Toxicol. Appl. Pharmacol. 62: 65-72 (1982).

139. J. Goodman and P. Hochstein. Generation of free radicals and lipid peroxidation by redox cycling of adriamycin and daunomycin. Biochem. Biophys. Res. Comm. 77: 797-803 (1977).

140. J.H. Doroshow, G.Y. Locker and C.E. Myers. Enzymatic defenses of the mouse heart against reactive oxygen metabolites.1. Alterations produced by doxorubicin. J. Clin. Invest. 65: 128-135 (1980).

141. E.G. Mimnaugh, T.E. Gram and M.A. Trush. Stimulation of mouse heart and liver microsomal lipid peroxidation by anthracycline anticancer drugs: Characterization and effects of reactive oxygen scavengers. J. Pharmacol. Exper. Ther. 226: 806-816 (1983).

142. L. Sterrenberg, R.H.M. Julicher, A. Bast and J. Noordhoek. Adriamycin stimulates NADPH-dependent lipid peroxidation in liver microsomes not only by enhancing the production of O_2^- and H_2O_2, but also by potentiating the catalytic activity of ferrous ions. Toxicol. Lett. 22: 153-159 (1984).

143. H. Muliwan, M.E. Scheulen and H. Kappus. Adriamycin stimulates only the iron ion-induced, NADPH-dependent microsomal alkane formation. Biochem. Pharmacol. 31: 3147-3150 (1982).

144. E.G. Mimnaugh, M.A. Trush and T.E. Gram. Enhancement of rat heart microsomal lipid peroxidation following doxorubicin treatment in vivo. Cancer Treat. Rep. 67: 731-733 (1983).

145. E.H. Herman, A.M. El-Hage, V.J. Ferrans and T. Witiak. Reduction by ICRF-187 of acute daunorubicin toxicity in Syrian golden hamsters. Res. Comm. Chem. Pathol. Pharmacol. 40: 217-231 (1983).

146. K. Sugioka, H. Nakano, T. Noguchi, J. Tsuchiya and M. Nakano. Decomposition of unsaturated phospholipid by iron-ADP-adriamycin co-ordination complex. Biochem. Biophys. Res. Comm. 100: 1251-1258 (1981).

147. K. Sugioka and M. Nakano. Mechanism of phospholipid peroxidation induced by ferric ion-ADP-adriamycin co-ordination complex. Biochim. Biophys. Acta 713: 333-343 (1982).

148. K. Sugioka, H. Nakano, M. Nakano, S. Tero-Kubota and Y. Ikegami. Generation of hydroxyl radicals during the enzymatic reductions of the Fe^{3+}-ADP-phosphate-adriamycin and Fe^{3+}-ADP-EDTA systems. Biochim. Biophys. Acta 753: 411-421 (1983).

149. N. Nakano, K. Ogita, J.M.C. Gutteridge and M. Nakano. Inhibition by the protein ceruloplasmin of lipid peroxidation stimulated by an Fe^{3+}-ADP-adriamycin complex. FEBS Lett. 166: 232-236 (1984).

150. D.A. Rowley and B. Halliwell. DNA damage by superoxide-generating systems in relation to the mechanism of action of the anti-tumor antibiotic adriamycin. Biochim. Biophys. Acta 761: 86-93 (1983).

151. H. Eliot, L. Gianni and C. Myers. Oxidative destruction of DNA by the adriamycin-iron complex. Biochem. 23: 928-936 (1984).

152. E.J.F. Demant. Transfer of ferritin-bound iron to adriamycin. FEBS Lett. 176: 97-100 (1984).

153. E.J.F. Demant and N. Norskov-Lavritsen. Binding of transferrin-iron by adriamycin at acidic pH. FEBS Lett. 196: 321-324 (1986).

CHAPTER I

FERRITIN AND SUPEROXIDE-DEPENDENT LIPID PEROXIDATION

ABSTRACT

Intracellular, low molecular weight iron complexes are proposed to promote membrane lipid peroxidation. However, hepatic cytosol prepared from iron-loaded rats contained little iron associated with proteins of less than 10,000 daltons as judged by gel filtration chromatography and ultrafiltration studies. Nearly 70% of the total cytosolic iron was found in ferritin, which promoted the peroxidation of phospholipid liposomes when incubated with xanthine oxidase, xanthine, and ADP. Activity was inhibited by SOD but markedly stimulated by the addition of catalase. Xanthine oxidase-dependent iron release from ferritin was also inhibited by SOD suggesting that $O_2^{\cdot-}$ can mediate the reductive release of iron from ferritin. Catalase had little effect on the rate of iron release from ferritin, thus H_2O_2 appears to inhibit lipid peroxidation by preventing the formation of an initiating species rather than by inhibiting iron release from ferritin. ESR spin trapping with DMPO was used to observe free radical production in this system. Addition of ferritin to the xanthine oxidase system resulted in loss of the $O_2^{\cdot-}$ spin trap adduct suggesting an interaction between $O_2^{\cdot-}$ and ferritin. If DMPO was added 2 min after ferritin addition the resultant spectrum was that of a $\cdot OH$ spin trap adduct which was abolished by the addition of catalase. These data suggest that ferritin may function *in vivo* as a source of iron for promotion of $O_2^{\cdot-}$-dependent lipid peroxidation. Stimulation of lipid peroxidation but inhibition of $\cdot OH$ formation by catalase suggests that, in this system, initiation is not via an iron-catalyzed Haber-Weiss reaction.

INTRODUCTION

Cellular damage resulting from ischemia (1), hyperoxia (2), redox cycling (3), and other oxygen associated toxicities is often attributed to enhanced production of $O_2^{\cdot-}$ with biochemical alterations reported to include peroxidation of membrane phospholipids (4) and DNA degradation (5). However, in aqueous media $O_2^{\cdot-}$ is relatively unreactive towards most organic compounds (6) thus its proposed deleterious effects may be the result of its participation in reactions leading to other more reactive species. Superoxide-dependent formation of more reactive radicals such as ·OH requires the presence of transition metal ions such as copper or iron (4). The most widely proposed mechanism is the iron-catalyzed Haber-Weiss reaction (7):

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

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

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

Although ·OH is highly reactive, its <u>in vivo</u> formation is contingent upon the availability of physiological iron. The ability of both lactoferrin (8) and transferrin (9) to generate ·OH in the presence of a $O_2^{\cdot-}$ generating system has been demonstrated. Winterbourn (10) and others (11) have reported the ability of iron in extracellular fluids such as synovial and cerebrospinal fluid to catalyze ·OH formation and promote low rates of lipid peroxidation. However, the ability of intracellular iron to participate in redox

reactions is relatively unknown although investigators have postulated that cells may contain low molecular weight iron complexes capable of promoting lipid peroxidation (4, 12). Nucleotide chelated iron complexes have been isolated from erythrocytes (13, 14) but definitive identification of similar iron chelates in liver is lacking with suggested chelators ranging from ADP (15) to citrate (16).

Although the existence of low molecular weight complexes remains controversial, it is known that the majority of intracellular iron is stored within ferritin, a large multi-subunit protein found predominately in the liver, spleen, and bone marrow (17). Ferric iron (up to 4,500 atoms/ferritin complex) is stored within the central core of ferritin as ferric hydroxide complexed with phosphate (18). The spherical ferritin complex contains a central core and six shallow 'pockets' through which iron is deposited or mobilized (19). While it is accepted that mobilization of ferritin iron requires reduction to the ferrous state (20), physiological reductant(s) remain unknown. Release of iron from the core may necessitate passage of the reductant through the narrow pockets, therefore potential biological reductants may be limited by size constraints.

Reducing agents such as dithionite and thioglycolate readily release ferritin iron <u>in vitro</u> (21, 22) while reductants with potential physiological significance (ascorbate, cysteine, and GSH) do so much more slowly (23). Chelating agents (EDTA and 2,2'-dipyridyl) can facilitate iron release from ferritin but only in the presence of a reductant (24). The most effective mobilizers of ferritin iron appear to be reduced flavins (25). Mazur (26,27) originally proposed that xanthine oxidase, in its NAD-requiring

dehydrogenase form, may promote the release of iron from ferritin by direct reduction of the ferric iron. More recent work by Topham et al. (28) has also provided evidence that xanthine dehydrogenase may participate in the mobilization of iron from ferritin.

The similarities in the conditions under which ferritin iron is mobilized and lipid peroxidation occurs, i.e., the presence of reducing equivalents and chelating agents, is striking. Nonetheless, relatively few studies have investigated the ability of ferritin to supply iron for the promotion of lipid peroxidation. Gutteridge (29) reported that ferritin was able to facilitate in vitro ascorbate-dependent lipid peroxidation. Similarily, Wills (30) demonstrated that ferritin may promote non-enzymatic lipid peroxidation, however, it was subsequently suggested that ferritin iron was not responsible for the enhancement of lipid peroxidation noted in microsomes isolated from iron-loaded rats (31).

Xanthine oxidase, which generates $O_2^{\cdot-}$ and H_2O_2 during its conversion of xanthine to urate (32), is often used for in vitro lipid peroxidation studies as $O_2^{\cdot-}$ readily reduces ferric iron. The small size of $O_2^{\cdot-}$, in conjunction with its ability to reduce chelated iron, suggests it to be an excellent candidate for mobilization of iron from ferritin. The existing controversy over the ability of xanthine oxidase to directly reduce and release ferritin iron provides further rationale for these studies.

In this paper, we report our findings which demonstrate that $O_2^{\cdot-}$, as generated by xanthine oxidase, reductively releases ferritin bound iron. Once released this iron can promote the peroxidation of phospholipid liposomes. Catalase markedly stimulates MDA formation in

this system, suggesting that initiation is not dependent upon H_2O_2. These results were further supported by the use of ESR spin trapping which demonstrated a negative correlation between ·OH formation and lipid peroxidation.

MATERIALS AND METHODS

Materials

Xanthine, cytochrome c (Type VI), 2-TBA, ADP, butylated hydroxytoluene, mannitol, 4,7-diphenyl-1,10-phenanthroline, potassium superoxide and crown ether were purchased from Sigma Chemical Company (St. Louis, MO). Thioglycolate and 2,2'-dipyridyl were from Fisher Scientific (Fairlawn, NJ). DMPO and dimethyl sulfoxide were obtained from Aldrich Chemical Company (Milwaukee, WI) and H_2O_2 was a product of Mallinckrodt Chemical (Paris, KY). Sephadex G-200, Sephadex G-25 and Sepharose 6B were from Pharmacia (Piscataway, NJ) while Ultragel AcA 44 was purchased from LKB (Bromma, Sweden). Imferon was a gift from Merrell Dow Pharmaceuticals (Cincinnati, OH). DMPO was vacuum distilled prior to use while all other chemicals were of analytical grade or better and used without further purification. All buffers and reagents were passed through Chelex 100 (Bio-Rad Laboratories) ion-exchange resin to free them of contaminating transition metal ions.

Enzymes

Bovine erythrocyte SOD (EC 1.15.1.1) and buttermilk xanthine oxidase (EC 1.2.3.2) were obtained from Sigma Chemical Company. Catalase (EC 1.11.1.6) was purchased from Millipore (Freehold, NJ). Gel filtration chromatography on Sephadex G-25 was utilized to remove the antioxidant thymol and ammonium sulfate from the commercial preparations of catalase and xanthine oxidase, respectively. After chromatography, xanthine oxidase activity was measured by aerobic reduction of cytochrome c (33), with a unit of activity defined as 1 μmol cytochrome c reduced/min/ml. Superoxide dismutase activity was

measured by the method of McCord and Fridovich (33) and catalase by the procedure of Beers and Sizer (34).

Fractionation of Rat Liver Cytosol

Male, Sprague-Dawley rats (275-300g) received 25 mg of Imferon (iron-dextran) intraperitoneally 24 h prior to sacrifice. Livers were excised, perfused, and homogenized in an equal amount (w/v) of 50 mM NaCl. The homogenate was centrifuged at 8,000 x g for 20 min and the resulting supernatant was subsequently centrifuged at 105,000 x g for 90 min. The supernatant was carefully decanted and applied to an Ultragel AcA 44 gel filtration column (8 x 85 cm) and eluted with 50 mM NaCl. Following an initial elution of 1,500 ml, fractions (19 ml) were collected and analyzed for total iron content. Five distinct iron-containing peaks were revealed and the appropriate fractions pooled for further analysis.

Rat hepatic cytosol, prepared from both control and iron loaded rats as detailed above, was fractionated by ultrafiltration on an Amicon ultrafiltration apparatus equipped with a PM 10 membrane. The filtrate and the retained fractions were then analyzed for total iron content.

Purification of Rat Liver Ferritin

Ferritin was purified from the livers of Imferon treated rats essentially as described by Halliday (35). Male Sprague-Dawley rats (250-300 g) received 12.5 mg of elemental iron as Imferon peritoneally on alternate days for one week (50 mg total dose). Rats were terminated by decapitation and livers were excised, perfused and homogenized in two volumes of distilled water. The homogenate was quickly heated to 70°C with continuous stirring for 5 minutes,

immediately cooled to 4°C, and centrifuged at 1,500 x g for 20 minutes to precipitate denatured protein. The supernatant was decanted and ferritin precipitated by 50% saturated ammonium sulfate and allowed to stir overnight. The pellet obtained after centrifugation at 1,500 x g for 30 minutes was then resuspended in PBS (0.02 M phosphate buffer, pH 7.4, containing 0.1 M sodium chloride) and dialyzed for 24 hours against 4 liters of the same buffer with one change of buffer. After dialysis the solution was centrifuged at 30,000 x g for 30 minutes. The resultant supernatant was then centrifuged at 100,000 x g for 2 hours. The pellet obtained was resuspended in PBS and chromatographed on Sepharose 6B (2.5 x 60 cm column), with PBS as the elution buffer. The readily visible ferritin peak was eluted near the void volume and fractions pooled based on spectrophotometric measurement of absorbance at 280 nm and total iron analysis. The combined fractions were then concentrated on an Amicon ultrafiltration apparatus equipped with a PM 30 membrane. The concentrated ferritin was applied to a Sephadex G-200 column (1.5 x 90 cm) and again eluted with PBS. Fractions were then pooled and purity determined by SDS gel electrophoresis on a 15% polyacrylamide gel. Prior to use, EDTA was added to the isolated ferritin to a final concentration of 10 mM and incubated on ice for 1 hour. This was done to ensure that no iron was loosely associated on the protein. Subsequent chromatography on Sephadex G-25 before use effectively separated the EDTA and ferritin.

Preparation of Microsomal Lipid and Liposomes

Male Sprague-Dawley rats (250-275 g) were obtained from Charles River (Boston, MA) from which liver microsomes were isolated as per Pederson and Aust (36). Microsomal lipid was extracted from freshly

isolated microsomes by the method of Folch et al. (37). All solvents utilized were purged with argon and all steps performed at 4°C to minimize autoxidation of unsaturated lipids. Phospholipid liposomes were subsequently prepared by indirect, anaerobic sonication (38) and lipid phosphate assays performed according to Bartlett (39).

Lipid Peroxidation Assays

Xanthine oxidase dependent peroxidation of liposomes was performed by incubating liposomes (1 μmol lipid phosphate/ml) with 0.33 mM xanthine and xanthine oxidase and rat liver ferritin as specified in figure legends. Reaction mixtures were constituted in 50 mM NaCl, pH 7.0, and incubated at 37°C in a Dubnoff metabolic shaker bath under an air atmosphere. Although unbuffered, incubations remained at pH 7.0 throughout the course of the experiments. Peroxidation was monitored by taking aliquots from the incubations at specified times and measuring MDA formation using the TBA test (40). Butylated hydroxytoluene (0.03 volumes in 2% ethenol) was added to the thiobarbituric acid reagent to prevent further peroxidation of lipid during the assay procedure.

Assays for Total Iron and Ferritin Iron Release

Total iron was determined essentially as described by Brumby and Massey (41). Aliquots of sample, or citrate-iron (8.5:0.5 mM) for a standard curve, were brought to 0.15 ml with water. To each sample was added 0.05 ml of thioglycolic acid (10% v/v) and 0.2 ml of glacial acetic acid followed by vigorous mixing. After allowing 30 minutes for protein denaturation, 0.32 ml of water, 0.28 ml of saturated sodium acetate, and 1 ml of 2.0 mM 4,7-diphenyl-1,10-phenanthroline in isoamyl alcohol were added. The mixture was agitated, centrifuged in

an IEC table top centrifuge and the absorbance of the upper pink layer read at 535 nm. Care was taken to extract endogenous iron from the saturated sodium acetate with the phenanthroline/isoamyl alcohol mixture prior to use.

Measurement of iron release from ferritin was performed according to Mazur et al. (27) with modification, relying upon absorbance of the ferrous-dipyridyl chromophore. Incubations in a final volume of 1 ml contained 0.025 units of xanthine oxidase, 0.33 mM xanthine, 500 μM ferritin iron, and 5.12 mM 2,2'-dipyridyl in 50 mM NaCl, pH 7.0. In the experiments involving potassium superoxide, xanthine oxidase and xanthine were replaced with 2 mM potassium superoxide prepared in crown ether essentially as described by Ruddock et al. (42). Catalase and SOD were included as indicated in figure legends. Reactions were continuously monitored at 520 nm in a Cary 219 dual beam recording spectrophotometer. The amount of iron released from ferritin was determined from a standard curve using ferric chloride reduced with 0.1 ml of 10% thioglycolate.

ESR Spin Trapping

Detection of radicals produced by xanthine oxidase in the presence or absence of ferritin was accomplished by ESR spin trapping experiments using 60 mM DMPO as outlined in the figure legends. Incubations were constituted and directly transferred to the cuvette of a Varian Century-112 EPR spectrometer. Spectrometer settings were: 3329.4 G magnetic field, 15 mW microwave power, 9.4232 GHz, 1000 KHz modulation frequency, 0.63 modulation amplitude, 2.0 second time constant and 8 minute scan time.

RESULTS

Fractionation Of Rat Liver Cytosol

The data in Table 1 demonstrate that 98.8% of the total cystolic iron was recovered in association with proteins in the range of molecular weight corresponding to 500-100 kdaltons. No iron was detected in the region expected to yield a low molecular weight form of iron (< 5,000 daltons). Similarly, ultrafiltration of rat liver cytosol from both control and iron-loaded rats confirmed that less than 1% of the total iron in the homogenates was recovered in the filtrate (< 10,000 daltons) and 64% in the retained fraction (> 10,000 daltons) (Table 2). The remainder of the homogenate iron was recovered in the pellets obtained from the centrifugation at 8,000 x g and 105,000 x g.

Table 2. Total Iron Analysis of Rat Liver Homogenate and Ultrafiltrated Cytosol

Fraction	nmol Fe^{3+}/ml (Range) Control	Iron loaded
Homogenate	640-880	1600-1920
Retained (> 10 kdaltons)	410-560	840-870
Filtrate (< 10 kdaltons)	8-8	5-6

Livers from control and iron-loaded rats (12.5 mg Imferon, 12 hr. prior to sacrifice) were homogenized in 50 mM NaCl and cytosol prepared as indicated in Materials and Methods. The cytosol (10 ml) was ultrafiltrated using a PM 10 membrane to near dryness and the volume adjusted to 10 ml with Chelex-treated 50 mM NaCl. Aliquots of the homogenate, the filtrate, and the retained fractions were then analyzed for total iron content as described in Materials and Methods. Data given are for two animals in each group.

Table 1. Total Iron Analysis of Pooled Fractions Obtained from Chromatography of Rat Liver Cytosol

MW Range	nmol Fe^{3+}/ml	% of total sample applied
500,000	164	68.9
↓	57	23.9
	7	3.0
	7	3.0
100,000	0	0.0
Totals	235	98.8

Rat liver cytosol (185 ml), prepared as described in Materials and Methods, was chromatographed on an Ultragel AcA 44 column (8 x 85 cm) with 50 mM NaCl, pH 7.0. Fractions were collected and analyzed for total iron and pooled into five fractions covering a molecular weight range of 500-100 kdaltons.

The fraction containing nearly 70% of the cytosolic iron eluted in the 400-500 kdalton range. It was subsequently determined by SDS gel electrophoresis and Ouchterlony double diffusion analysis that this iron was associated with ferritin (results not shown).

Xanthine Oxidase-Dependent Lipid Peroxidation

As shown in Figure 1, MDA formation was observed in a lipid peroxidation system employing liposomes, purified rat liver ferritin, and xanthine oxidase. Increasing the amount of xanthine oxidase beyond 0.005 U/ml to generate greater rates of $O_2^{\cdot-}$ production had an inhibitory effect on MDA formation. The addition of catalase (10 and 25 U/ml) markedly increased the rate of lipid peroxidation observed at all xanthine oxidase concentrations used (Figure 1). The maximum rate of MDA formation occurred at greater xanthine oxidase activity as the concentration of catalase was increased with the maxima occurring at 0.015 and 0.02 U/ml of xanthine oxidase with 10 and 25 U/ml of catalase, respectively. Similarly, overall rates of lipid peroxidation increased with the shift to higher activities of the two enzymes. In the absence of xanthine oxidase or ferritin rates were 22 and 30% of the complete system in the absence of catalase. The inclusion of catalase had no effect on these rates (data not shown).

When xanthine oxidase concentration was held constant at 0.005 U/ml, where the rate of MDA formation was maximal in the absence of catalase, increasing concentrations of catalase up to 100 U/ml resulted in increased rates of MDA formation as shown in Figure 2.

To determine whether enhancement of lipid peroxidation by catalase was related to the use of ferritin as the source of iron, the

Figure 1. Ferritin and Xanthine Oxidase-Dependent Peroxidation of Phospholipids. Reaction mixtures (5 ml final volume) contained phospholipid liposomes (1 µmol lipid phosphate/ml), rat liver ferritin (200 µm Fe^{3+}), ADP (1 mM), xanthine (0.33 mM), catalase (100 U/ml) and varying concentrations of xanthine oxidase in 50 mM NaCl, pH 7.0. Peroxidation was initiated by the addition of xanthine oxidase and aliquots from the reactions mixtures were assayed for MDA content.

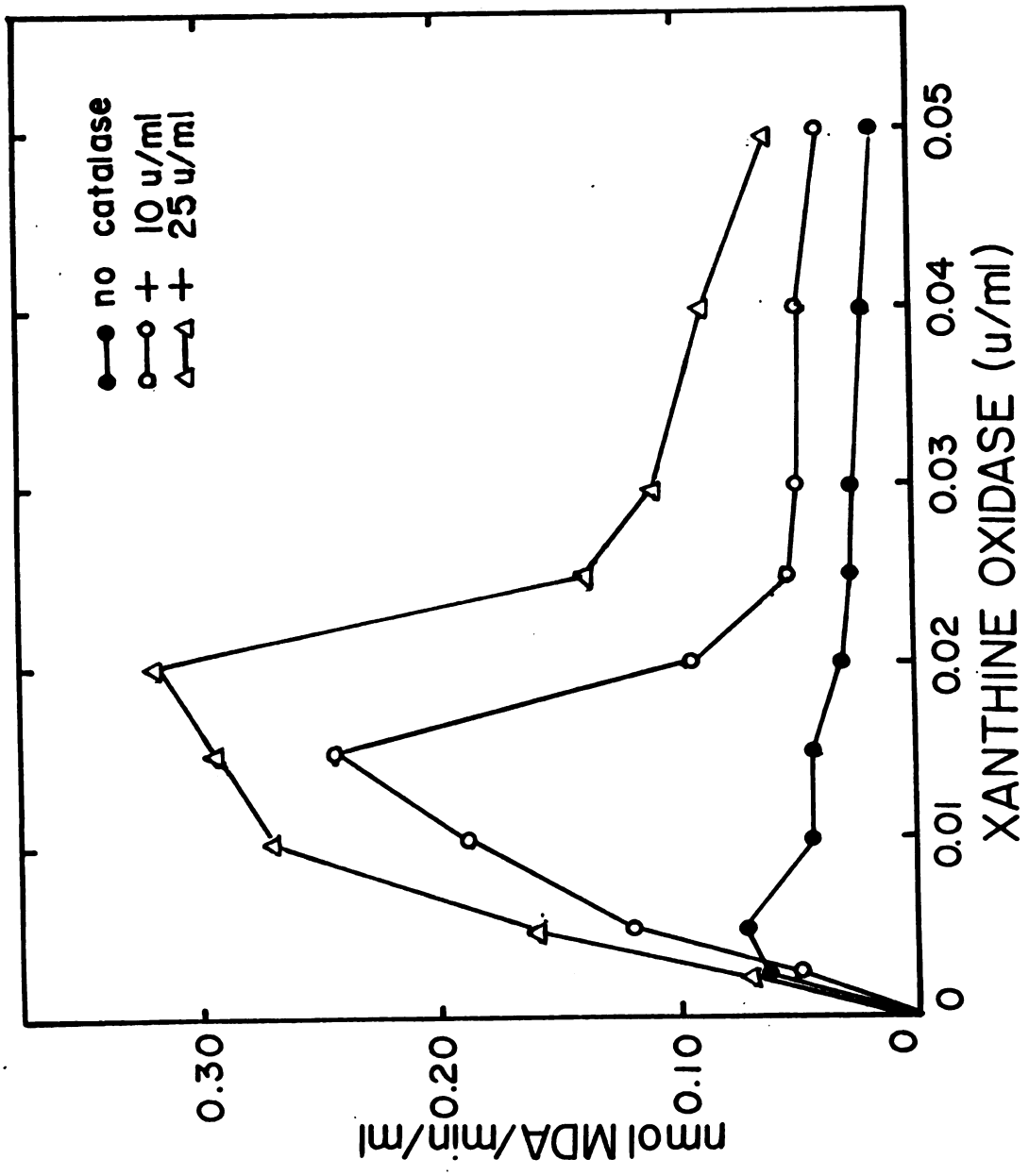
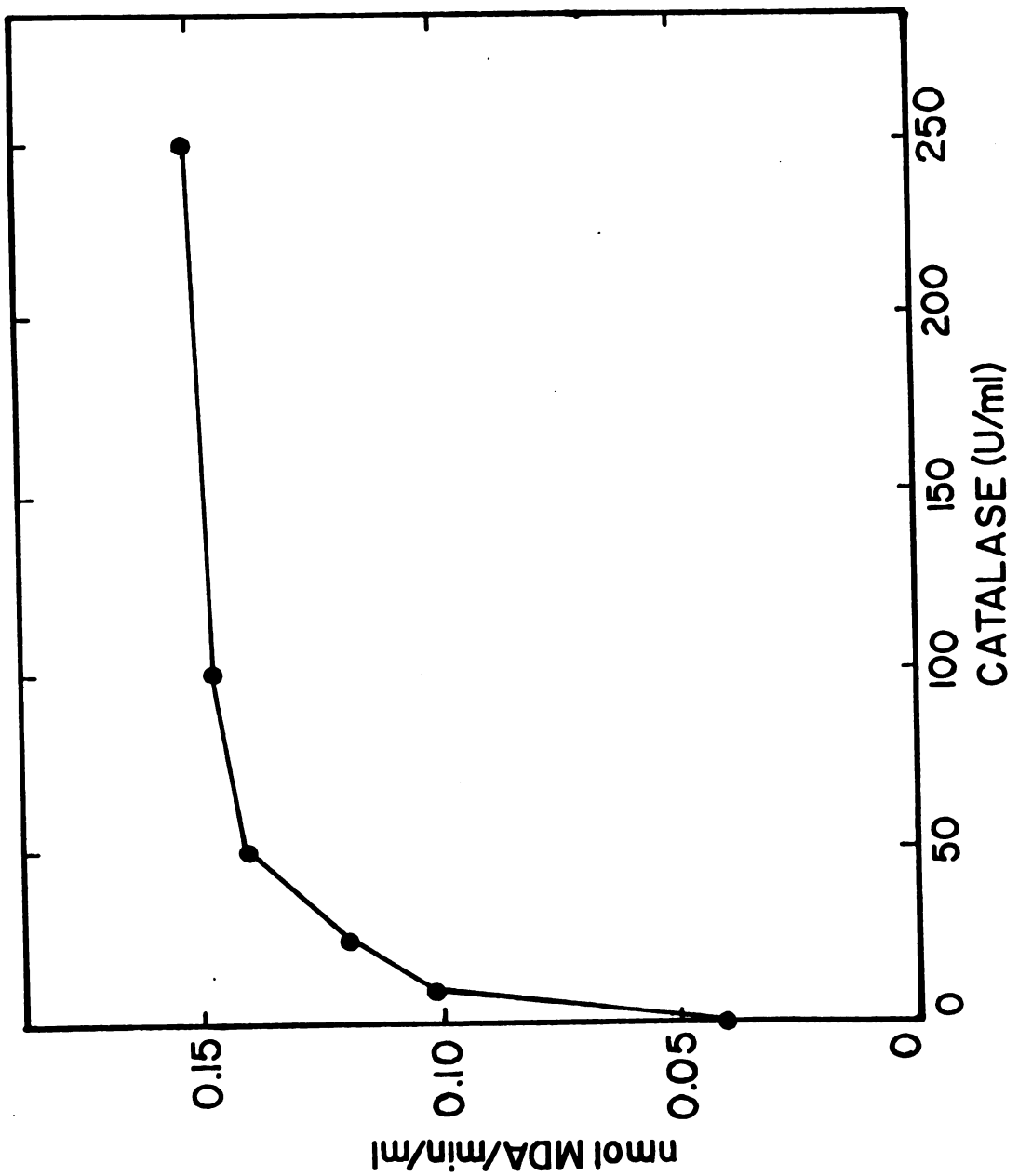

no catalase
+ 10 u/ml
+ 25 u/ml
nmol MDA/min/ml
0.30
0.20
0.10
0
0
0.01
0.02
0.03
0.04
0.05
XANTHINE OXIDASE (u/ml)

Figure 2. The Effect of Varying Catalase Concentrations on Ferritin and Xanthine Oxidase-Dependent Peroxidation of Phospholipids. Reaction mixtures (5 ml final volume) contained phospholipid liposomes (1 µmol lipid phosphate/ml), rat liver ferritin (200 uM Fe^{3+}), ADP (1 mM), xanthine (0.33 mM), xanthine oxidase (0.005 U/ml) and varying concentrations of catalase in 50 mM NaCl, pH 7.0. Peroxidation was initiated by the addition of xanthine oxidase and aliquots from the reaction mixtures were assayed for MDA content.


nmol MDA/min/ml
0.15
0.10
0.05
0
0
50
100
150
200
250
CATALASE (U/ml)

effect of catalase was assessed when iron was supplied directly as ADP-Fe^{3+}. As shown in Figure 3 catalase (100 U/ml) was found to stimulate the rate of MDA formation approximately two-fold at a xanthine oxidase concentration of 0.01 U/ml.

Effect of SOD and Mannitol

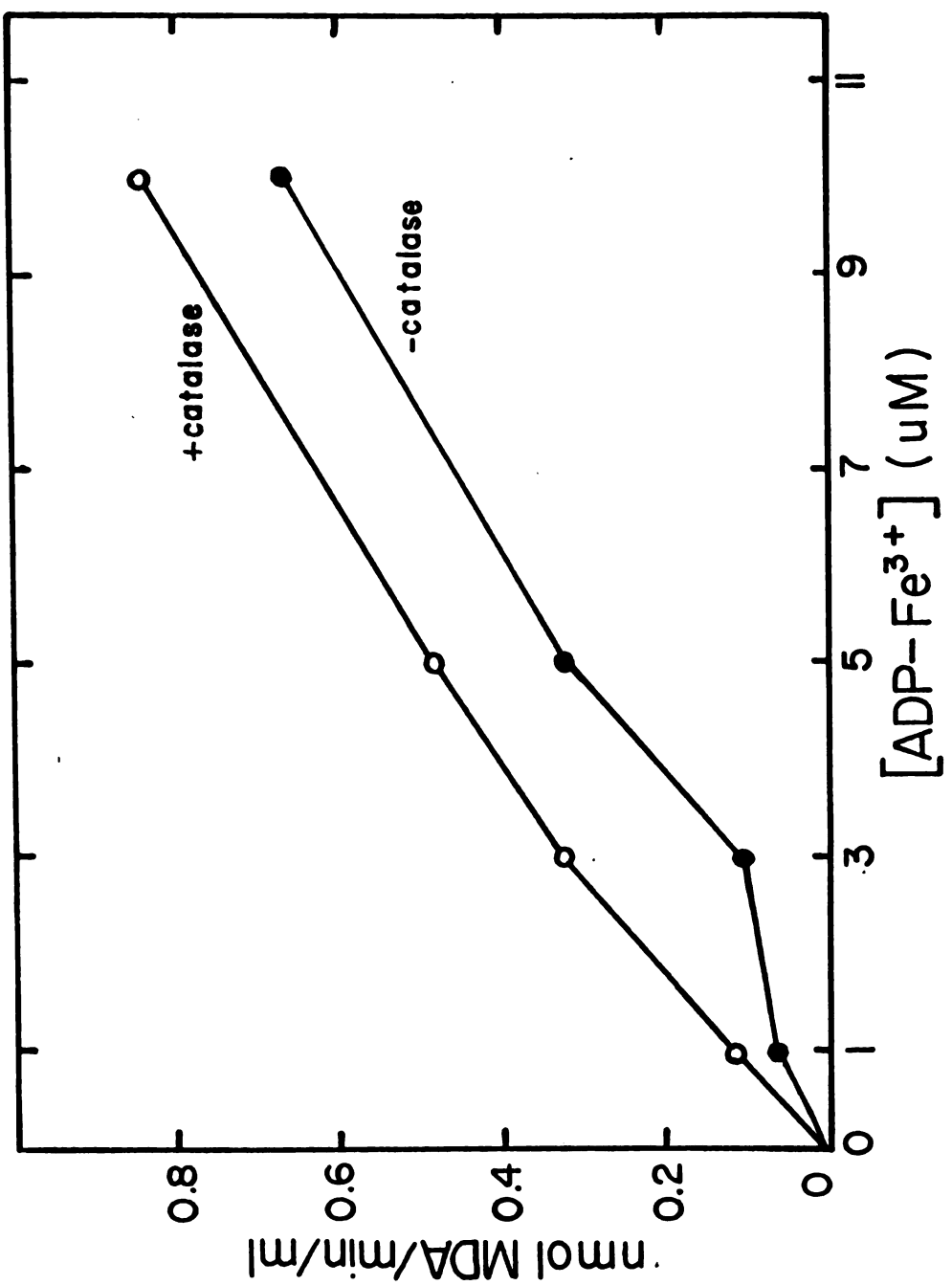
The stimulation of MDA formation by catalase suggested that initiation of lipid peroxidation in this system is inhibited by H_2O_2. To further characterize lipid peroxidation in this system, the effect of SOD and mannitol was also investigated. As can be seen in Table 3 SOD significantly inhibited MDA formation both in the presence and absence of catalase while mannitol stimulated activity slightly. The omission of xanthine oxidase or ferritin resulted in rates of lipid peroxidation being 23 and 18% of control in the absence of catalase, with the addition of catalase having no effect on these incubations.

Table 3. The Effect of SOD and Mannitol on Xanthine Oxidase-Dependent Peroxidation of Phospholipids

	nmol MDA/min/ml	
	- catalase	+ catalase
no additions	0.038	0.480
+ SOD	0.007	0.015
+ mannitol	0.044	0.510
- xanthine oxidase	0.009	-----
- ferritin	0.007	-----

Reaction mixtures (5 ml final volume) contained phospholipid liposomes (1 μmol lipid phosphate/ml), rat liver ferritin (200 μM Fe^{3+}), ADP (1 mM), xanthine (0.33 mM) and xanthine oxidase (0.0025 U/ml) in 50 mM NaCl, pH 7.0. Where indicated, reaction mixtures contained SOD (100 U/ml), mannitol (10 mM) or catalase (200 U/ml). Peroxidation was initiated by the addition of xanthine oxidase and aliquots from the reaction mixtures were assayed for MDA content.

Figure 3. The Effect of Catalase on ADP-Fe^{3+} and Xanthine Oxidase-Dependent Peroxidation of Phospholipids. Reaction mixtures (5 ml final volume) contained phospholipid liposomes (1 µmol lipid phosphate/ml), ADP-Fe^{3+} (5:1 at 1, 3, 5 and 10 µM Fe), xanthine (0.33 mM), xanthine oxidase (0.01 U/ml) and catalase (100 U/ml) in 50 mM NaCl, pH 7.0. Peroxidation was initiated by the addition of xanthine oxidase and aliquots from the reaction mixtures were assayed for MDA content.


nmol MDA/min/ml
0
0.2
0.4
0.6
0.8
0
1
3
5
7
9
11
[ADP-Fe3+] (uM)
+catalase
-catalase

Release of Iron From Ferritin

Inhibition of lipid peroxidation by SOD in this system suggests that $O_2^{-\cdot}$ may function in the reductive release of iron from ferritin. To test this hypothesis, iron release from ferritin was directly measured with 2,2'-dipyridyl which readily complexes with ferrous iron, forming a chromophore absorbing maximally at 520 nm.

As shown in Figure 4, iron release from ferritin was strictly dependent upon xanthine oxidase. Greater rates of iron release occurred when the amount of ferritin added to the system was increased (results not shown). Importantly, SOD completely inhibited the release of iron from ferritin. To further confirm that $O_2^{-\cdot}$ can reductively release ferritin iron, potassium superoxide in crown ether was found to also result in the release of iron from ferritin (Figure 5). Again, addition of SOD resulted in a decreased rate of iron release.

Inclusion of catalase in either the xanthine oxidase or potassium superoxide system resulted in an apparent stimulation of iron release. The results in Figure 4 show that increasing concentrations of catalase cause an apparent increase in the rate of iron release with 1000 U/ml of catalase apparently effectively scavenging all H_2O_2 produced by xanthine oxidase (0.025 U/ml) or $O_2^{-\cdot}$ dismutation as 2000 U/ml of catalase gave virtually identical rates.

ESR Spin Trapping

ESR spin trapping was utilized to observe radical production in this system. This technique has been shown to be effective for trapping of both $O_2^{-\cdot}$ and $\cdot OH$ using DMPO as the radical trap (43). In the absence of ferritin, xanthine oxidase generated $O_2^{-\cdot}$ which reacted

Figure 4. The Effect of SOD and Catalase on Xanthine Oxidase-Dependent Iron Release from Ferritin. Reaction mixtures (1 ml final volume) contained rat liver ferritin (500 μM Fe^{3+}), 2,2'-dipyridyl (5.12 mM), xanthine (0.33 mM) and xanthine oxidase (0.025 U) in 50 mM NaCl, pH 7.0. Catalase and SOD were included as indicated. Reactions were initiated by the addition of xanthine oxidase and monitored continuously at 520 nm. Δ minus xanthine oxidase, o no additions, □ plus SOD (100 U), ● plus catalase (varying amounts).

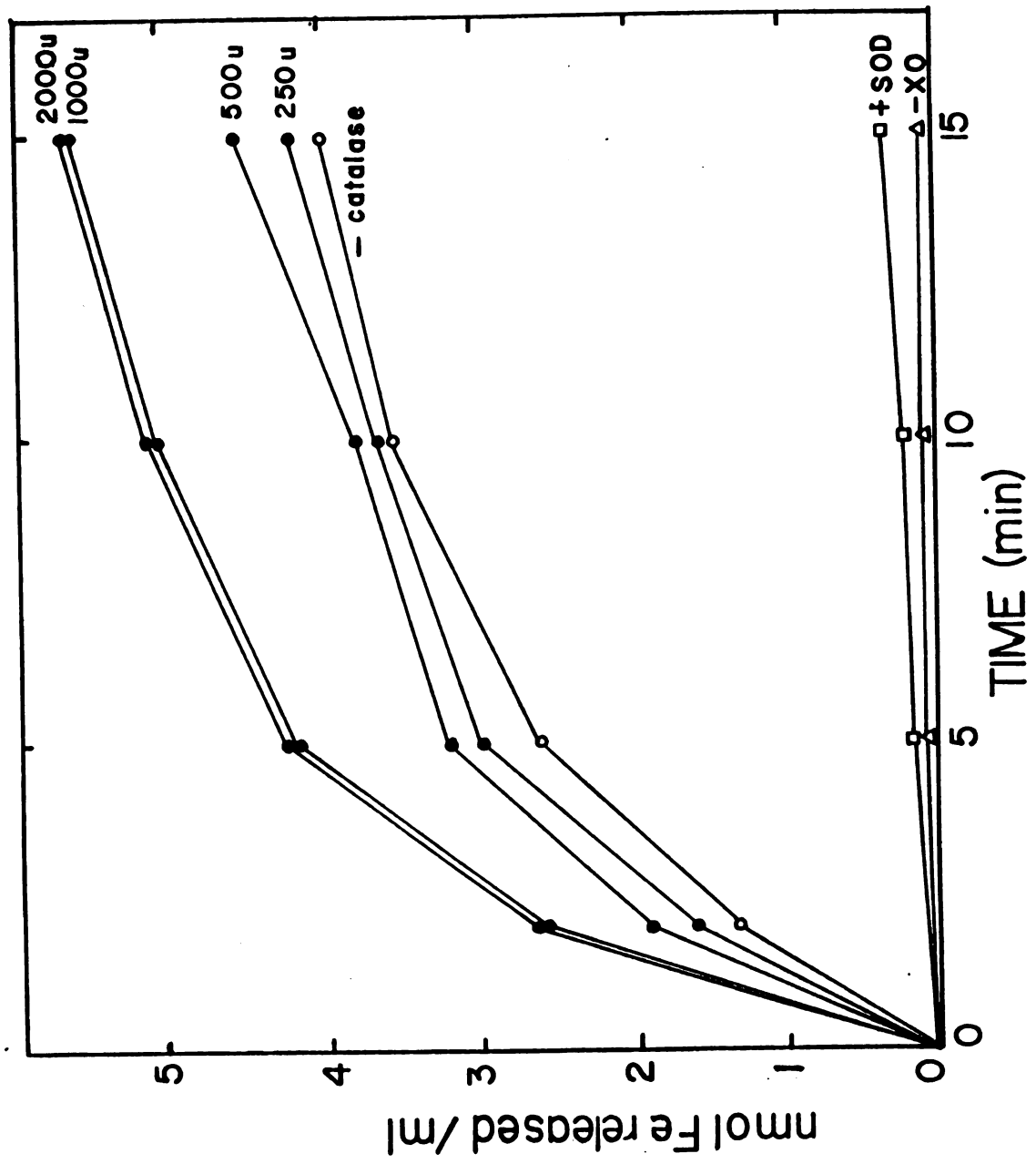
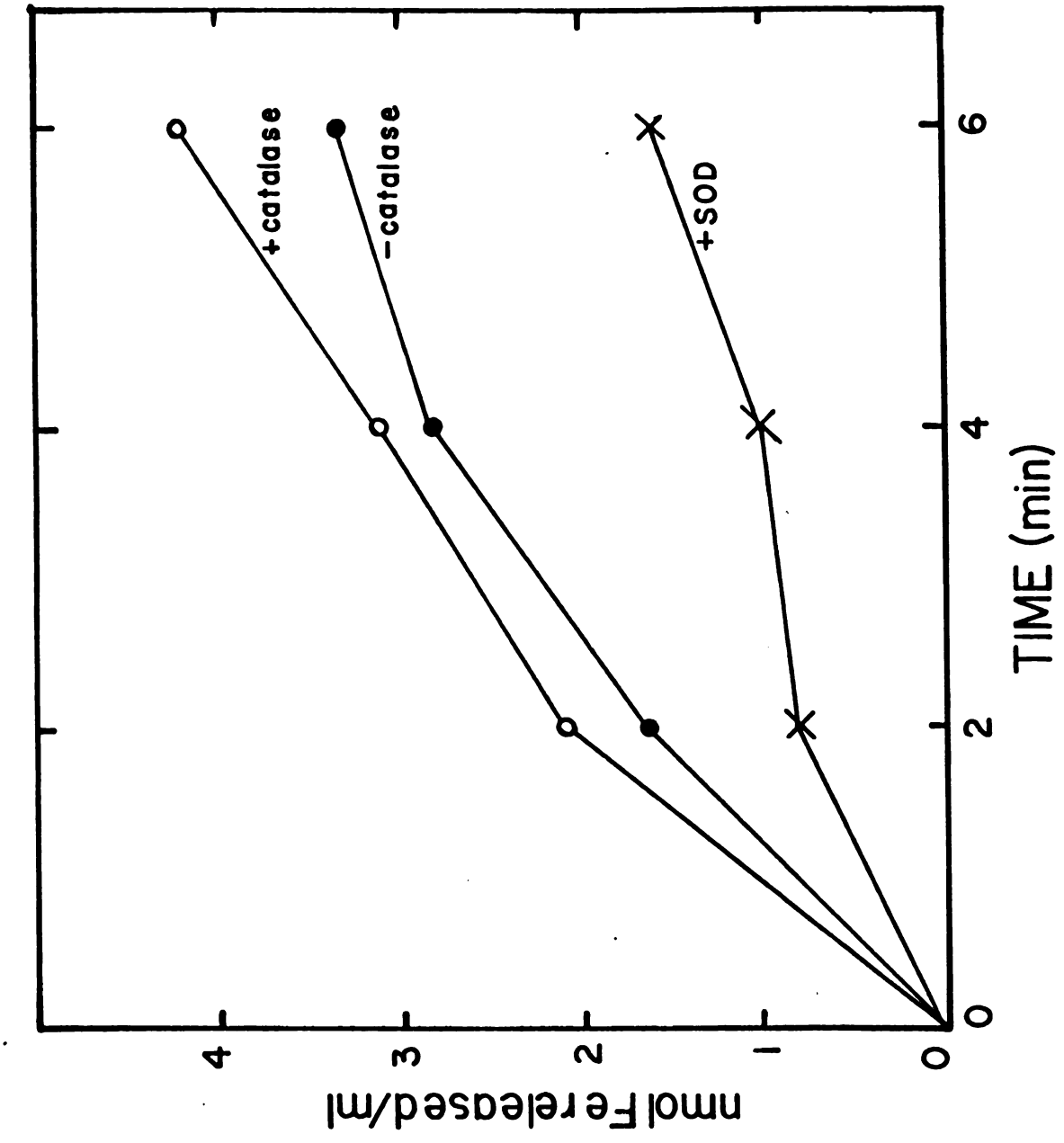

nmol Fe released /ml
TIME (min)
0
1
2
3
4
5
0
5
10
15
2000u
1000u
500u
250u
– catalase
□ +SOD
△ –XO

Figure 5. The Effect of SOD and Catalase on Potassium Superoxide-Dependent Iron Release from Ferritin. Reaction mixtures (1 ml final volume) contained rat liver ferritin (500 μM Fe^{3+}), 2,2'-dipyridyl (5.12 mM), and potassium superoxide (2 mM) in 50 mM NaCl, pH 7.0. Where indicated catalase (1000 U) and SOD (100 U) were included.


+catalase
−catalase
+SOD
nmol Fe released/ml
TIME (min)
0
1
2
3
4
0
2
4
6

with DMPO yielding the DMPO-OOH adduct signal (hfs constants A_N = 13.1 G, A_β H = 11.0 G and A_γ H = 1.3 G) (Figure 6). Addition of ADP to the incubation had no effect on the intensity of the signal while catalase appeared to prevent the decay of the signal adduct. Formation of the DMPO-OOH adduct was dependent upon enzymatic activity as no signal was observed in the absence of xanthine.

When ferritin was present in this system no DMPO-OOH signal was observed, suggesting that $O_2^{\cdot-}$ was interacting with the ferritin molecule (Figure 7A). When DMPO was added two minutes after xanthine oxidase additions, DMPO-OH (hfs constants $A_N = A_H$ = 14.8 G) but no DMPO-OOH signal adduct was observed (Figure 7B). The inclusion of ADP had little effect on the DMPO-OH signal intensity. Catalase (200 U/ml) prevented DMPO-OH adduct formation in this system, indicating that $\cdot OH$ formation required H_2O_2.

Figure 6. ESR Spin Trapping of Superoxide Generated by Xanthine Oxidase. Reaction mixtures (1 ml final volume) contained xanthine oxidase (0.05 U) and DMPO (60 mM) in 50 mM NaCl, pH 7.0. Additions were as follows: (A) no xanthine; (B) plus xanthine (0.33 mM); (C) plus xanthine (0.33 mM), plus ADP (1.25 mM); (D) plus xanthine (0.33 mM), plus catalase (200 U).

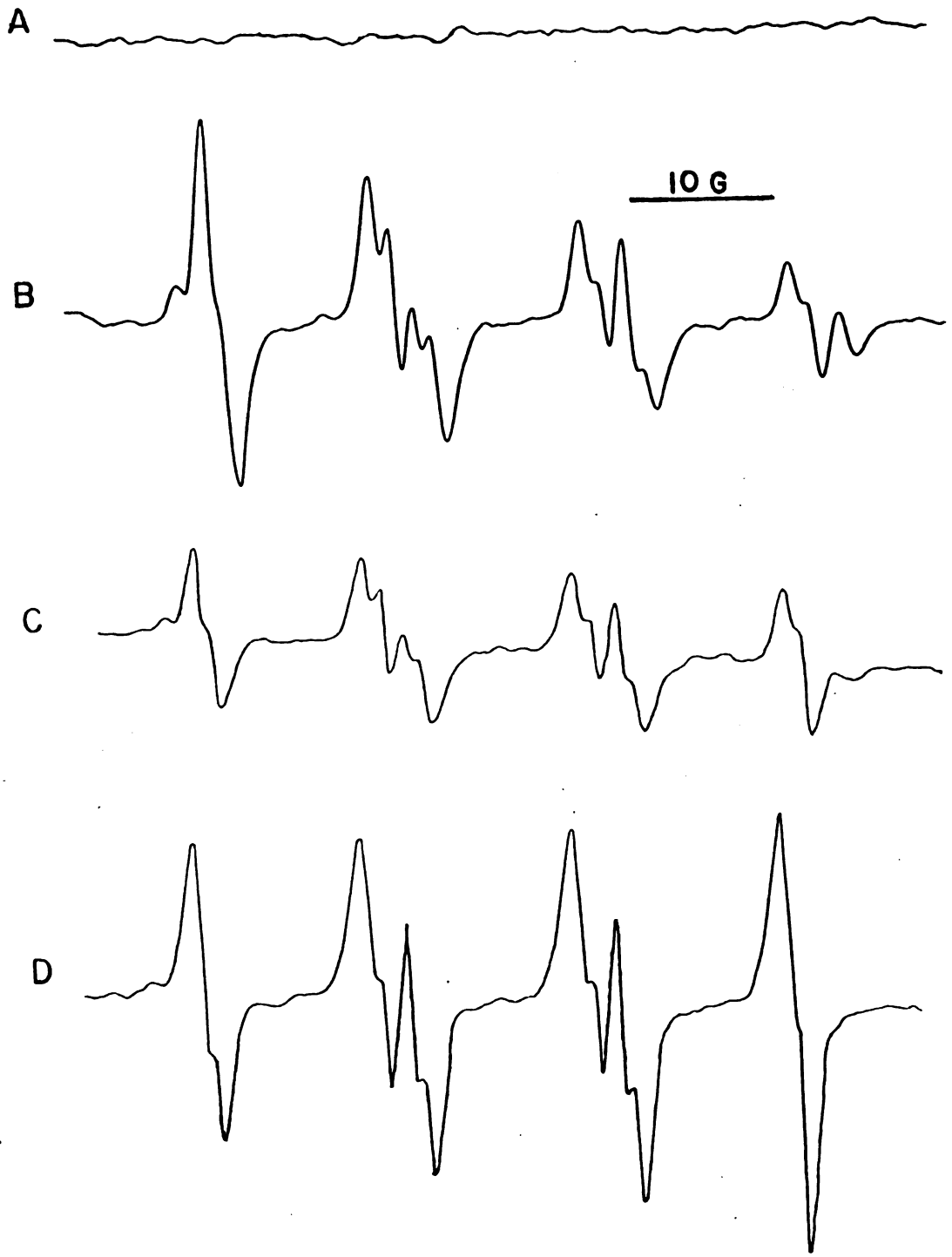
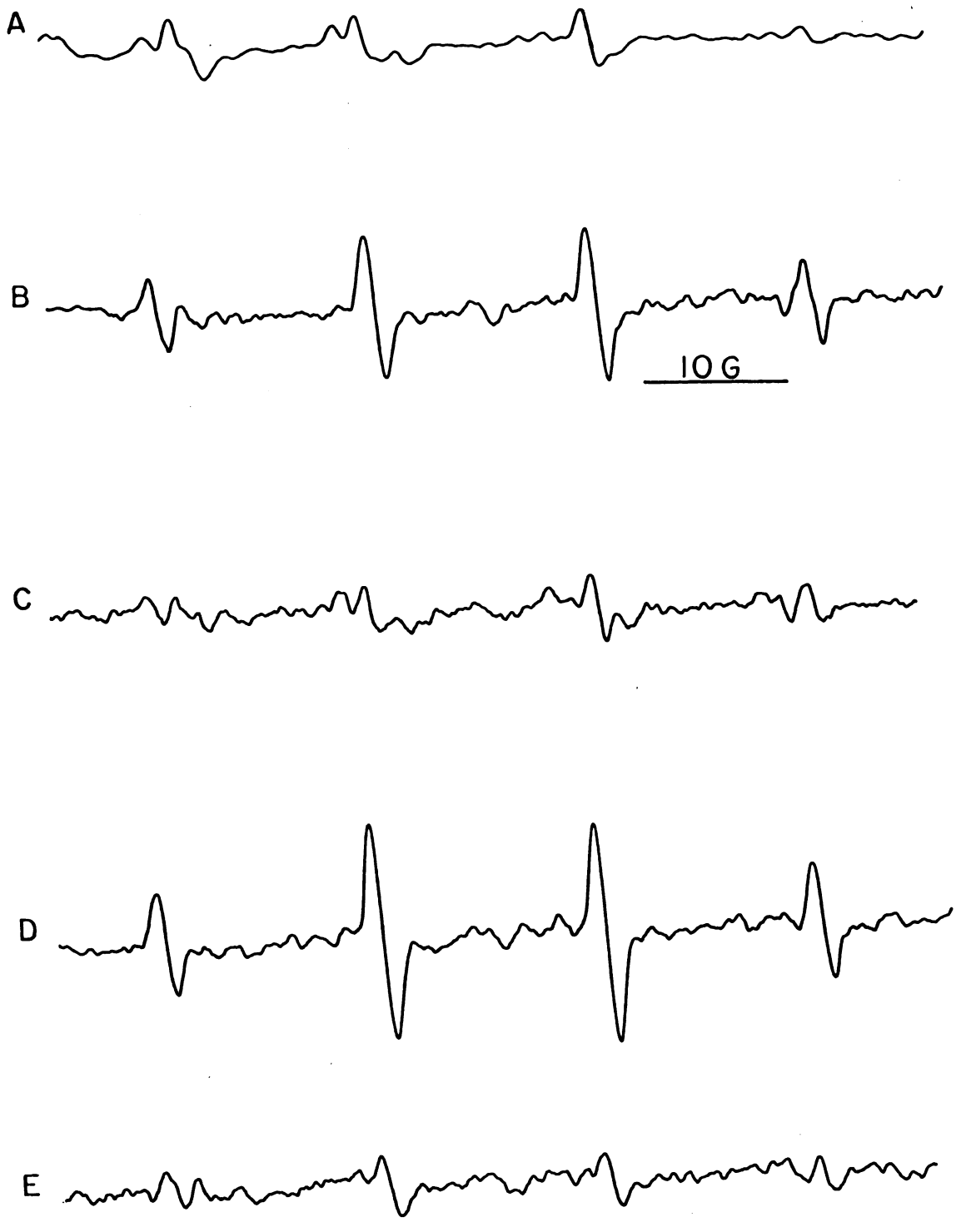

A
B
10 G
C
D

Figure 7. ESR Spin Trapping of Hydroxyl Radical Generated by Xanthine Oxidase and Rat Liver Ferritin. Reaction mixtures (1 ml final volume) contained rat liver ferritin (3 mM Fe^{3+}), xanthine (0.33 mM) and 60 mM DMPO added at two minutes after initiation except where indicated in 50 mM NaCl, pH 7.0. Additions were as follows: (A) plus 0.10 U xanthine oxidase with DMPO added at zero time; (B) plus 0.10 U xanthine oxidase; (C) plus 0.10 U xanthine oxidase, plus 200 U catalase; (D) plus 0.10 U xanthine oxidase, plus 1.25 mM ADP; (E) plus 0.05 U xanthine oxidase, plus 1.25 mM ADP, plus 200 U catalase.


A
B
10 G
C
D
E

DISCUSSION

The results of this study demonstrate that only 1-2% of the total cytosolic iron from iron-loaded rats is present in a low molecular weight form. The existence of such iron complexes in erythrocytes has been documented but the demonstration of similar complexes in other tissues, including liver, has not been achieved. In spite of the largely hypothetical nature of such complexes they are generally proposed to be a source of iron for the promotion of lipid peroxidation *in vivo*. The majority of the iron was recovered in ferritin, in agreement with recent reports that demonstrate an increased synthesis of ferritin in response to iron loading (44).

As iron overload is known to result in enhanced lipid peroxidation and toxicity it was of interest to assess the potential for ferritin to serve as a source of iron for the formation of complexes capable of initiating oxidative damage. Accordingly, it was demonstrated that rat liver ferritin can provide the iron necessary to support lipid peroxidation in a model system consisting of phospholipid liposomes and xanthine oxidase. In the absence of either ferritin or xanthine oxidase little MDA formation was observed indicating that maximal activity required both xanthine oxidase and a source of iron. It was found that rates of lipid peroxidation were directly proportional to the amount of ferritin present. The data depicted utilize ferritin containing 200 μM iron so that rates in the absence of catalase are evident. Superoxide dismutase (100 U/ml) inhibited peroxidation by 82 and 97% in the absence and presence of catalase respectively, indicating that $O_2^{\cdot-}$ was required for

peroxidation to occur. Other investigators had previously proposed that xanthine oxidase was capable of releasing iron from ferritin directly (26, 27), however this was before it was recognized that xanthine oxidase produces $O_2^{\cdot-}$ (32).

Several lines of evidence indicate that the primary function of $O_2^{\cdot-}$ in promoting ferritin-dependent lipid peroxidation is the release of iron from ferritin. The release of iron from ferritin was continuously monitored spectrophotometrically by complexing the iron with 2,2'-dipyridyl. This chelator was chosen since it has high affinity for iron, does not mobilize iron from ferritin in the absence of a reductant, and forms a stable ferrous complex with a characteristic absorbance maxima at 520 nm (24). Incubation of xanthine oxidase, xanthine, and ferritin with dipyridyl resulted in an increase in A_{520} with time, indicative of ferrous iron release from ferritin. Superoxide dismutase inhibited this iron release by 95%. Potassium superoxide prepared in crown ether was similarly capable of mobilizing iron from ferritin and SOD also inhibited this reaction. Lastly, results of ESR spin trapping experiments also demonstrate an interaction between ferritin and $O_2^{\cdot-}$ generated by xanthine oxidase. The DMPO-OOH adduct signal was observed in incubations containing xanthine, xanthine oxidase and DMPO. However, the addition of ferritin resulted in a significant diminuition of the EPR signal intensity, suggesting that ferritin effectively competes with DMPO for $O_2^{\cdot-}$.

Superoxide-dependent initiation of lipid peroxidation is generally thought to be via an iron-catalyzed Haber-Weiss mechanism. The role of $O_2^{\cdot-}$ in this process is two-fold, generation of H_2O_2 by

nonenzymatic dismutation (Reaction 2) and reduction of ferric iron to ferrous (Reaction 1). However, it is believed that the concentration of free or low molecular weight chelated forms of iron within cells is miniscule, or perhaps nonexistent, therefore whether this mechanism is operative in vivo remains speculative. In fact, one of the major cellular protective mechanisms against oxidative cytolysis may be the sequestration of redox active metals such as iron or copper in unreactive states. In this context, this work suggests a novel role for $O_2^{\cdot-}$ in promoting toxicity, that is the reductive release of iron from ferritin, thereby potentially increasing the low molecular weight iron pool capable of undergoing redox reactions leading to the formation of stronger oxidants.

The hydroxyl radical is the most widely proposed initiator of lipid peroxidation and can be formed by Fenton's chemistry (Reaction 3). This would be a likely mechanism for initiation of lipid peroxidation in the xanthine oxidase system as H_2O_2 is generated directly by the enzyme (32) as well as by $O_2^{\cdot-}$ dismutation and could react with the ferrous iron released from ferritin. Hydroxyl radical-dependent initiation of lipid peroxidation is often inferred from the ability of catalase to inhibit lipid peroxidation by scavenging H_2O_2 (45), thus preventing ·OH formation. However, the addition of catalase to the xanthine oxidase and ferritin system used in this study resulted in significant stimulation of rates of MDA formation. Increasing xanthine oxidase activity, which would result in greater H_2O_2 production, inhibited lipid peroxidation. This was in spite of the greater rates of iron release from ferritin occurring at increased xanthine oxidase activity. Even in the presence of catalase high

levels of xanthine oxidase activity still resulted in a decrease in the rate of MDA formation, presumably because the generation of H_2O_2 exceeded the catalatic capacity of catalase. Although the amount of H_2O_2 produced by xanthine oxidase is dependent upon factors such as substrate concentrations (xanthine and oxygen) and pH (46), the data demonstrate that with 0.005 U/ml of xanthine oxidase activity no further stimulation of lipid peroxidation was observed above 100 U/ml of catalase.

Similarly, when catalase was added to another xanthine oxidase-dependent system in which ferritin was replaced with ADP-Fe^{3+} (at iron concentrations similar to that released from ferritin by xanthine oxidase) enhancement of lipid peroxidation was also noted. These results indicated that the effect of catalase was not unique to ferritin per se (i.e., due to the facilitation of iron release from ferritin) but rather that H_2O_2 is inhibitory to lipid peroxidation when iron is supplied at low concentrations. This premise was supported by the results of studies of iron release from ferritin. The addition of catalase caused a further increase in dipyridyl-Fe^{2+} formation. Catalase may cause this apparent increase in iron release by preventing H_2O_2-dependent oxidation of released ferrous iron or of the dipyridyl-Fe^{2+} complex (47) as dipyridyl-Fe^{3+} has little absorbance at 520 nm. To distinguish between these possibilities varying concentrations of dipyridyl-Fe^{2+} (0-100 nmol Fe^{2+}/ml) were incubated with xanthine and xanthine oxidase (0.025 U/ml) or H_2O_2 (1mM). No oxidation of the ferrous complex was observed in either case over a 30 minute period (results not shown). Therefore it appears that the apparent increase in the amount of iron released from

ferritin, in the presence of catalase, is attributable to its prevention of oxidation of released ferrous iron prior to its complexation by dipyridyl rather than preventing oxidation of the dipyridyl-Fe^{2+} complex. When dipyridyl was included at saturating concentrations no further increase in apparent iron release was observed at catalase concentrations above 1000 U/ml.

The fate of the released iron following oxidation is not known. As iron incorporation into ferritin appears to require oxidation (48) it may be that, in the presence of H_2O_2, the released iron is oxidized and reincorporated into ferritin. Alternatively, H_2O_2 may oxidize the ferrous iron after its chelation by ADP used in these studies. Neither of these possibilities can be ruled out by this work, however, we have demonstrated that H_2O_2 does not affect iron release from ferritin but appears to oxidize ferrous iron, preventing the formation of an initiator of lipid peroxidation.

Although catalase stimulated MDA formation, indicating that initiation of lipid peroxidation is not dependent upon ·OH, it is conceivable that catalase was unable to scavenge all H_2O_2 and some ·OH may still have been generated. To investigate this possibility mannitol, an ·OH trap, was included in the lipid peroxidation system. In either the presence or absence of catalase, mannitol slightly stimulated rates of MDA formation, therefore initiation appears to be dependent upon a species other than ·OH.

Further evidence against ·OH dependent lipid peroxidation was noted in the results of ESR spin trapping experiments. The addition of ferritin to the xanthine oxidase system resulted in the appearance of a DMPO-OH adduct signal when DMPO was added several minutes after

initiation of the reaction to allow ferritin to interact with $O_2^{\cdot-}$. The intensity of the signal was found to correlate with ferritin concentrations and therefore with the amount of iron released. Importantly, the addition of catalase to the incubation resulted in a marked decrease in DMPO-OH signal intensity, indicating that under conditions in which •OH formation is prevented, lipid peroxidation is maximal.

The present studies demonstrate that ferritin can provide the iron necessary for initiation of lipid peroxidation. Furthermore, the data indicate that rapid oxidation of released ferrous iron produces •OH but apparently precludes the formation of a more efficient initiating species. However, irrespective of the mechanism, these findings suggest that the proposed toxicity of $O_2^{\cdot-}$ may be a result of its ability to release iron from ferritin, with the released iron functioning in the iron-oxygen redox reactions leading to the initiation of lipid peroxidation.

LIST OF REFERENCES

1. J.M. McCord. Oxygen-derived free radicals in postischemic tissue injury. New England J. of Medicine 312: 159-163 (1985).

2. B.A. Freeman, M.K. Topolsky, and J.D. Crapo. Hyperoxia increases oxygen radical production in rat lung homogenates. Arch. Biochem. Biophys. 216: 477-484 (1982).

3. P. Hochstein. Futile redox cycling: Implications for oxygen radical toxicity. Fund. Appl. Toxicol. 3: 215-217 (1983).

4. B. Halliwell and J.M.C. Gutteridge. Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem. J. 219: 1-14 (1984).

5. J.M.C. Gutteridge, G.J. Quinlan, and S. Wilkins. Mitomycin C-induced deoxyribose degradation inhibited by superoxide dismutase. FEBS Lett. 167: 37-41 (1984).

6. I. Fridovich. Superoxide radical: An endogenous toxicant. Ann. Rev. Pharmacol. Toxicol. 23: 239-257 (1983).

7. J.M. McCord and E.D. Day, Jr. Superoxide-dependent production of hydroxyl radical catalyzed by the iron-EDTA complex. FEBS Lett. 86: 139-142 (1978).

8. J.V. Bannister, W.H. Bannister, H.A.O. Hill, and P.J. Thornalley. Enhanced production of hydroxyl radicals by the xanthine-xanthine oxidase reaction in the presence of lactoferrin. Biochim. Biophys. Acta 715: 116-120 (1982).

9. J.V. Bannister, P. Bellavite, A. Davioli, P.J. Thornalley, and F. Rossi. The generation of hydroxyl radicals following superoxide production by neutrophil oxidase. FEBS Lett. 150: 300-302 (1982).

10. C.C. Winterbourn. Hydroxyl radical production in body fluids. Roles of metal ions, ascorbate, and superoxide. Biochem. J. 198: 125-131 (1981).

11. J.M.C. Gutteridge, D.A. Rowley, and B. Halliwell. Superoxide-dependent formation of hydroxyl radicals and lipid peroxidation in the presence of iron salts. Biochem. J. 206: 605-609 (1982).

12. A. Jacobs. Low molecular weight intracellular iron transport compounds. Blood 50: 433-439 (1977).

13. G.R. Bartlett. Phosphate compounds in rat erythrocytes and reticulocytes. Biochem. Biophys. Res. Comm. 70: 1055-1062 (1976).

14. S. Pollack and T. Campana. Low molecular weight non-heme iron and a highly labeled heme pool in the reticulocyte. Blood 56: 564-566 (1980).

15. K.L. Fong, P.B. McCay, J.L. Poyer, H.P. Misra and B.B. Keele. Evidence for superoxide-dependent reduction of Fe^{3+} and its role in enzyme-generated hydroxyl radical formation. Chem. Biol. Inter. 15: 77-89 (1976).

16. C.G.D. Morley and A. Bezkorovainy. Identification of the iron chelate in hepatocyte cytosol. IRCS Med. Sci. 11: 1106-1107 (1983).

17. P.M. Harrison. Ferritin: An iron-storage molecule. Sem. Hematol 14: 55-70 (1977).

18. P. Aisen and I. Listowsky. Iron transport and storage proteins. In: Annual Review of Biochemistry, Volume 49 (E.E. Snell, P.D. Boyer, A. Meister and C.C. Richardson, eds.), pp. 357-393, Annual Reviews, Inc., Pala Alto, CA (1980).

19. R.R. Crichton. Iron uptake and utilization by mammalian cells. II. Intracellular iron utilization. Trends Biochem. Sci. 9: 283-285 (1984).

20. E.C. Theil. Ferritin: Structure, function, and regulation. In: Iron Binding Proteins Without Cofactors or Sulfur Clusters (E.C. Theil, G.L. Eichhorn, and L.G. Marzilli, eds.), pp. 1-38, Elsevier, New York (1983).

21. S. Granick and L. Michaelis. Ferritin II. Apoferritin of horse spleen. J. Biol. Chem. 147: 91-97 (1943).

22. R.R. Crichton. Ferritin. Struct. Bond 17: 67-134 (1973).

23. A. Mazur, S. Baez, and E. Shorr. The mechanism of iron release from ferritin as related to its biological properties. J. Biol. Chem. 213: 147-160 (1955).

24. R.R. Crichton, F. Roman, and F. Roland. Iron mobilization from ferritin by chelating agents. J. Inorg. Biochem. 13: 305-316 (1980).

25. J. Dognin and R.R. Crichton. Mobilization of iron from ferritin fractions of defined iron content by biological reductants. FEBS Lett. 54: 234-236 (1975).

26. A. Mazur and A. Carleton. Hepatic xanthine oxidase and ferritin iron in the developing rat. Blood 26: 317-322 (1965).

27. A. Mazur, S. Green, A. Saha and A. Carleton. Mechanism of release of ferritin iron in vivo by xanthine oxidase. J. Clin. Invest. 37: 1809-1817 (1958).

28. R.W. Topham, M.C. Walker, and M.P. Calisch. Liver xanthine dehydrogenase and iron mobilization. Biochem. Biophys. Res. Comm. 109: 1240-1246 (1982).

29. J.M.C. Gutteridge, B. Halliwell, A. Treffry, P.M. Harrison, and D. Blake. Effect of ferritin-containing fractions with different iron loading on lipid peroxidation. Biochem. J. 209: 557-560 (1983).

30. E.D. Wills. Mechanisms of lipid peroxide formation in animal tissues. Biochem. J. 99: 667-676 (1966).

31. E.D. Wills. Lipid peroxide formation in microsomes. The role of non-haem iron. Biochem. J. 113: 325-332 (1969).

32. J.M. McCord and I. Fridovich. The reduction of cytochrome c by milk xanthine oxidase. J. Biol. Chem. 243: 5753-5760 (1968).

33. J.M. McCord and I. Fridovich. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J. Biol. Chem. 244: 6049-6055 (1969).

34. R.F. Beers, Jr. and I.W. Sizer. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. 195: 133-140 (1952).

35. J.W. Halliday. Immunoassay of ferritin in plasma. In: Methods in Enzymology, Volume 84 (J. Langone and J. Van Vunakis, eds.) pp. 148-171, Academic Press, New York (1982).

36. T.C. Pederson and S.D. Aust. Aminopyrine demethylase. Kinetic evidence for multiple microsomal activities. Biochem. Pharmacol. 19: 2221-2230 (1970).

37. J. Folch, M. Lees, and G.H. Sloane-Stanley. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226: 497-509 (1957).

38. T.C. Pederson, J.A. Buege and S.D. Aust. Microsomal electron transport. The role of reduced nicotinamide adenine dinucleotide phosphate-cytochrome c reductase in liver microsomal lipid peroxidation. J. Biol. Chem. 248: 7134-7141 (1973).

39. G.R. Bartlett. Phosphorus assay in column chromatography. J. Biol. Chem. 234: 466-468 (1959).

40. J.A. Buege and S.D. Aust. Microsomal lipid peroxidation. In: Methods in Enzymology, Volume 52 (S. Fleischer and L. Packer, eds.) pp. 302-310, Academic Press, New York (1978).

41. P.E. Brumby and V. Massey. Determination of non-heme iron, total iron, and copper. In: Methods in Enzymology, Volume 10 (R.W. Estabrook and M.D. Pullman, eds.) pp. 463-474, Academic Press, New York (1967).

42. G.W. Ruddock, J.A. Raleigh, and C.L. Greenstock. Reactivity of chemically generated superoxide radical anion with peroxides as determined by competition kinetics. Biochem. Biophys. Res. Comm. 102: 554-560 (1981).

43. G.R. Buettner. The spin trapping of superoxide and hydroxyl radicals. In: Superoxide Dismutase, Volume II (L.W. Oberley, ed.) pp. 63-81, CRC Press, Boca Raton (1982).

44. G.E. Shull and E.C. Theil. Regulation of ferritin mRNA: A possible gene-sparing phenomenon. J. Biol Chem. 258: 7921-7923 (1983).

45. E.W. Kellogg, III and I. Fridovich. Superoxide, hydrogen peroxide and singlet oxygen in lipid peroxidation by a xanthine oxidase system. J. Biol. Chem. 250: 8812-8817 (1975).

46. I. Fridovich. Quantitative aspects of the production of superoxide anion radical by milk xanthine oxidase. J. Biol. Chem. 245: 4053-4057 (1970).

47. G.W. Winston, W. Harvey, L. Berl and A.I. Cederbaum. The generation of hydroxyl and alkoxyl radicals from the interaction of ferrous bipyridyl with peroxides. Biochem. J. 216: 415-421 (1983).

48. M. Wauters, A.M. Michelson, and R.R. Crichton. Studies on the mechanism of ferritin formation. FEBS Lett. 91: 276-280 (1978).

CHAPTER II

PARAQUAT AND FERRITIN-DEPENDENT LIPID PEROXIDATION

ABSTRACT

A lipid peroxidation system consisting of phospholipid liposomes, paraquat, ADP, and NADPH-cytochrome P450 reductase was constituted using ferritin as the sole source of iron. Lipid peroxidation was inhibited by SOD, essentially not affected by mannitol, but markedly stimulated by catalase. Similar effects of these scavenging agents were observed in incubations void of ADP. These data suggest that $O_2^{\cdot-}$, produced by the redox cycling of paraquat, can release iron from ferritin and thereby promote lipid peroxidation. The effects of catalase and mannitol suggest that the initiation of peroxidation, in either the presence or absence of ADP, is not significantly dependent upon the hydroxyl radical produced via an iron-catalyzed Haber-Weiss reaction.

INTRODUCTION

Paraquat (methyl viologen, 1-1-dimethyl-4,4'-bipyridylium dichloride), is a broad spectrum herbicide widely used as a non-selective weed killer which has been shown to be highly lethal to man and animals (1-4). The target organ appears to be the lung, most probably because of active uptake (5) but biochemical changes are also seen in kidney, thymus, and adrenals of paraquat-treated animals (6-9). Damage is thought to be related to increased $O_2^{\cdot-}$ production, formed as a result of NADPH-cytochrome P450 reductase catalyzed reduction and autoxidation of the herbicide (10-17). As $O_2^{\cdot-}$ is relatively unreactive towards most organic compounds in aqueous media (18), others have suggested NADPH or GSH depletion as mechanisms of toxicity (19-20).

However, it must be considered that in the presence of transition metal ions such as copper or iron, $O_2^{\cdot-}$ is converted to a more reactive, partially reduced oxygen species capable of initiating lipid peroxidation. The nature of the initiating species is controversial but is generally thought to be $\cdot OH$ or an iron-oxygen complex (21-25). Irrespective of mechanism, there is a requirement for metal ions for formation of an initiator. Thus, while it is likely that iron may have an important role in mediating paraquat toxicity, the ability of physiological iron to participate in such redox reactions is relatively unknown. Most investigators envision an intracellular low molecular weight iron chelate pool, however, the identity of this complex(es) remains to be established. Nucleotide-chelated iron

complexes have been isolated from erythrocytes (26,27), however, their existence in other tissues has not been conclusively demonstrated.

The majority of iron in most cells is stored within ferritin as ferric hydroxide complexed with phosphate (28).Mobilization of iron from within ferritin appears to require reduction (29), but biological reductants which release iron at rates considered to be of physiological significance have not been identified (30). Previous work in this laboratory demonstrated that $O_2^{\cdot-}$, generated by xanthine oxidase, can release iron from ferritin and promote the peroxidation of phospholipid liposomes (31).

As paraquat also produces $O_2^{\cdot-}$ by redox cycling, we have investigated the ability of paraquat and NADPH-cytochrome P-450 reductase to initiate the peroxidation of liposomes using ferritin as the sole source of iron. The results show that the redox cycling of paraquat promotes ferritin-dependent lipid peroxidation. Inhibition of peroxidation by SOD suggests that $O_2^{\cdot-}$ is required for ferritin iron release, as was observed with xanthine oxidase (31). These data indicates that ferritin may function *in vivo* as a source of iron for the potentiation of peroxidative damage associated with paraquat intoxication.

MATERIALS AND METHODS

Materials

Paraquat dichloride, NADPH (Type III), ADP (Grade III), cytochrome c (Type VI), 2-TBA, butylated hydroxytoluene, mannitol and 4,7-diphenyl-1,10-phenanthroline were purchased from Sigma Chemical Company (St. Louis, MO). Horse spleen ferritin was a product of United States Biochemical Corporation (Cleveland, OH). EDTA and H_2O_2 were obtained from Mallinckrodt Chemical Company (Paris, KY) and ferric chloride was purchased from Baker Chemical Company (Phillipsburg, NJ). Sephadex G-25 was obtained from Pharmacia (Piscataway, NJ) and Chelex 100 from BioRad Laboratories (Richmond, CA). All other chemicals were of analytical grade or better and used without further purification. All buffers and reagents were passed through Chelex 100 ion-exchange resin to free them of contaminating transition metal ions.

Enzymes

Bovine erythrocyte SOD (EC 1.15.1.1) was obtained from Sigma Chemical Company and catalase (EC 1.11.1.6) was purchased from Millipore (Freehold, NJ). Protease-solubilized NADPH-cytochrome P450 reductase (EC 1.6.2.4) was prepared from liver microsomes of male Sprague-Dawley rats (250-300 g), previously pretreated with 0.1% phenobarbital in their drinking water for 10 days, as previously described (32). The reductase was desalted on Sephadex G-25 columns equilibrated with 0.3 M NaCl, pH 7.0 prior to use with a unit of

activity defined as 1 μmol cytochrome c reduced/min/ml. Superoxide dismutase activity was measured by the method of McCord and Fridovich (33) and catalase activity by the procedure of Beers and Sizer (34).

Preparation of Ferritin and Assay for Total Iron

Horse spleen ferritin was incubated on ice in 10 mM EDTA for 1 hour and passed over a Sephadex G-25 column equilibrated with 0.3 M NaCl, pH 7.0 to remove loosely associated iron. Total iron was determined essentially by the method of Brumby and Massey (35) with minor modification (31).

Preparation of Microsomal Lipid and Liposomes

Rat liver microsomes were isolated by the procedure of Pederson and Aust (36) and microsomal lipid was extracted by the method of Folch et al. (37). All solvents utilized were purged with argon and all steps performed at 4°C to minimize autoxidation of unsaturated lipids. Phospholipid liposomes were subsequently prepared by indirect, anaerobic sonication (38) and lipid phosphate determined by the method of Bartlett (39).

Lipid Peroxidation Assays

Paraquat-dependent peroxidation of liposomes was performed by incubating liposomes (1 μmol lipid phosphate/ml) with NADPH, NADPH-cytochrome P450 reductase, paraquat, ferritin and ADP as specified in the figure legends. In some experiments lipid peroxidation assays were performed without ADP. Reaction mixtures were constituted in 0.25 M NaCl,pH 7.0, and incubated at 37°C in a Dubnoff metabolic shaker under an air atmosphere. Although unbuffered, incubations remained at pH 7.0 throughout the course of

the experiments. Reactions were initiated by the addition of NADPH and peroxidation was monitored by taking aliquots from the incubations at 0, 10, 20, and 30 min. and measuring MDA formation by the TBA assay (40).Butylated hydroxytoluene (0.03 volumes of 2% BHT in ethanol) was added to the TBA reagent to prevent further peroxidation of lipid during the assay procedure.

RESULTS

Paraquat and Ferritin-Dependent Lipid Peroxidation

As shown in Figure 1, incubation of NADPH-cytochrome P450 reductase, paraquat, and ferritin promoted peroxidation of phospholipid liposomes as evidenced by MDA production. Increasing the activity of NADPH-cytochrome P450 reductase in the reaction mixture beyond 0.025 U/ml had an inhibitory effect on MDA formation. The addition of catalase (50 U/ml) markedly promoted the rate of lipid peroxidation at all NADPH-cytochrome P450 reductase activities used. In addition, the activity of NADPH-cytochrome P450 reductase yielding the greatest rate of lipid peroxidation shifted from 0.025 U/ml to 0.05 U/ml when catalase was added.

As shown in Figure 2A, increasing the concentration of paraquat beyond 1.0 mM also had an inhibitory effect on rates of lipid peroxidation. In the absence of paraquat, only very low rates of peroxidation were observed even when ferritin was present in the reaction mixtures. On the other hand, increasing the ferritin concentration (in the presence of paraquat) resulted in a concomitant increase in rates of lipid peroxidation (Figure 2B).

Effect of SOD, Catalase, and Mannitol on Paraquat and Ferritin-Dependent Lipid Peroxidation

Ten units/ml of SOD inhibited the paraquat and ferritin-dependent lipid peroxidation (data not shown) indicating that initiation of lipid peroxidation in this system was $O_2^{\cdot-}$-dependent. As catalase stimulated the rate of paraquat and ferritin-dependent lipid peroxidation (Figure 1), the effect of increasing catalase

Figure 1. The Effect of Varying NADPH-Cytochrome P450 Reductase Activity on Paraquat and Ferritin-Dependent Peroxidation of Phospholipids in the Presence or Absence of Catalase. Reaction mixtures (2.5 ml final volume) contained phospholipid liposomes (1 μmol lipid phosphate/ml), ferritin (1 mM Fe^{3+}), ADP (2.5 mM), paraquat (0.5 mM), catalase (50 U/ml where indicated), NADPH (0.2 mM) and varying activities of NADPH-cytochrome P450 reductase in 0.25 M NaCl, pH 7.0. Aliquots from the reaction mixture were assayed for MDA as in Materials and Methods.

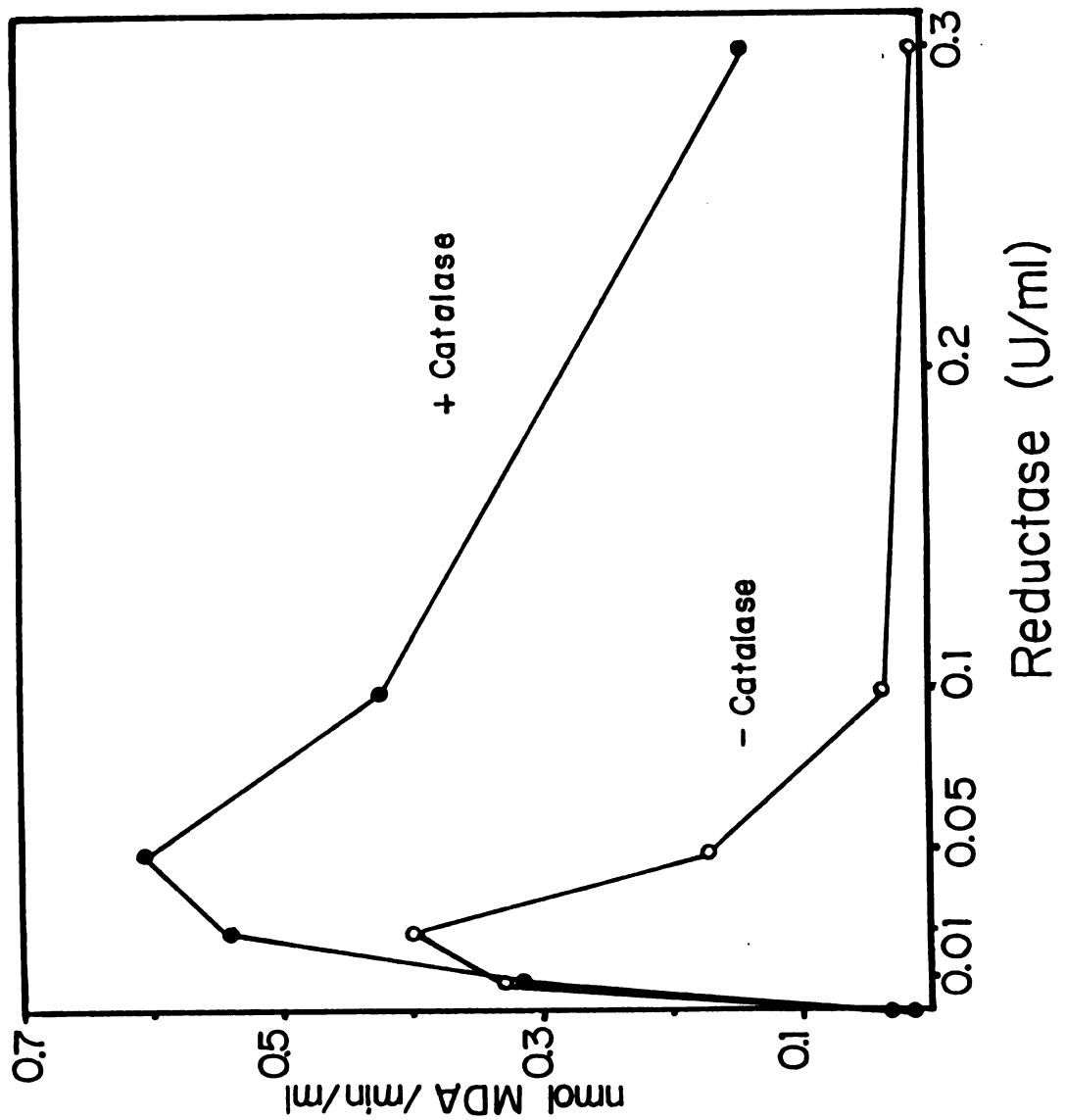
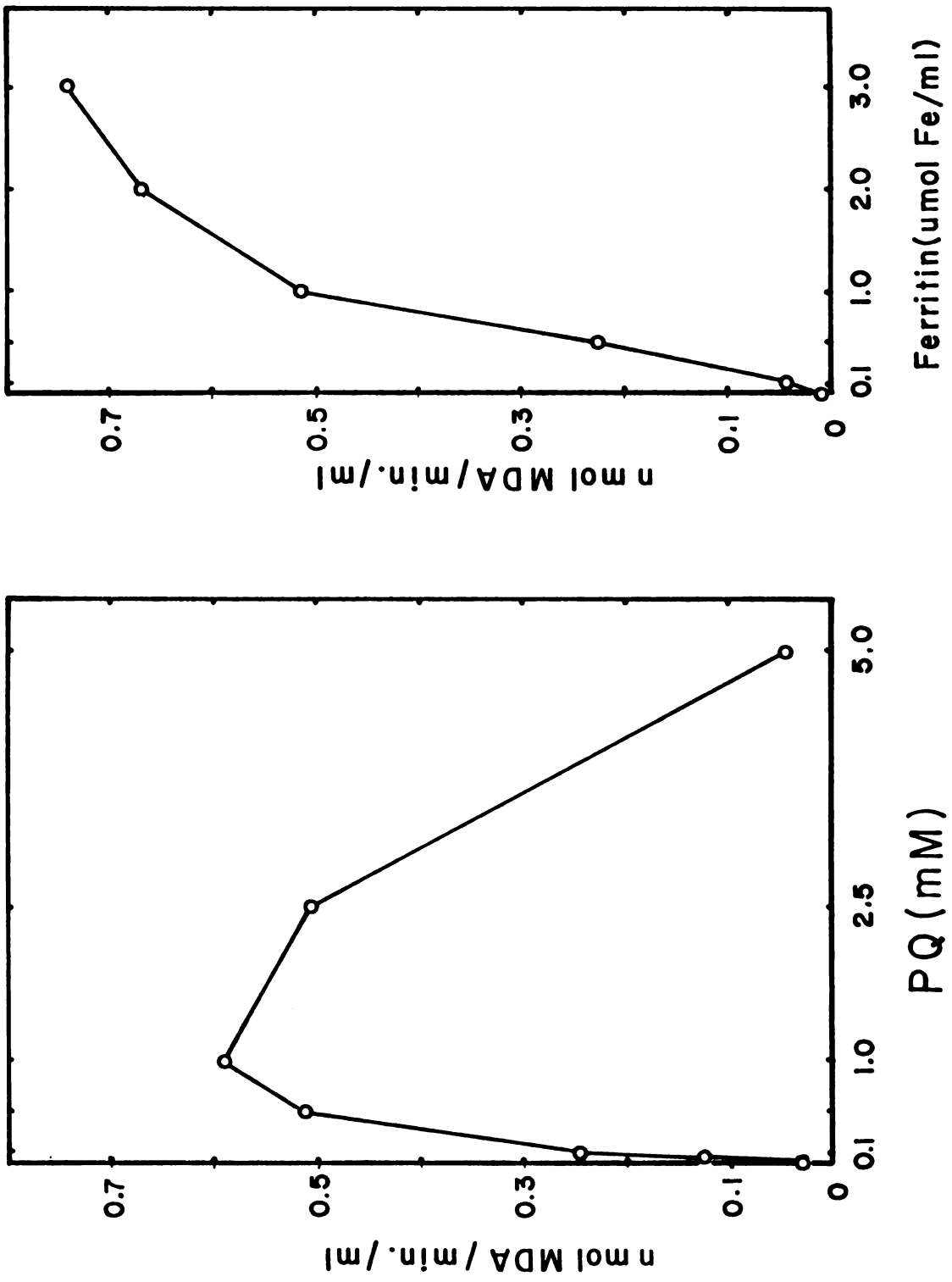

nmol MDA /min/ml
0.7
0.5
0.3
0.1
0.01
0.05
0.1
0.2
0.3
Reductase (U/ml)
+ Catalase
- Catalase

Figure 2. The Effect of Varying Paraquat (A) or Ferritin (B) Concentration on Peroxidation of Phospholipids. Reaction mixtures (2.5 ml final volume) contained phospholipid liposomes (1 µmol lipid phosphate/ml), ADP (2.5 mM), NADPH (0.2 mM) and NADPH-cytochrome P450 reductase (0.025 U/ml) in 0.25 M NaCl, pH 7.0. In (A) ferritin was 1 mM Fe^{3+} and in (B) paraquat was 0.5 mM. MDA content was assayed as indicated in Materials and Methods.


n mol MDA / min. / ml
0
0.1
0.3
0.5
0.7
0.1
1.0
2.5
5.0
PQ (mM)
n mol MDA / min. / ml
0
0.1
0.3
0.5
0.7
0.1
1.0
2.0
3.0
Ferritin(umol Fe/ml)

concentrations was assessed. As shown in Figure 3, 50 U/ml of catalase gave maximum rates of lipid peroxidation with 0.025 U/ml of NADPH-cytochrome P450 reductase activity. The marked stimulation of peroxidation by catalase suggests that ·OH is not involved in the peroxidative process. These results were supported by the inability of mannitol, in the absence of catalase, to inhibit peroxidation up to 50 mM mannitol (Figure 4).

Paraquat and Ferritin-Dependent Lipid Peroxidation in the Absence of ADP

The effect of varying ADP concentration on paraquat and ferritin-dependent lipid peroxidation is shown in Figure 5. Increasing the ADP concentration resulted in a decrease in the rates of lipid peroxidation with the greatest rate of lipid peroxidation obtained in the absence of ADP. Others have suggested that the chelation of iron with ADP may affect the mechanism of initiation of lipid peroxidation (41). Therefore, the effects of SOD, catalase and mannitol on paraquat and ferritin-dependent lipid peroxidation in the absence of ADP were also investigated. Superoxide dismutase again inhibited the paraquat and ferritin-dependent lipid peroxidation at 10 units/ml, (data not shown) indicating again that initiation of lipid peroxidation in this system is $O_2^{\cdot-}$-dependent. Catalase also stimulated the rate of lipid peroxidation but to a lesser extent than was observed in the same system with ADP (Figure 6).

The effect of varying mannitol concentration on paraquat and ferritin-dependent lipid peroxidation in the absence of ADP, either in the presence or absence of catalase, is shown in Figure 7. As shown previously (Figure 6) catalase stimulated activity slightly.

Figure 3. The Effect of Varying Catalase Activity on Peroxidation of Phospholipids Catalyzed by Paraquat and Ferritin in the Presence of ADP. Reaction mixtures (2.5 ml final volume) contained phospholipid liposomes (1 µmol lipid phosphate/ml), NADPH (0.2 mM), NADPH-cytochrome P450 reductase (0.025 U/ml), ADP (2.5 mM), paraquat (0.5 mM), ferritin (1 mM Fe^{3+}) and varying activities of catalase in 0.25 M NaCl, pH 7.0. MDA was quantitated as described in Materials and Methods.

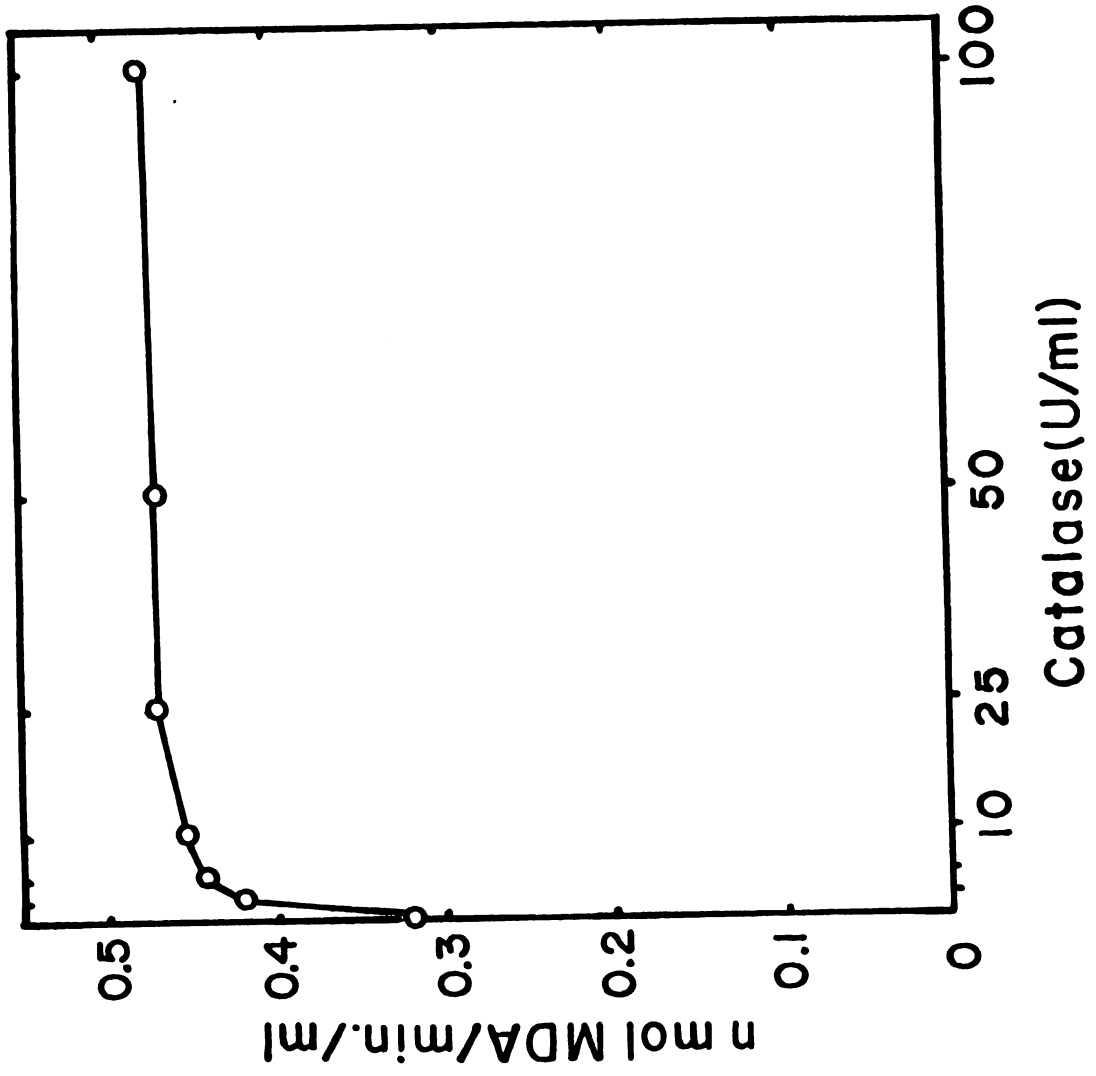

n mol MDA/min./ml
0.5
0.4
0.3
0.2
0.1
0
10
25
50
100
Catalase(U/ml)

Figure 4. The Effect of Varying Mannitol Concentration on Paraquat and Ferritin-Dependent Peroxidation of Phospholipids in the Presence of ADP and Absence of Catalase. Reaction mixtures (2.5 ml final volume) contained phospholipid liposomes (1 μmol lipid phosphate/ml), ADP (2.5 mM), ferritin (1 mM Fe^{3+}), paraquat (0.5 mM), NADPH (0.2 mM), NADPH-cytochrome P450 reductase (0.025 U/ml) and varying concentrations of mannitol in 0.25 M NaCl, pH 7.0. Aliquots from the reaction mixtures were assayed for MDA content.

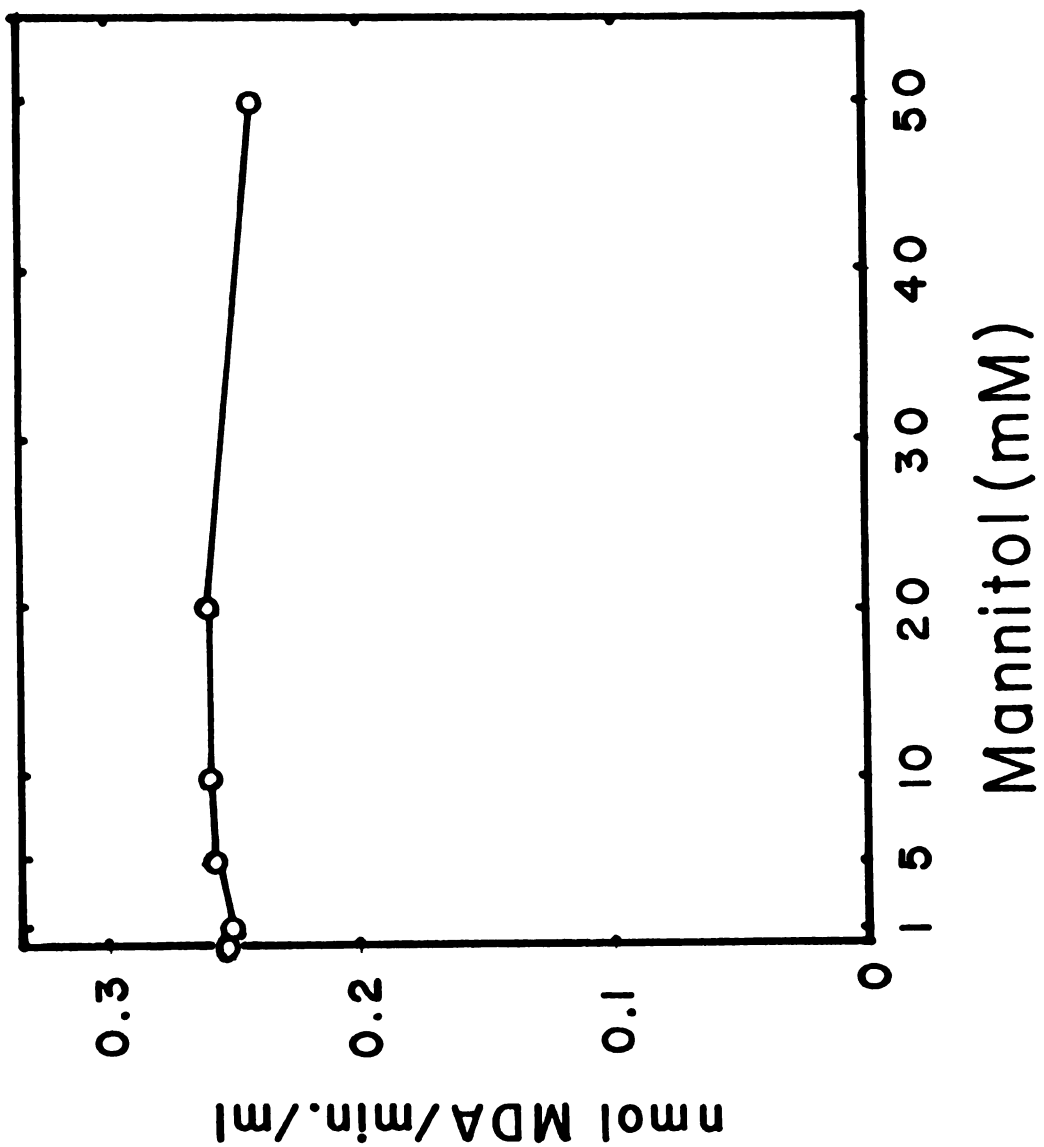

nmol MDA/min./ml
0.3
0.2
0.1
0
1
5
10
20
30
40
50
Mannitol (mM)

Figure 5. The Effect of Varying ADP Concentration on Paraquat and Ferritin-Dependent Peroxidation of Phospholipids. Reaction mixtures (2.5 ml final volume) contained phospholipid liposomes (1 μmol lipid phosphate/ml), ferritin (1 mM Fe^{3+}), paraquat (0.5 mM), NADPH (0.2 mM), NADPH-cytochrome P450 reductase (0.025 U/ml) and varying concentrations of ADP in 0.25 M NaCl, pH 7.0. MDA content was measured as in Materials and Methods.

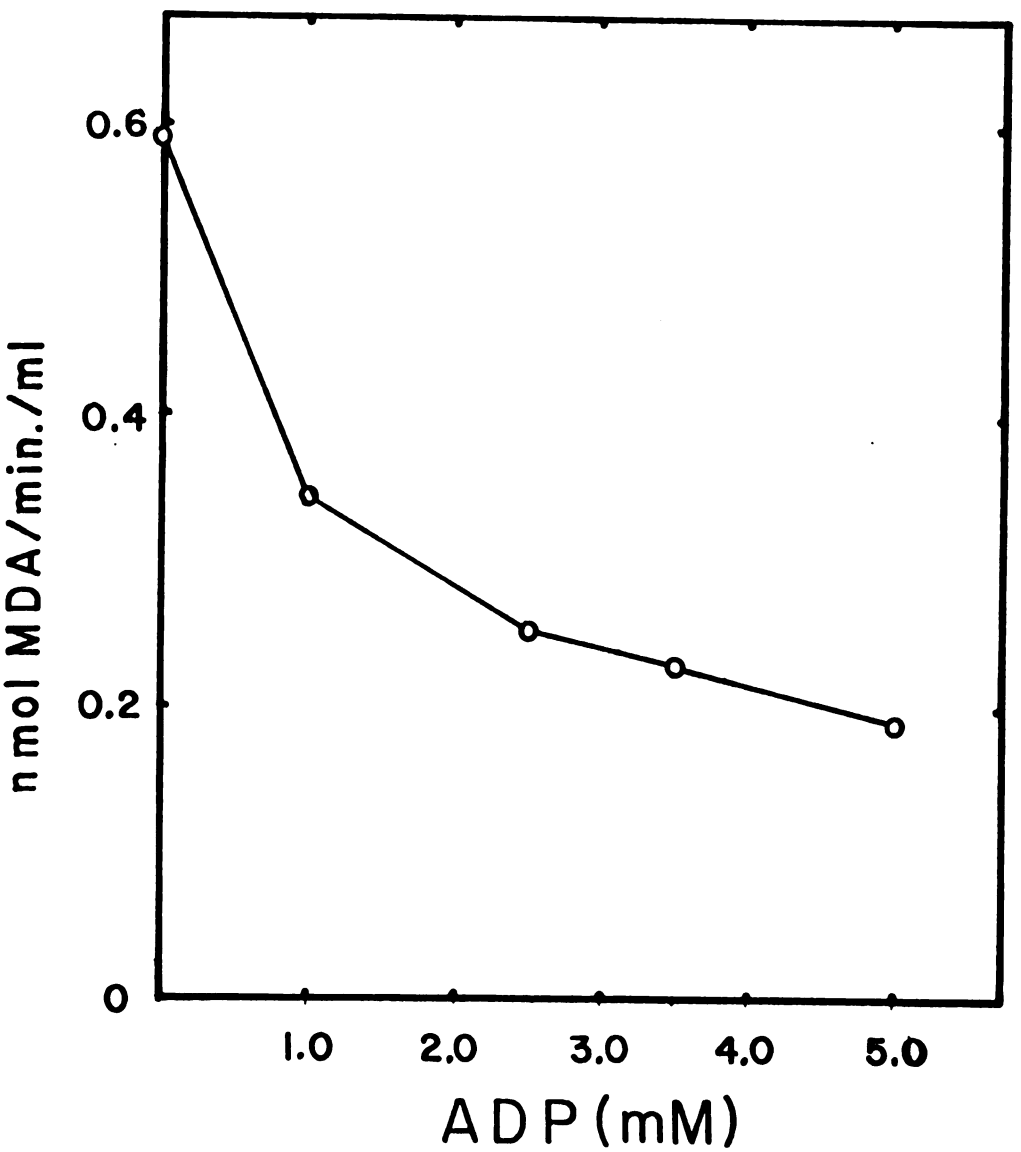

0.6
0.4
0.2
0
n mol MDA/min./ml
1.0
2.0
3.0
4.0
5.0
ADP (mM)

Figure 6. The Effect of Varying Catalase Activity on Paraquat and Ferritin-Dependent Peroxidation of Phospholipids in the Absence of ADP. Reaction mixtures (2.5 ml final volume) contained phospholipid liposomes (1 μmol lipid phosphate/ml), ferritin (1 mM Fe^{3+}), paraquat (0.5 mM), NADPH (0.2 mM), NADPH-cytochrome P450 reductase (0.025 U/ml), and varying activities of catalase in 0.25 M NaCl, pH 7.0. Aliquots of incubations were assayed for MDA as outlined in Materials and Methods.

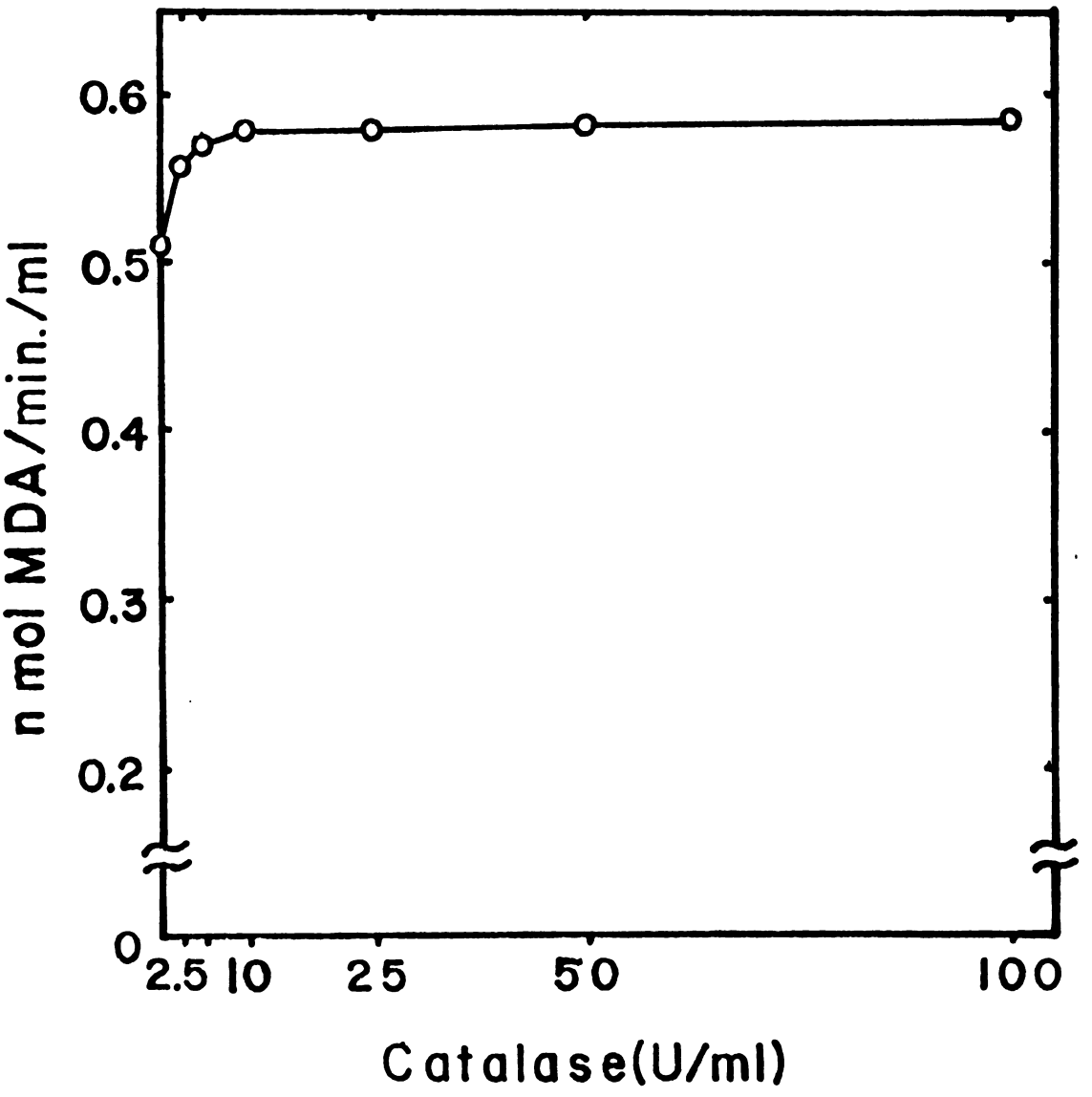
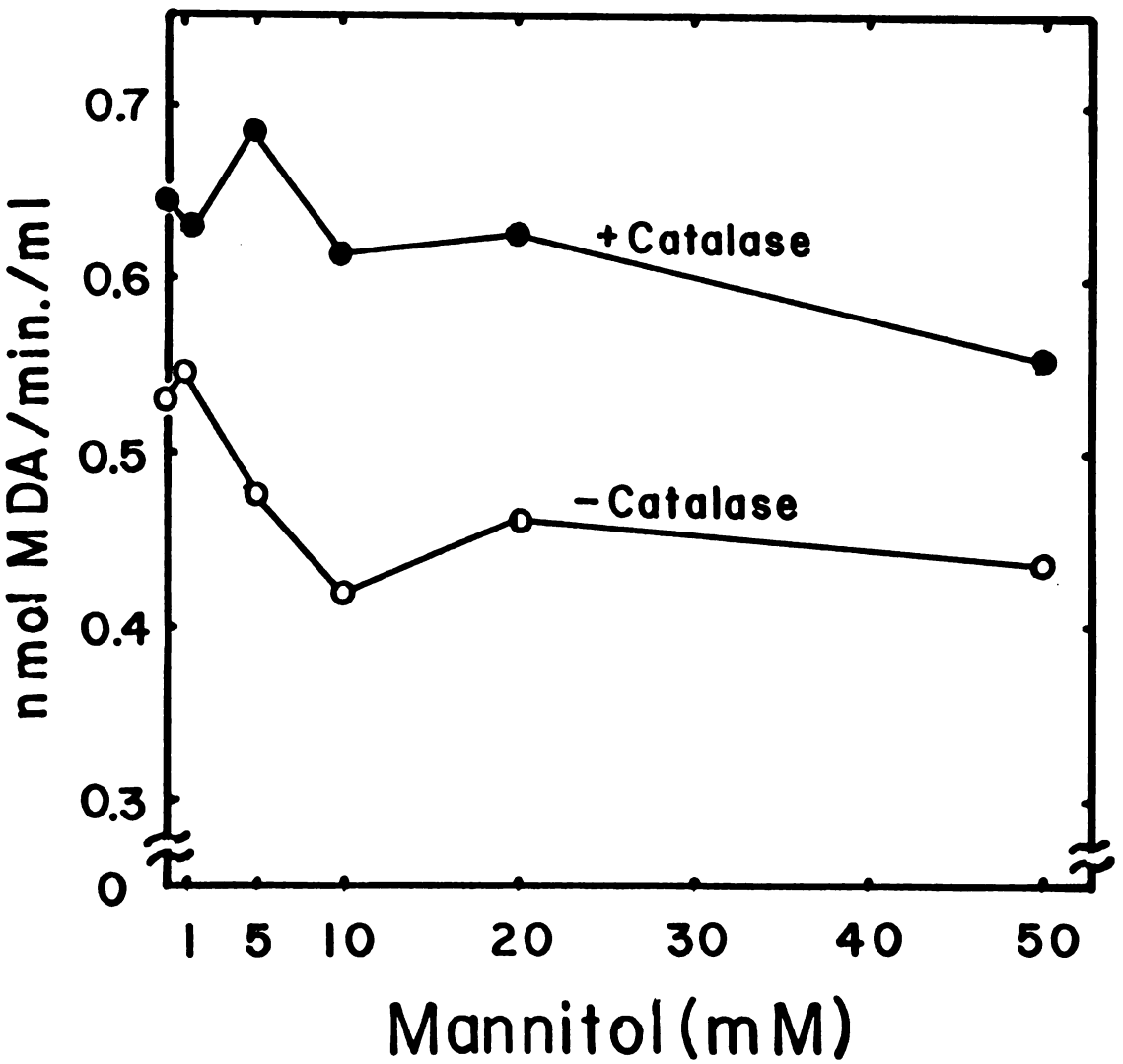

0.6
0.5
0.4
0.3
0.2
0
n mol MDA/min./ml
2.5
10
25
50
100
Catalase(U/ml)

Figure 7. The Effect of Varying Mannitol Concentration on Paraquat and Ferritin-Dependent Peroxidation of Phospholipids in the Presence or Absence of Catalase. Reaction mixtures (2.5 ml final volume) contained phospholipid liposomes (1 μmol lipid phosphate/ml), ferritin (1 mM Fe^{3+}), paraquat (0.5 mM), catalase (10 U/ml, where indicated), NADPH (0.2 mM), NADPH-cytochrome P450 reductase (0.025 U/ml) and varying concentrations of mannitol in 0.25 M NaCl, pH 7.0. Aliquots of reaction mixtures were assayed for MDA content as described in Materials and Methods.


0.7
0.6
0.5
0.4
0.3
0
nmol MDA/min./ml
+Catalase
-Catalase
1
5
10
20
30
40
50
Mannitol (mM)

The effects of mannitol were somewhat variable at low concentration but it was essentially without effect in both the presence and absence of catalase.

DISCUSSION

These studies demonstrate that the redox cycling of paraquat can promote lipid peroxidation in a model system consisting of phospholipid liposomes, NADPH-cytochrome P450 reductase, and ferritin. In the absence of either ferritin, NADPH-cytochrome P450 reductase, or paraquat almost no MDA formation was observed, demonstrating a requirement for both $O_2^{\cdot-}$ and a source of iron. Previous work using a xanthine oxidase system had shown that the primary function of $O_2^{\cdot-}$ in initiating ferritin-dependent lipid peroxidation is the release of ferrous iron from ferritin (31). The inability of NADPH-cytochrome P450 reductase to directly reduce ADP-Fe^{3+} (32), and the inhibition of peroxidation by SOD, provides further evidence that $O_2^{\cdot-}$ produced by reduced paraquat autoxidation releases ferritin bound iron.

As iron is released from ferritin in the ferrous state (31), and H_2O_2 is formed by the non-enzymatic dismutation of $O_2^{\cdot-}$, the generation of $\cdot OH$ by Fenton's reaction in this system seemed likely. Thus the effect of mannitol, an $\cdot OH$ trap, on liposomal peroxidation was assessed. A wide range of mannitol concentrations up to 50 mM had no effect on rates of MDA formation. Therefore, initiation of peroxidation in this system appeared to be independent of $\cdot OH$.

To examine this possibility further, catalase was added to scavenge H_2O_2 and prevent $\cdot OH$ formation. Interestingly, catalase markedly stimulated paraquat and ferritin-dependent lipid peroxidation. Increasing either NADPH-cytochrome P450 reductase activity or paraquat concentration, both of which increase $O_2^{\cdot-}$ production, inhibited

peroxidation presumably by increasing H_2O_2 production. Even in the presence of catalase, high NADPH-cytochrome P450 reductase activity still resulted in a decrease in the rate of MDA formation, presumably as the catalatic activity of catalase was exceeded. Accordingly, at NADPH-cytochrome P450 reductase activities greater than 0.05 U/ml increasing the concentration of catalase resulted in a further stimulation of lipid peroxidation (results not shown). These data demonstrate that with 0.025 U/ml of NADPH-cytochrome P450 reductase activity and 0.5 mM paraquat no further stimulation of peroxidation was observed beyond 50 U/ml of catalase, suggesting that all H_2O_2 was effectively scavenged.

Stimulation of liposomal peroxidation by catalase was previously reported using ferritin and xanthine oxidase (31). A negative correlation between $\cdot OH$ production and lipid peroxidation was demonstrated with ESR spin trapping. Iron release studies suggested that H_2O_2 did not affect iron release from ferritin but inhibited peroxidation by facilitating oxidation of the released ferrous iron. It is likely that in the present system the H_2O_2, generated by dismutation of $O_2^{\cdot-}$ or perhaps directly by paraquat radical autoxidation (42), similarly functions to prevent the more efficient formation of an initiator other than $\cdot OH$.

The greatest rate of MDA formation was observed in the absence of ADP. Thus, it was of interest to determine whether ADP affected the mechanism of initiation of peroxidation. Therefore, the effects of SOD, catalase and mannitol on lipid peroxidation in the absence of ADP were also examined. As obtained in the presence of ADP, SOD significantly inhibited peroxidation. Therefore, $O_2^{\cdot-}$ remains an

absolute requirement for peroxidation, even in the absence of ADP. Catalase again stimulated rates of peroxidation, however the degree of stimulation was less than in the presence of ADP. This effect suggests that ADP affects the rate of $O_2^{\cdot-}$ dismutation, or that oxidation of free ferrous is slower than that of ADP-Fe^{2+} as has been shown (43), thus peroxidation would not be as markedly enhanced by catalase. Since catalase still stimulated MDA formation in the absence of ADP, initiation of lipid peroxidation still appeared to be independent of ·OH. Contribution of ·OH to initiation of lipid peroxidation in the absence of chelators (EDTA or ADP) was recognized by Tien et al. (41) using a Fenton-type reaction. In the present system, the inability of mannitol to inhibit lipid peroxidation indicated that the ·OH may not be involved. Thus, it appears that the addition of ADP does not significantly affect the apparent mechanism(s) of initiation of lipid peroxidation.

The toxicity of paraquat has been attributed to its enhancement of cellular $O_2^{\cdot-}$ production with concomitant increased lipid peroxidation (10,13-17). Superoxide is relatively unreactive towards most organic compounds (18) in aqueous media, thus it likely participates in the formation of other more reactive species capable of initiating peroxidation. Superoxide is generally proposed to function in the iron-catalyzed Haber-Weiss reaction to generate ·OH; however, the indiscriminate reactivity of ·OH and the potentially limited availability of free iron has led to suggestions that ·OH may not be the initiator in vivo. This hypothesis is supported by the inability of ·OH scavengers to inhibit in vitro microsomal peroxidation (44) or to ameliorate toxicity when given to paraquat

treated animals (45). In most instances, SOD effectively inhibits paraquat mediated lipid peroxidation thus its participation must be via a mechanism other than the Haber-Weiss reaction. The above findings can be rationalized by these studies which suggest that paraquat may enhance lipid peroxidation by generating $O_2^{\cdot-}$ which can release iron from ferritin. Thus, what normally provides a secure means of sequestering iron (ferritin) now may serve as a source of iron for the initiation of peroxidation.

While these studies demonstrate that paraquat can stimulate NADPH and ferritin-dependent lipid peroxidation, the role of lipid peroxidation in the *in vivo* toxicity of paraquat remains to be determined. Certainly, lipid peroxidation is not the only potentially destructive reaction and may not wholly account for the observed pulmonary toxicity. We have shown, by Ouchterlony double diffusion analysis, that rat lung microsomes contain significant quantities of ferritin (C.E. Thomas and S.D. Aust, unpublished). Therefore, interaction of $O_2^{\cdot-}$ with ferritin in the lung may provide the iron necessary for the initiation of lipid peroxidation.

LIST OF REFERENCES

1. C.M. Bullivant. Accidental poisoning by paraquat: Report of two cases in man. Br. Med. J. 1: 1272-1273 (1966).

2. D.F. Kelly, D.G. Morgan, P.G.G. Darke, H. Pearson, and B.M.G. Weaver. Pathology of acute respiratory distress in the dog associated with paraquat poisoning. J. Comp. Pathol. 88: 275-294 (1978).

3. R.D. Kimbrough and T.B. Gaines. Toxicity of paraquat to rats and its effect on rat lungs. Toxicol. Appl. Pharmacol. 17: 679-690 (1970).

4. R.E. Murray and J.E. Gibson. A comparative study of paraquat intoxication in rats, guinea pigs and monkeys. Exp. Mol. Pathol. 17: 317-325 (1972).

5. M.S. Rose, L.L. Smith and J. Wyatt. Evidence for energy-dependent accumulation of paraquat into rat lung. Nature (London) 252: 314-315 (1974).

6. D.G. Clark, T.F. McElligott and E.W. Hurst. The toxicity of paraquat. Br. J. Ind. Med. 23: 126-132 (1966).

7. D.M. Conning, K. Fletcher and A.A.B. Swan. Paraquat and related bipyridyls. Br. Med. Bull. 25: 245-249 (1969).

8. G.S. Vijeyaratnam and B. Corrin. Experimental paraquat poisoning: A histological and electron-optical study of the changes in the lung. J. Pathol. 103: 123-129 (1971).

9. A.P. Autor. Reduction of paraquat toxicity by superoxide dismutase. Life Sci. 14: 1309-1319 (1974).

10. J.S. Bus, S.D. Aust and J.E. Gibson. Superoxide- and singlet oxygen-catalyzed lipid peroxidation as a possible mechanism for paraquat (methyl viologen) toxicity. Biochem. Biophys. Res. Commun. 58: 749-755 (1974).

11. R.E. Brooks. Ultrastructure of lung lesions produced by ingested chemicals. I. Effect of the herbicide paraquat on mouse lung. Lab. Invest. 25: 536-545 (1971).

12. H.M. Hassan and I. Fridovich. Superoxide radical and the oxygen enhancement of the toxicity of paraquat in Escherischia coli. J. Biol. Chem. 253: 8143-8148 (1978).

13. K.F. Ilett, B. Stripp, K.H. Menard, W.D. Reid and J.R. Gillette. Studies on the mechanism of the lung toxicity of paraquat: Comparison of tissue distribution and some biochemical parameters in rats and rabbits. Toxicol. Appl. Pharmacol. 28: 216-226 (1974).

14. J.S. Bus, S.Z. Cagen, M. Olggard and J.E. Gibson. A mechanism of paraquat toxicity in mice and rats. Toxicol. Appl. Pharmacol. 35: 501-513 (1976).

15. J.S. Bus, S.D. Aust and J.E. Gibson. Lipid peroxidation: A possible mechanism for paraquat toxicity. Res. Commun. Chem. Pathol. Pharmacol. 11: 31-38 (1975).

16. M.A. Trush, E.G. Mimnaugh, E. Ginsburg and T.E. Gram. In vitro stimulation by paraquat of reactive oxygen-mediated lipid peroxidation in rat lung microsomes. Toxicol. Appl. Pharmacol. 60: 279-286 (1981).

17. H.K. Fisher. Importance of oxygen and of pulmonary alveolar surfactant in lung injury by paraquat. In: Biochemical Mechanisms of Paraquat Toxicity (A.P. Autor, ed.) pp. 57-65. Academic Press, New York (1977).

18. I. Fridovich. Superoxide radical: An endogenous toxicant. Ann. Rev. Pharmacol. Toxicol. 23: 239-257 (1983).

19. L.L. Smith, M.S. Rose and I. Wyatt. The pathology and biochemistry of paraquat. In: Oxygen Free Radicals in Tissue Damage (Ciba Foundation Symposium 65) pp. 321-341, Excerpta Medica, New York (1979).

20. H. Sies, R. Brigelius, H. Wefers, A. Muller and E. Cadenas. Cellular redox changes and response to drugs and toxic agents. Fund. Appl. Toxicol. 3: 200-208 (1983).

21. B. Halliwell and J.M.C. Gutteridge. Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem. J. 219: 1-14 (1984).

22. S.E. Fridovich and N.A. Porter. Oxidation of arachidonic acid in micelles by superoxide and hydrogen peroxide. J. Biol. Chem. 256: 260-265 (1981).

23. J.S. Bus and J.E. Gibson. Lipid peroxidation and its role in toxicology. In: Reviews in Biochemical Toxicology, Volume 1 (E. Hodgson, J.R. Bend and R.M. Philpot, eds.) pp. 125-149, Elsevier, New York (1979).

24. S.D. Aust and B.A. Svingen. The role of iron in enzymatic lipid peroxidation. In: Free Radicals in Biology, Volume 5 (W.A. Pryor, ed.) pp. 1-28, Academic Press, New York (1982).

25. J.M.C. Gutteridge, D.A. Rowley and B. Halliwell. Superoxide-dependent formation of hydroxyl radicals and lipid peroxidation in the presence of iron salts. Detection of catalytic iron and antioxidant activity in extracellular fluids. Biochem. J. 206: 605-609 (1982).

26. G.R. Bartlett. Phosphate compounds in rat erythrocytes and reticuloytes. Biochem. Biophys. Res. Commun. 70: 1055-1062 (1976).

27. G.R. Bartlett. Iron nucleotide in human and rat red cells. Biochem. Biophys. Res. Commun. 70: 1063-1070 (1976).

28. P.M. Harrison. Ferritin: An iron-storage molecule. Sem. Hematol. 14: 55-70 (1977).

29. E.C. Theil. Ferritin: Structure, function and regulation. In: Iron Binding Proteins without Cofactors or Sulfur Clusters, Volume 5 (E.C. Theil, G.L. Eichhorn and L.G. Marzilli, eds.) pp. 1-38, Elsevier, New York (1983).

30. A. Mazur, S. Baez and E. Shorr. The mechanism of iron release from ferritin as related to its biological properties. J. Biol. Chem. 213: 147-160 (1955).

31. C.E. Thomas, L.A. Morehouse and S.D. Aust. Ferritin and superoxide-dependent lipid peroxidation. J. Biol. Chem. 260: 3275-3280 (1985).

32. L.A. Morehouse, C.E. Thomas and S.D. Aust. Superoxide generation by NADPH-cytochrome P450 reductase: The effect of iron chelators and the role of superoxide in microsomal lipid peroxidation. Arch. Biochem. Biophys. 232: 366-377 (1984).

33. J.M. McCord and I. Fridovich. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J. Biol. Chem. 244: 6049-6055 (1969).

34. R.F. Beers, Jr. and I.W. Sizer. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. 195: 133-140 (1952).

35. P.E. Brumby and V. Massey. Determination of nonheme iron, total iron, and copper. Methods Enzymol. 10: 463-474 (1967).

36. T.C. Pederson and S.D. Aust. Aminopyrine demethylase. Kinetic evidence for multiple microsomal activities. Biochem. Pharmacol. 19: 2221-2230 (1970).

37. J. Folch, M. Lees and G.H. Sloane-Stanley. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226: 497-509 (1957).

38. T.C. Pederson, J.A. Buege and S.D. Aust. Microsomal electron transport. The role of reduced nicotinamide adenine dinucleotide phosphate-cytochrome c reductase in liver microsomal lipid peroxidation. J. Biol. Chem. 248: 7134-7141 (1973).

39. G.R. Bartlett. Phorphorus assay in column chromatography. J. Biol. Chem. 234: 466-468 (1959).

40. J.A. Buege and S.D. Aust. Microsomal lipid peroxidation. Methods Enzymol. 52: 302-310 (1977).

41. M. Tien, B.A. Svingen and S.D. Aust. An investigation into the role of hydroxyl radical in xanthine oxidase-dependent lipid peroxidation. Arch. Biochem. Biophys. 216: 142-151 (1982).

42. A. Ledwith. Electron transfer reactions of paraquat. In: Biochemical Mechanism of Paraquat Toxicity (A.P. Autor, ed.) pp. 21-38, Academic Press, New York (1977).

43. J.R. Bucher, M. Tien, L.A. Morehouse and S.D. Aust. Influence of superoxide dismutase and catalase on strong oxidant formation during autoxidation of ferrous chelates. In: Oxy Radicals and Their Scavenger Systems, Volume I: Molecular Aspects (G. Cohen and R.A. Greenwald, eds.) pp. 292-295, Elsevier Science Publishing Co., Inc., New York (1983).

44. H.P. Misra and L.D. Gorsky. Paraquat and NADPH-dependent lipid peroxidation in lung microsomes. J. Biol. Chem. 256: 9994-9998 (1981).

45. R.D. Fairshter, N.D. Vaziri, L.C. Dearden, K. Malott and M. Caserio. Effect of dimethylthiourea on paraquat toxicity in rats. Toxicol. Appl. Pharmacol. 74: 150-154 (1984).

CHAPTER III

REDUCTIVE RELEASE OF IRON FROM FERRITIN BY CATION FREE RADICALS OF PARAQUAT AND OTHER BIPYRIDYLS

ABSTRACT

NADPH-cytochrome P450 reductase catalyzed reduction of paraquat promoted the release of iron from ferritin. Aerobically, iron release was inhibited approximately 60% by SOD while xanthine oxidase-dependent iron release was inhibited nearly 100%. This suggests that both $O_2^{\cdot-}$ and the paraquat cation radical can catalyze the release of iron from ferritin. Accordingly, under anaerobic conditions, the paraquat radical mediated a very rapid, complete release of iron from ferritin. Similarly, the cation free radicals of the closely related chemicals, diquat and benzyl viologen, also promoted iron release. ESR studies demonstrated that electron transfer from the paraquat cation radical to ferritin accounts for the reductive release of iron. The ferritin structure was not altered by exposure to the paraquat radical and also retained its ability to reincorporate iron. These studies indicate that release of iron from ferritin may be a common feature contributing to free radical mediated toxicities.

INTRODUCTION

The toxicities associated with numerous drugs and chemicals is often attributed to their ability to be metabolized to free radical species. The free radicals of many of these compounds, such as paraquat and related bipyridyls,undergo cyclic reduction and autoxidation resulting in the production of partially reduced forms of dioxygen such as $O_2^{\cdot-}$ and H_2O_2 (1). Many studies have linked excessive $O_2^{\cdot-}$ and/or H_2O_2 production with oxidative damage to numerous biological macromolecules including lipids, proteins, and DNA (2). However, neither $O_2^{\cdot-}$ nor H_2O_2 is considered to be of sufficient reactivity to directly initiate and/or promote oxidation of cellular constituents (3). Therefore oxidants capable of initiating lipid peroxidation and other deleterious oxidative processes are generally proposed to result from reactions between these species and transition metals (4).

Although the initiating species has not been clearly identified, numerous studies indicate that iron can be intimately involved in the peroxidative process. Thus, the in vivo formation of an initiator is likely to be contingent upon the availability of physiological iron. Although iron metabolism has been the subject of considerable study the possibility that iron may participate in these deleterious oxidations in vivo remains largely unexplored. At present the most likely catalyst for mediating oxidative processes is thought to be a low molecular weight intermediate functioning to transport iron between transferrin and ferritin or between ferritin and other iron-requiring biochemical processes (5). Some evidence for such

complex(es) in erythrocytes has been presented (6,7) although in other tissues such as liver a variety of potential chelators such as ADP (8) or citrate (9) have been proposed but not conclusively identified.

While the existence of low molecular weight iron chelates remains speculative, it is clear that the vast majority of iron, other than heme iron as in hemoglobin, is present in the high molecular weight proteins transferrin, ferritin, and hemosiderin (10). The relative insolubility of ferric iron and its potential toxicity necessitates its complexation by these proteins so it is likely that release of iron from the protein(s) is necessary for catalysis of the aforementioned reactions. Most of the intracellular iron is stored within ferritin, a large spherical protein consisting of 24 subunits arranged symmetrically around a hollow core in which ferric iron is held in crystalline aggregates (11). Access to the central core is via six narrow, hydrophobic channels through which the iron can be shuttled.

The release of ferric iron from ferritin appears to require reduction (12), however positive identification of the physiological reductant(s) has not been achieved. Harrison and colleagues (13) suggest that reductants pass through the channels and interact directly with the ferric hydroxide core, while others envison the existence of oxido-reduction sites on the interior of the channels (14). It has been demonstrated that reduced flavins, which are among the most potent reductants for releasing ferritin iron, must at least partially traverse the channels in order to release iron (15,16). Furthermore, reductive mobilization of iron from ferritin appears to be dependent upon formation of a complex at the surface of the iron

hydroxide core by the reductant (16). Thus, biological agents capable of releasing iron from ferritin may be limited by their size as well as their potential to form complexes. Another factor that must be taken into consideration is the redox potential of the reductant as recent work reports the reduction potential for ferritin iron to be approximately -230 mV at pH 7.0 (17).

We have previously demonstrated that $O_2^{\cdot-}$, generated by xanthine oxidase, can reductively release iron from ferritin and promote the peroxidation of phospholipid liposomes (18). Superoxide-dependent iron release from ferritin by stimulated polymorphonuclear leukocytes has also been demonstrated (19). Subsequent studies demonstrated that the redox cycling of paraquat promoted ferritin-dependent lipid peroxidation (20). These results support the reports which indicate that paraquat-induced lipid peroxidation is a result of enhanced cellular $O_2^{\cdot-}$ production (21,22). However, exposure of Escherichia coli to hyperbaric oxygen had little effect on paraquat induced lethality (23) and toxicity has even been shown to be enhanced by low oxygen concentrations (24). There also exists conflicting data on the ability of SOD to prevent or protect against paraquat toxicity (25,26).

These apparent anomalous results suggest that the toxicity of these compounds may not be just a function of redox cycling alone, or of the free radical itself, but rather a combination of the two. The paraquat cation radical has been reported to reduce ferric iron (27,28), which, in conjunction with its relatively small size, suggests it to be an excellent candidate for mediating iron release from ferritin. Therefore, we have investigated the ability of

paraquat, and other chemicals capable of undergoing one electron redox cycling, to reductively release iron from ferritin. In this paper we demonstrate that several cation free radicals rapidly release virtually all of the iron from within ferritin under anaerobic conditions. Aerobically, iron release is mediated by both $O_2^{\cdot-}$ and the paraquat free radical. These studies suggest that radical-dependent release of iron from ferritin may contribute to the free radical mediated toxicities of several chemicals by providing the iron necessary for the formation of potentially harmful oxidants.

MATERIALS AND METHODS

Materials

Paraquat dichloride, benzyl viologen, NADPH (Type III), cytochrome c (Type IV), bathophenanthroline sulfonate, 4-7-diphenyl-1,10-phenanthroline, Sephadex G-25, and xanthine were purchased from Sigma Chemical Company (St. Louis, MO). Glucose and ferric chloride were obtained from Baker Chemical Company (Phillipsburg, NJ) and thioglycolate from Fisher Chemical Company (Fairlawn, NJ). Ultragel AcA 44 was purchased from LKB (Bromma, Sweden) and Chelex 100 from Bio-Rad Laboratories (Richmond, CA). Diquat was a generous gift from Dr. Lewis Smith of Imperial Chemical Industries Ltd. (Macclesfield, Cheshire, United Kingdom) and Imferon was a gift from Merrell Dow Pharmaceuticals (Cincinnati, OH).

Enzymes

Glucose oxidase (EC 1.1.3.4), xanthine oxidase (EC 1.2.3.2), and bovine erythrocyte SOD (EC 1.15.1.1) were obtained from Sigma and catalase (EC 1.11.1.6) was purchased from Millipore (Freehold, NJ). The glucose oxidase contained significant adventitious iron (2.7 nmol Fe^{3+}/Unit of activity), therefore it was incubated with 10 mM EDTA for one hour on ice and chromatographed over a Sephadex G-25 column equilibrated with 0.3 M NaCl, pH 7.0 to remove contaminating iron. The catalase was also chromatographed on Sephadex G-25 and was assayed as per Beers and Sizer (29) and xanthine oxidase was similarly treated to remove ammonium sulfate and activity measured by aerobic reduction of cytochrome c (30). Superoxide dismutase activity was measured by the method of McCord and Fridovich (30). Protease solubilized NADPH-cytochrome P450 reductase (EC 1.6.2.4) was purified from

microsomes isolated from the livers of male Sprague-Dawley rats (250-300 g), previously pretreated with 0.1% phenobarbital in their drinking water for 10 days, as previously described (31) with the modification that enzyme eluting from the Sephadex G-100 column was subjected to affinity chromatography on 2',5'-ADP-Sepharose (32) using buffers containing no detergent. The final preparation of NADPH-cytochrome P450 reductase was chromatographed on a Sephadex G-25 column with 0.3 M NaCl, pH 7.0 just prior to use, assayed, and activity defined as 1 μmol cytochrome c reduced/min/ml.

Preparation of Ferritin and Iron Release Assays

Ferritin was purified from the livers of either control rats or those given an intraperitoneal injection of Imferon (iron-dextran) as described by Halliday (33) with slight modification (18). Additionally, Ultragel AcA 44 was used in place of Sepharose 6B and, following purification, the ferritin was incubated on ice in 10 mM EDTA for 1 hr and chromatographed on Sephadex G-25 to remove traces of adventitious iron. Total iron was determined essentially by the bathophenanthroline method of Brumby and Massey (34) with minor modification (18).

Iron release from ferritin under aerobic conditions was determined according to Thomas et al. (18) with modification. The ferrous iron chelator used was bathophenanthroline sulfonate (E = 22.14 mM^{-1} cm^{-1}) (35) and the increase in absorbance at 530 nm was continuously monitored in a Cary 219 dual beam recording spectrophotometer. For anaerobic experiments all solutions were purged with argon, and glucose and glucose oxidase were added to the incubation mixtures to scavenge any remaining oxygen. To prevent

ferrous oxidation by H_2O_2, catalase was also included. Incubation mixtures (1 ml final volume) were constituted in 50 mM NaCl, pH 7.0 and reactions were started by the addition of NADPH (0.5 mM) with other chemicals at concentrations specified in the Figure Legends. The oxidation of NADPH was measured at 340 nm under conditions identical to those utilized for iron release assays except that NADPH was included at 0.2 mM.

Analysis of the Structure and Iron Uptake Ability of Ferritin Following Iron Release by Paraquat

To assess the effect of iron release by paraquat on the protein and its subsequent ability to reincorporate iron, reaction mixtures containing ferritin (500 µg), NADPH-cytochrome P450 reductase (0.2 U), paraquat (0.5 mM), ADP (5 mM), and NADPH (0.5 mM) in 50 mM NaCl, pH 7.0, (final volume 200 µl) were incubated either aerobically or anaerobically for 30 min. In addition, one reaction mixture contained no enzyme or paraquat, another contained enzyme but no paraquat, and in one the iron was reduced using dithionite (1 mM) in place of enzyme and paraquat. Following incubation the reaction mixtures were chromatographed on Sephadex G-25 (1 x 25 cm) in 0.1 M Hepes, pH 9.6. One ml fractions were collected and aliquots (50 µl) were analyzed for ferritin by ELISA as per Thomas and Aust (36). Appropriate fractions were pooled and total protein determined by the BCA method (37).

Iron uptake by ferritin was assayed using the procedure of Collawn *et al.* (38) with minor modification. Incubation mixtures (5 ml final volume, 0.1 M Hepes) contained aliquots of the pooled ferritin fractions such that the final concentration of ferritin was 80 µg/ml and the pH of the incubations was adjusted to 6.8. The

reaction was started by the addition of ferrous ammonium sulfate (0.5 mM) and at 0, 2, 4, and 6 min, 1 ml aliquots of the reaction mixtures were mixed with 0.3 g of Chelex 100 in a 13 x 100 mm test tube. Following vigorous mixing to facilitate chelation of free ferrous iron, the aliquots were transferred to a 1 ml plastic Eppendorf tube, into which pin holes had been made, and which rested atop a 13 x 100 mm test tube. Low speed centrifugation was used to separate the ferritin from the Chelex 100 and the absorbance of the recovered protein at 310 nm was recorded as an index of incorporation of iron into ferritin.

Evaluation of the nature of the ferritin protein structure following iron release by paraquat, both aerobic and anaerobic, was done using gel electrophoresis. Aliquots of reaction mixtures similar to those utilized for the iron uptake studies were subjected to native gel electrophoresis (5.45% polyacrylamide) and SDS-gel electrophoresis on a 15% polyacrylamide gel. The concentration of ferritin applied to each lane was 7.5 µg and the gels were stained for protein using Coomassie Blue. Ferritin not treated with paraquat was also run for comparative purposes.

Paraquat Radical Detection

Formation of the paraquat cation radical resulting from NADPH-cytochrome P450 reductase catalyzed reduction was monitored at 603 nm (39) under anaerobic conditions with reactants as specified in the Figure Legend. Detection of the paraquat radical in the presence and absence of ferritin was also done under anaerobic conditions using ESR spectroscopy (40). Incubations were constituted at room temperature and directly transferred to the cuvette of a Varian Century-112 ESR

spectrometer. Spectrometer settings were: 3320.0 G magnetic field, 15 mW microwave power, 9.4275 GHz frequency, 1000 KHz modulation frequency, 0.63 modulation amplitude, 0.5 sec time constant, 4×10^4 gain, and a 100 G scan over 4 min. Concentrations of reactants are specified in the Figure Legends.

Effect of SOD and O_2 on Iron Release from Ferritin

The results in Table 1 demonstrate that the release of iron from ferritin by NADPH-cytochrome P450 reductase and paraquat is only inhibited 62% by SOD. This is in contrast to the 97% inhibition, observed when SOD is added to a system containing xanthine and xanthine oxidase. A low rate of iron release was observed with NADPH-cytochrome P450 reductase in the absence of paraquat and this activity was almost completely inhibited by the addition of SOD.

Table 1. Effect of SOD on Xanthine Oxidase and Paraquat-Dependent Release of Iron from Ferritin.

System	(nmol Fe^{2+}/min) -SOD	+ SOD
Xanthine oxidase	0.64	0.02
NADPH-cytochrome P450 reductase	0.19	0.03
NADPH-cytochrome P450 reductase plus paraquat	0.66	0.25

Reaction mixtures (1ml final volume) contained rat liver ferritin (1600 nmol Fe^{3+}), bathophenanthroline sulfonate (1 mM), catalase (500 U) and, where indicated, xanthine (0.33 mM), xanthine oxidase (0.025 U), NADPH (0.5 mM), NADPH-cytochrome P450 reductase (0.10 U), paraquat (1 mM), and SOD (500 U) in 50 mM NaCl, pH 7.0. Reactions were initiated by the addition of appropriate enzyme and continuously monitored at 530 nm.

The data in Table 2 demonstrate that the rate of iron release from ferritin isolated from the livers of control animals (native) is much greater than that from ferritin obtained from animals pretreated

Table 2. Effect of SOD on Aerobic, Paraquat-Dependent Release of Iron from Ferritin.

Ferritin (nmol Fe^{3+})	(nmol Fe^{2+}/min) -SOD	+SOD
I. Native Ferritin		
0.1 mg (94)	0.21	0.04
0.25 mg (235)	0.42	0.07
0.50 mg (470)	0.80	0.16
1.00 mg (940)	1.54	0.38
1.50 mg (1410)	1.76	0.54
II. Iron-Induced Ferritin		
0.1 mg (320)	0.12	0.05
0.25 mg (800)	0.38	0.14
0.50 mg (1600)	0.74	0.29
1.00 mg (3200)	0.91	0.45
1.50 mg (4800)	1.44	0.72

Reaction mixtures (1 ml final volume) contained NADPH-cytochrome P450 reductase (0.10 U), NADPH (0.5 mM), paraquat (1 mM), catalase (500 U), bathophenanthroline sulfonate (1 mM), varying amounts of rat liver ferritin, and, where indicated, SOD (500 U) in 50 mM NaCl, pH 7.0. Reactions were initiated with NADPH and continuously monitored at 530 nm.

with iron-dextran (iron-induced). However it can also be seen that, even when the ferritin concentration was varied greatly, paraquat-dependent iron release from both ferritin preparations was consistently inhibited only 50-60% by SOD.

In Figure 1 it can be seen that when NADPH-cytochrome P450 reductase and paraquat were incubated with purified rat liver ferritin, a lag period of approximately 7 min was observed before a rapid release of iron from ferritin occurred, as indicated by formation of the Fe^{2+}-bathophenanthroline complex absorbing at 530 nm (curve A). When the reaction mixture was purged with argon,to decrease the O_2 content prior to the addition of NADPH, the lag period was shortened to approximately 3 min (curve B). When glucose oxidase, glucose, and catalase were included to further reduce the concentration of O_2, iron release was observed immediately (curve C).

Effect of Varying Concentrations of Ferritin, NADPH-cytochrome P450 Reductase, and Paraquat on Iron Release from Ferritin by Paraquat.

The quantity of iron released by paraquat anaerobically was assessed by continuously monitoring iron release from ferritin until no further formation of the bathophenanthroline sulfonate - ferrous complex was observed. The data in Table 3 demonstrate that all of the iron was released from ferritin over a range of ferritin concentrations when NADPH-cytochrome P450 reductase and paraquat were incubated with ferritin anaerobically.

Figure 1. Effect of Oxygen on Release of Iron from Ferritin by Paraquat. Reaction mixtures (1 ml final volume) contained rat liver ferritin (25 μM Fe^{3+}), bathophenanthroline sulfonate (1 mM) and catalase (1000 U) in 50 mM NaCl, pH 7.0 and were initiated by the addition of NADPH (0.5 mM) and monitored at 530 nm. (A) paraquat (1 mM), NADPH-cytochrome P450 reductase (0.2 U); (B) paraquat (0.25 mM), NADPH-cytochrome P450 reductase (0.1 U) and Ar purge; and (C) paraquat (0.25 mM), NADPH-cytochrome P450 reductase (0.1 U), glucose (5 mM), and glucose oxidase (10 U).

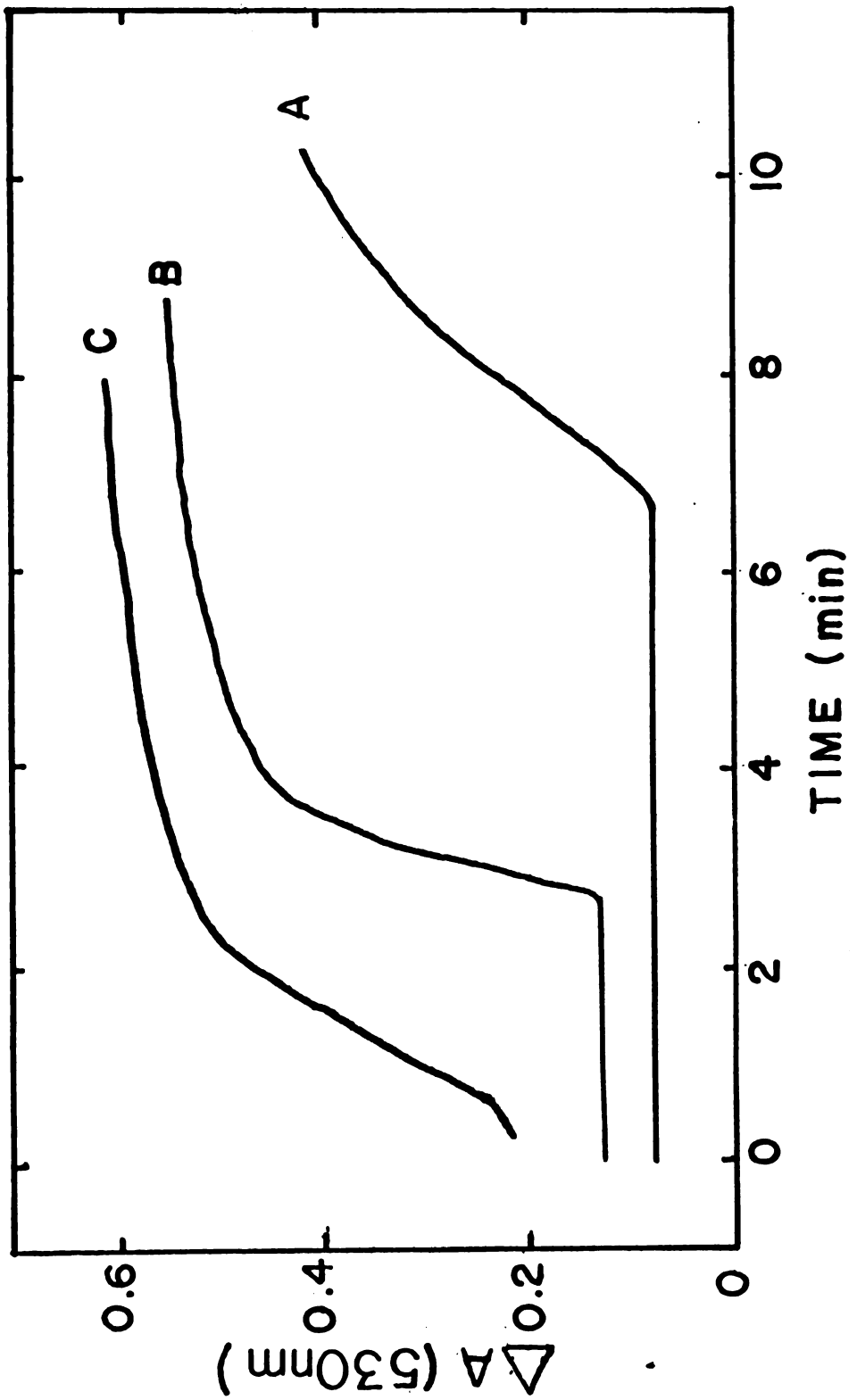

A
B
C
0
2
4
6
8
10
TIME (min)
0
0.2
0.4
0.6
ΔA (530nm)

Table 3. Anaerobic Iron Release from Ferritin by Paraquat at Varying Ferritin Concentrations.

Ferritin Fe^{3+} Added (nmol)	Total Fe^{3+} Reduced (nmol)
0	0.21
25	26.1
50	50.4
75	74.9
100	96.1

Reaction mixtures (1 ml final volume) contained NADPH-cytochrome P450 reductase (0.1 U), paraquat (0.25 mM), bathophenanthroline sulfonate (1 mM), glucose (5 mM), glucose oxidase (10 U), catalase (500 U), and varying amounts of rat liver ferritin as indicated in 50 mM NaCl, pH 7.0. Reactions were initiated by the addition of NADPH (0.5 mM) and continously monitored at 530 nm until no further formation of the bathophenanthroline sulfonate-Fe^{2+} complex was observed. Results are the averages of two separate experiments.

As shown in Figure 2, the time required to release all of the iron from ferritin was decreased by increasing NADPH-cytochrome P450 reductase activity. A marked decrease in the amount of time required for complete release of iron was also observed when the concentration of paraquat was increased, up to approximately 0.10 mM paraquat (Figure 3).

Release of Ferritin Iron and NADPH Oxidation by Other Bipyridyls

Other bipyridyls which undergo one electron reduction were also investigated for their ability to mediate the release of iron from ferritin under anaerobic conditions. As shown in Table 4 diquat and benzyl viologen, which are also reduced to cation radicals, also

Figure 2. Effect of Varying NADPH-Cytochrome P450 Reductase Activity on the Time Required for Complete Release of Iron from Ferritin by Paraquat. Reaction mixtures (1 ml final volume) contained rat liver ferritin (25 μM Fe^{3+}), paraquat (0.25 mM), bathophenanthroline sulfonate (1 mM), catalase (1000 U), glucose (5 mM), glucose oxidase (10 U) and varying amounts of NADPH-cytochrome P450 reductase as indicated in 50 mM NaCl, pH 7.0. Reactions were initiated by the addition of NADPH (0.5 mM) and continously monitored to completion at 530 nm.

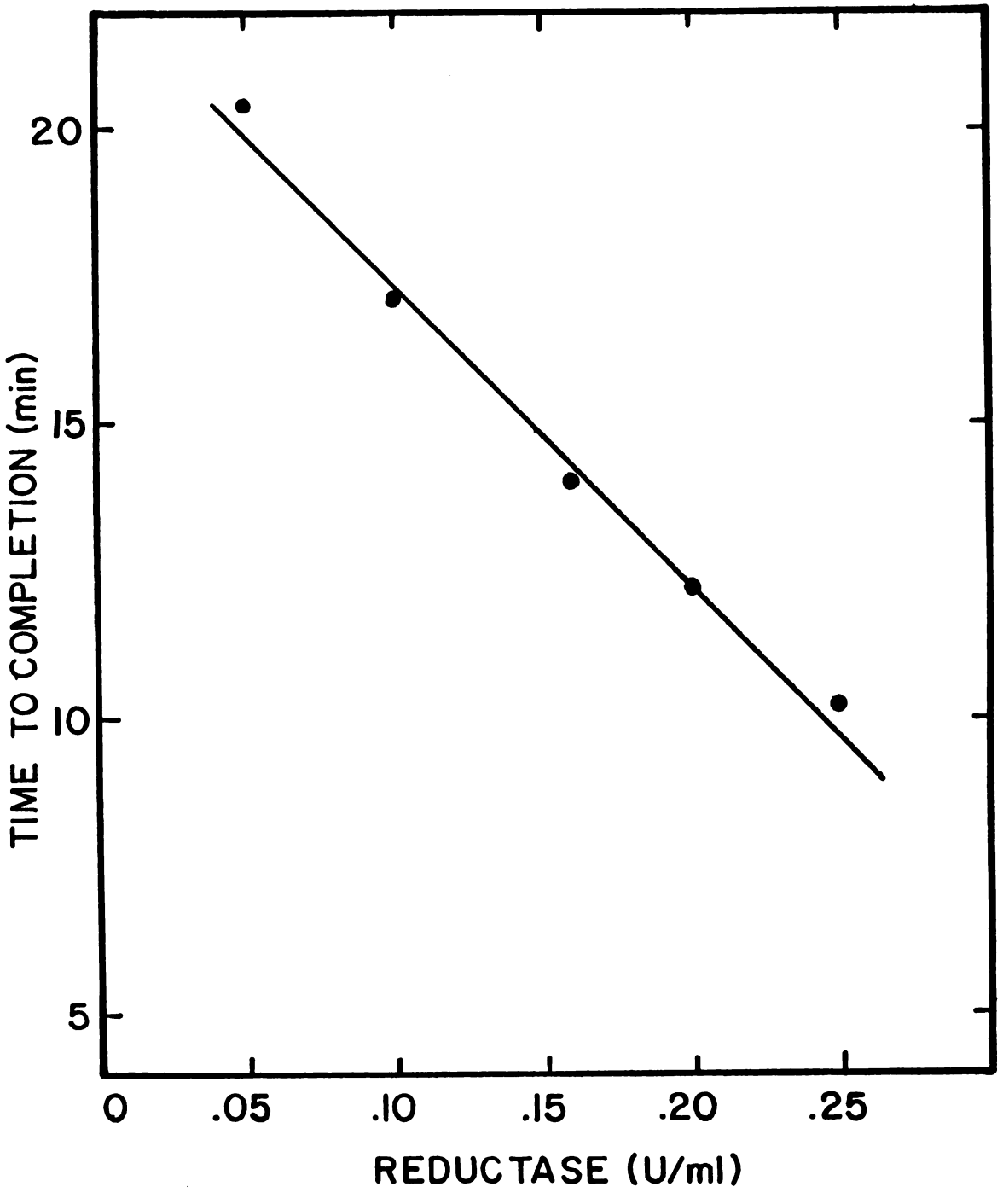
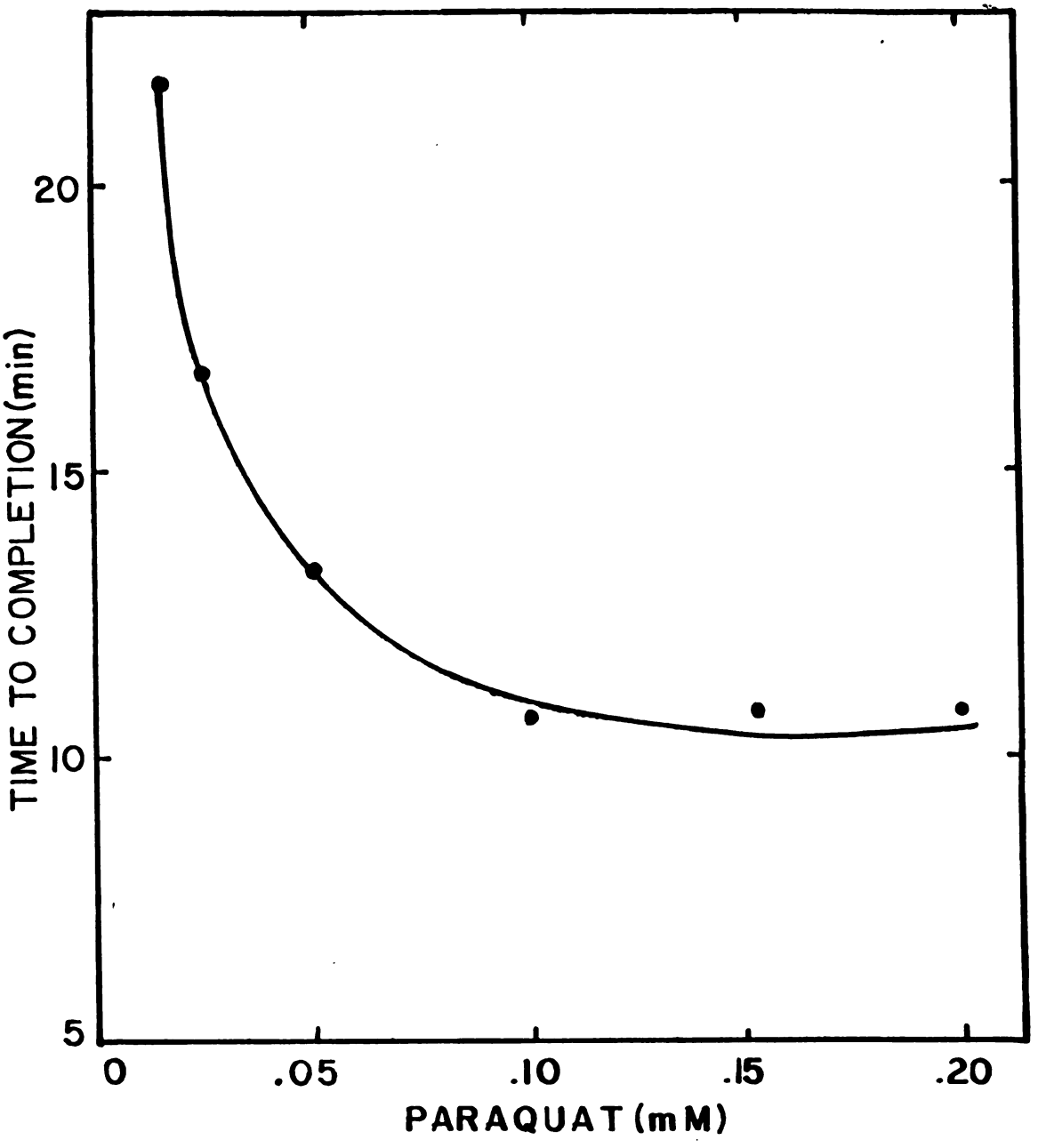

20
15
10
5
TIME TO COMPLETION (min)
0
.05
.10
.15
.20
.25
REDUCTASE (U/ml)

Figure 3. Effect of Varying Paraquat Concentration on the Time Required for Complete Release of Iron from Ferritin by Paraquat. Reaction mixtures (1 ml final volume) contained rat liver ferritin (25 μM Fe^{3+}), NADPH-cytochrome P450 reductase (0.1 U), bathophenanthroline sulfonate (1 mM), catalase (1000 U), glucose (0.5 mM), glucose oxidase (10 U) and varying amounts of paraquat as indicated in 50 mM NaCl, pH 7.0. Reactions were initiated by the addition of NADPH (0.5 mM) and continously monitored to completion at 530 nm.


TIME TO COMPLETION (min)
20
15
10
5
0
.05
.10
.15
.20
PARAQUAT (mM)

released iron from ferritin at a rapid rate. The data presented were calculated from the initial linear portion of the curve, such as is shown in Figure 1 (curve C).

Table 4. Release of Iron from Ferritin and NADPH Oxidation by Redox Active Bipyridyls.

compound	iron released (nmol/min)	NADPH oxidation (nmol/min)	nmol NADPH oxidized / nmol iron released
Paraquat	4.88	11.58	2.37
Diquat	5.05	12.22	2.42
Benzyl Viologen	3.43	9.00	2.62

Reaction mixtures for iron release (1 ml final volume) contained NADPH-cytochrome P450 reductase (0.1 U), rat liver ferritin (25 μM Fe^{3+}), bathophenanthroline sulfonate (1 mM), glucose (5 mM), glucose oxidase (10 U), catalase (500 U), and the various bipyridyls (all at 0.25 mM) in 50 mM NaCl, pH 7.0. Reactions were initiated by the addition of NADPH (0.5 mM) and monitored continously at 530 nm. For NADPH oxidation studies the chelator was omitted, NADPH was 0.2 mM, and the decrease in absorbance at 340 nm was monitored.

The rates of NADPH oxidation in the presence of the various compounds were also determined and compared to the rates at which the chemicals catalyzed the release of iron from ferritin. These data indicated that approximately two nmol of NADPH were oxidized per nmol of iron released for the three chemicals tested.

Effect of Ferritin on Detection of the Paraquat Radical

As shown in Figure 4, as little as 8 μg/ml of ferritin (25 μM Fe^{3+}) reduced the 603 nm absorbance of the paraquat radical (curve B). When the amount of ferritin was continually increased, up to 64 μg/ml protein containing 200 μM Fe^{3+}, (curves C-E), a lag period proceeding

Figure 4. Effect of Ferritin on Spectrophotometric Detection of the Paraquat Cation Radical. Reaction mixtures (1 ml final volume) contained NADPH-cytochrome P450 reductase (0.1 U), paraquat (0.25 mM), catalase (500 U), glucose (5 mM), glucose oxidase (10 U) and varying amounts of rat liver ferritin as indicated in 50 mM NaCl. Reaction mixtures were initiated by the addition NADPH (5 mM) and continuously monitored at 603 nm. (A) no additions; (B) 8 µg (25 µM Fe^{3+}); (C) 16 µg (50 µM Fe^{3+}); (D) 32 µg (100 µM Fe^{3+}); and (E) 64 µg (200 µM Fe^{3+}) rat liver ferritin.

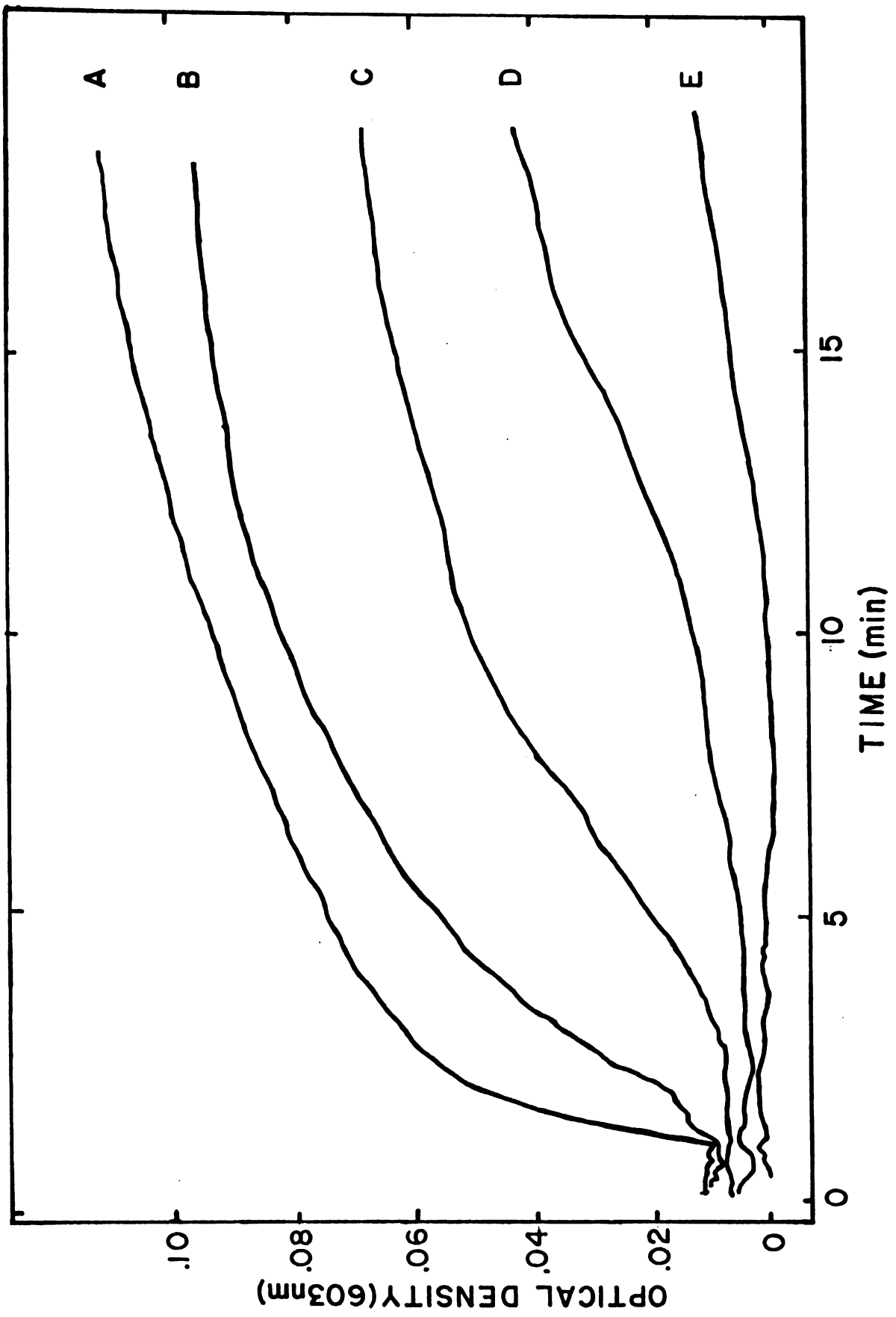

A
B
C
D
E
0
5
10
15
TIME (min)
0
.02
.04
.06
.08
.1
OPTICAL DENSITY (603nm)

Figure 5. Effect of Ferritin on Detection of the Paraquat Cation Radical by ESR. Reaction mixtures (1 ml final volume) contained NADPH-cytochrome P450 reductase (0.1 U), catalase (500 U), paraquat (0.25 mM), glucose (5 mM), and glucose oxidase (10 U) in 50 mM NaCl, pH 7.0. Reactions were initiated by the addition of NADPH (0.5 mM) and scanned immediately with ESR conditions as outlined in Materials and Methods. Rat liver ferritin was included at the following concentrations: (A) no additions; (B) 8 µg (25 µM Fe^{3+}); (C) 16 µg (50 µM Fe^{3+}); (D) 32 µg (100 µM Fe^{3+}); and (E) 64 µg (200 µM Fe^{3+}).

Figure 6. Repetitive Scan of the Effect of Ferritin on Paraquat Cation Radical Detection by ESR. Reaction mixtures (1 ml final volume) contained NADPH-cytochrome P450 reductase (0.1 U), catalase (500 U), paraquat (0.25 mM), glucose (5 mM), glucose oxidase (10 U) and rat liver ferritin (50 μM Fe^{3+}). Reactions were initiated by the addition of NADPH (0.5 mM) and scanned repetitively with ESR conditions as outlined in Materials and Methods.

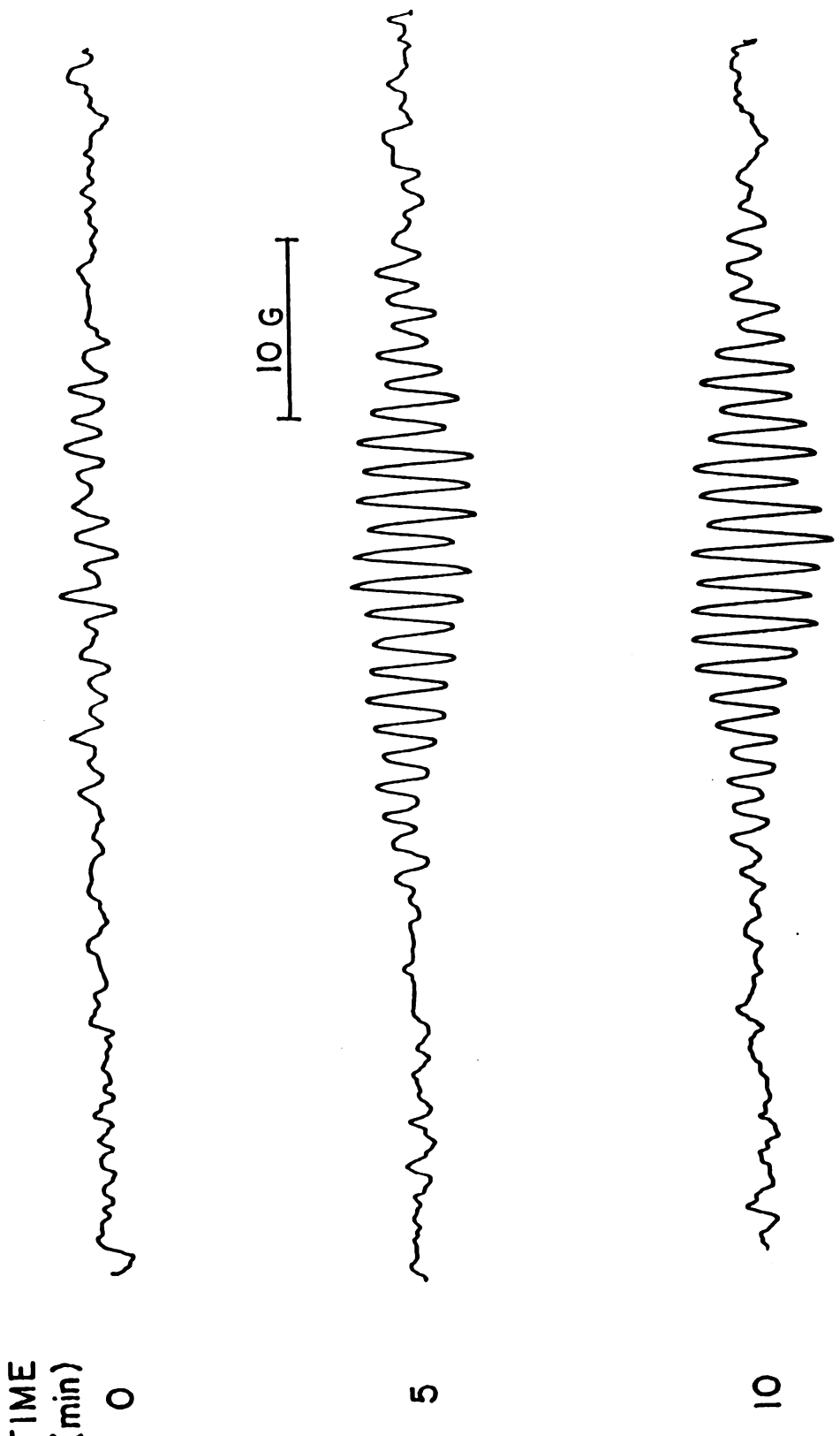
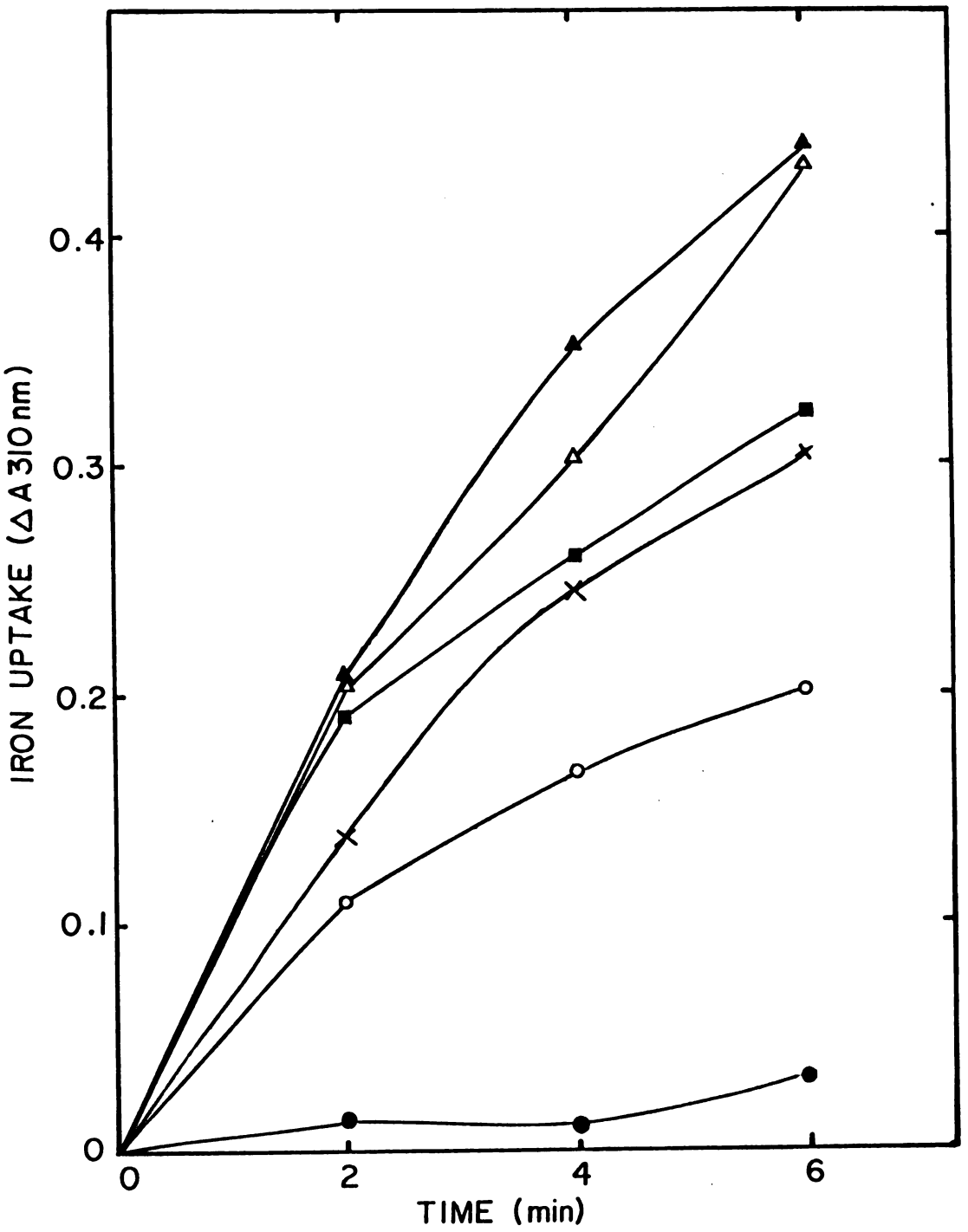

TIME
(min)
0
5
10
10 G

Figure 7. Iron Uptake by Ferritin. Reaction mixtures (5 ml final volume) contained ferritin (80 µg/ml) in 0.1 Hepes, pH 6.8. Reactions were started by the addition of ferrous ammonium sulfate (0.5 mM) and quenched in 0.3 g Chelex 100 as described in Materials and Methods with the absorbance at 310 nm recorded as an index of iron uptake. The ferritin preparations were recovered from incubation mixtures detailed in Materials and Methods and are as follows: ● = no ferritin, Fe^{2+} only; 0 = no treatment; X = NADPH-cytochrome P450 reductase; ■= NADPH-cytochrome P450 reductase and paraquat, aerobically; ▲= NADPH-cytochrome P450 reductase and paraquat, anaerobically; Δ= dithionite.


IRON UPTAKE (ΔA 310 nm)
0.4
0.3
0.2
0.1
0
0
2
4
6
TIME (min)

non-treated ferritin sample. Likewise, the individual subunits exhibited mobility identical to that of the non-treated ferritin and no subunit fragments of less than approximately 18,000 molecular weight were detected (results not shown).

DISCUSSION

The results of this study demonstrate that free radicals produced by NADPH-cytochrome P450 reductase catalyzed reduction are capable of releasing iron from ferritin. The toxicities associated with these compounds is often attributed to their ability to undergo redox cycling resulting in the production of active oxygen species leading ultimately to oxidative damage of cellular constituents, including membrane unsaturated lipids, proteins and DNA (2). In general, it appears that oxidative damage requires, or is greatly potentiated by, the presence of transition metals therefore the identification of biological sources of iron is critical to an understanding of the mechanism(s) governing the toxicity of these chemicals. We had previously demonstrated the ability of $O_2^{\cdot-}$, generated by xanthine oxidase, to release iron from ferritin (18) suggesting that increased production of $O_2^{\cdot-}$ in biological systems may contribute to oxidative stress by increasing the amount of iron available to participate in redox reactions. Similarly, the redox cycling of paraquat promoted ferritin-dependent lipid peroxidation (20).

Xanthine oxidase-dependent iron release was inhibited 97% by SOD showing that the enzyme can prevent essentially all of the $O_2^{\cdot-}$-dependent iron release. However, the present data demonstrate that under aerobic conditions SOD only partially prevents paraquat-dependent release of iron from ferritin. Iron release from ferritin isolated from animals not pretreated with iron was even greater, as has been previously demonstrated using reduced flavins (41). This suggests that iron release from ferritin may be even more

significant *in vivo*. The difference between total iron release and SOD-sensitive iron release was used to assess the $O_2^{\cdot-}$-independent release of iron and indicated that 40-50% of the iron released could be attributed to reduction by a reductant other than $O_2^{\cdot-}$. The direct reduction of Fe^{3+} by the paraquat radical has been previously reported. (27,28) Recent work has indicated that the reduction potential required for ferritin iron release is approximately -230 mV at pH 7.0 (17), therefore the paraquat radical (-444 mV) (39) may be more effective at mediating the reductive release of iron from ferritin than $O_2^{\cdot-}$ (-330 mV) (42). The second order rate constant for the reaction of the paraquat radical with oxygen is 8×10^8 M^{-1} sec^{-1} (43) and 2.1×10^8 M^{-1} sec^{-1} with cytochrome c (28). In these studies, the ferric iron within ferritin appears to compete effectively with oxygen for the radical as evidenced by the ability of the radical to release nearly 50% of the iron from ferritin even under aerobic conditions.

In agreement with the above data, the results in Figure 1 demonstrate the efficiency with which the paraquat radical can release ferritin iron. Under aerobic conditions a very low rate of iron release occurs. However, at the onset of anaerobiosis, resulting from continued redox cycling (at approximately 7 min), a rapid rate of iron release was observed. Accordingly, lowering the oxygen concentration of the reaction mixture considerably shortened the time required to achieve rapid iron release while anaerobiosis at the initiation of the reaction resulted in an immediate release of iron from ferritin. Also, reductive release of ferritin iron by the paraquat radical was much more complete than with $O_2^{\cdot-}$, as virtually 100% of the iron could

be removed, whereas $O_2^{\cdot-}$ appeared to release only approximately 5% of the iron from purified rat liver ferritin (18). The dependency of the time required for complete release of iron from ferritin on both the activity of NADPH-cytochrome P450 reductase and the concentration of paraquat provide further evidence that iron release was mediated by the paraquat radical.

The remarkable ability of the paraquat radical to effectively release iron from ferritin suggested that perhaps other bipyridyl radicals would be similarly capable of mobilizing iron from ferritin. Accordingly, our data demonstrate that the cation radicals of benzyl viologen and diquat were also very effective at mediating release of iron from ferritin. It is difficult to assess the effectiveness of the various radicals to release ferritin iron solely on the basis of rates of iron release due to varying rates of reduction of the chemicals by the enzyme, therefore rates of NADPH oxidation were also monitored. NADPH oxidation rates in the presence of the enzyme and the chemicals are an indicator of the rate of formation of the radical form of the chemicals. The results demonstrated that two nmol of NADPH were oxidized per nmol iron released, thus the process was not tightly coupled. This finding is not totally surprising as iron release from ferritin is highly dependent upon a number of factors including redox potential and size of the reductant (15), complex formation at the iron core surface (16), and the amount of surface area on the iron core available for release (13). Conversely, paraquat can undergo di- or tetravalent reduction or even disproportionation (39) which would significantly affect NADPH oxidation.

The rapidity at which the paraquat cation radical mediates the complete release of iron from ferritin suggests that electron transfer from the radical to the ferric hydroxide iron core, or to oxido-reduction sites on the protein, occurs very rapidly under anaerobic conditions. Confirmation of this was provided by the ESR and UV-visible spectroscopy studies used to detect paraquat radical formation. The addition of as little as 8 μg/ml of ferritin protein (25 μM Fe^{3+}) resulted in a marked diminution of the ESR free radical signal of paraquat, with no detectable signal at 64 μg/ml of ferritin. Similar data were obtained when paraquat radical formation was monitored at 603 nm. As the incubations were monitored over time the paraquat radical could be detected once the iron was released from ferritin. Accordingly, if the ESR reaction mixtures were scanned repetitively over time, a reappearance of the signal was observed as the iron was released from ferritin, thereby allowing an accumulation of the paraquat radical. These data indicated that there was significant interaction between the radical form of paraquat and the protein and that loss of the signal was likely due to transfer of electrons from the cation free radical to ferritin.

To provide further evidence that paraquat-dependent release of iron from ferritin occurs by simple electron transfer, and does not alter the protein, gel filtration chromatography was used to recover ferritin following its incubation with paraquat and NADPH-cytochrome P450 reductase, under aerobic and anaerobic conditions. Enzyme linked immunosorbent analysis of fractions collected from the Sephadex G-25 column revealed essentially no change in the reactivity of rabbit, anti-rat liver ferritin IgG towards the ferritin (results not shown).

In agreement, native and denaturing gel electrophoresis of the ferritin demonstrated there was no effect on the assembled protein or on the individual subunits (data not shown).

It is known that ferritin does have sites sensitive to degradation by proteolysis (44) and pH (45). Preliminary experiments in this laboratory have indicated that iron-free ferritin, prepared by incubation of ferritin with the paraquat radical, was much more susceptible to proteolysis. These results agree with other studies (44,46) which reported that apoferritin is more readily degraded. Therefore, while the paraquat radical may not directly degrade the protein, paraquat intoxication in vivo may lead to disruption of the normal iron-storage capacity of cells.

Additionally, iron uptake by ferritin was determined to confirm that the biologic function of ferritin was not compromised by radical mediated iron release. Iron uptake was greatest for ferritin from which the most iron was released (the paraquat radical under anaerobic conditions or by dithionite). These results confirm previous work (13) which demonstrated that iron uptake was most rapid for those molecules containing the least iron. The data presented in Figure 6 have been corrected to account for the iron present in ferritin prior to addition of ferrous iron, thus while iron uptake was greatest for ferritin containing the least iron, by 6 min all ferritin preparations contained nearly the same amount of iron. Subsequently, the iron in all of the reloaded ferritin preparations could be reductively released by $O_2^{\cdot-}$ or the paraquat radical (data not shown).

These results provide evidence that the generation of organic free radicals in biological systems can serve to mobilize cellular

iron from its storage protein. This finding may help to explain the variable results obtained when using SOD to protect against paraquat intoxication (25,26) i.e., that the relative contributions of $O_2^{\cdot-}$ and the paraquat radical to paraquat toxicity may be highly dependent upon oxygen concentrations within the cell as has been proposed for carbon tetrachloride (47,48). It is conceivable that continuous redox cycling of the herbicide may lead to hypoxic conditions which could favor the rapid release of ferritin iron by the paraquat radical while still maintaining a concentration of oxygen sufficient to promote lipid peroxidation. In this regard, it has been reported that hypoxia may potentiate paraquat toxicity *in vivo* (24). Also, Cadenas *et al.* (49) observed a burst of chemiluminescence upon oxygenation of microsomes previously incubated anaerobically with NADPH and paraquat. The intensity of chemiluminescence was related to the amount of paraquat radical accumulated during anaerobic incubation.

Barry (50) has reported that human liver iron stores (ferritin) range from 10.8-14.3 mM Fe^{3+}, thus it is conceivable that interaction between the radical and the protein may occur *in vivo*. However, the lung is the primary target organ of paraquat toxicity, presumably due to an active uptake mechanism (51). Ferritin is present in almost all tissues at varying levels (52) but the present studies indicate that as little as 8 µg (25 µM Fe^{3+}) of ferritin can readily accept electrons from the paraquat radical, therefore similar interactions in the lung are feasible. Another factor to be considered with respect to physiologic significance is the subcellular distribution of ferritin. While ferritin is found predominantly in the cytosol, electron microscopy studies have demonstrated the existence of

ferritin in the endoplasmic reticulum (53) and, accordingly, ferritin is found in liver (36) and lung (C.E. Thomas and S.D. Aust, unpublished) microsomes prepared by differential centrifugation at 105,000 x g for 90 minutes. Very recent work indicates that ferritin bound iron remains tightly associated with the microsomal fraction even following sucrose gradient centrifugation (54). Therefore, it is likely that, in vivo, ferritin is in close proximity to the site of paraquat radical and $O_2^{\cdot-}$ generation on the endoplasmic reticulum and that little diffusion of these radical species through the cellular millieu to access ferritin is required.

While the biochemical mechanisms governing the toxicities of paraquat and other redox active chemicals are likely to be very diverse, these data suggest that release of iron from ferritin may be a common feature contributing to free radical mediated toxicities. It has been recently demonstrated that iron greatly potentiates the toxicity of paraquat in mice (55). If this iron is derived ultimately from ferritin remains to be determined, however. These findings would indicate that the administration of appropriate iron chelators may help to ameliorate the toxicity of these compounds. In agreement, EDTA and desferrioxamine prevented paraquat induced bacterial inactivation (56) and desferrioxamine treatment prolonged survival of paraquat treated mice (55). These results suggest that redox active metals such as iron may play an integral role in paraquat toxicity. Thus, the demonstration that the free radicals of paraquat and related toxic herbicides possess the capability to delocalize tissue iron may provide insight into the mechanisms which are involved in the toxicities of these compounds and lead ultimately to more effective prevention and treatment regimens.

LIST OF REFERENCES

1. H. Kappus, and H. Sies. Toxic drug effects associated with oxygen metabolism: redox cycling and lipid peroxidation. Experentia 37: 1233-1241 (1981).

2. B. Halliwell and J.M.C. Gutteridge. Lipid Peroxidation: a radical chain reaction. In: Free Radicals in Biology and Medicine, pp. 139-189, Claredon Press, Oxford (1985).

3. D.T. Sawyer and J.S. Valentine. How super is superoxide? Accts. Chem. Res. 14: 393-400 (1981).

4. S.D. Aust, L.A. Morehouse, and C.E. Thomas. Role of metals in oxygen radical reactions. J. Free Rad. Biol. Med. 1: 3-25 (1985).

5. A. Jacobs. Low molecular weight intracellular iron transport compounds. Blood 50: 433-439 (1977).

6. G.R. Bartlett. Phosphate compounds in rat erythrocytes and reticulocytes, Biochem. Biophys. Res. Commun. 70: 1055-1062 (1976).

7. S. Pollack, T. Campana and J. Weaver. Low molecular weight iron in guinea pig reticulocytes. Amer. J. Hematol. 19: 75-84 (1985).

8. K.L. Fong, P.B. McCay, P.B., J.L. Poyer, H.P. Misra, and B.B. Keele. Evidence for superoxide-dependent reduction of Fe^{3+} and its role in enzyme-generated hydroxyl radical formation. Chem. Biol. Inter. 15: 77-89 (1976).

9. C.G.D. Morley and A. Bezkorovainy. Identification of the iron chelate in hepatocyte cytosol. IRCS Med. Sci. Libr. Compend. 11: 1106-1107 (1983).

10. P. Aisen and I. Listowsky. Iron transport and storage proteins. In: Annual Review of Biochemistry, Volume 49 (E.E. Snell, P.D. Boyer, A. Meister and C.C. Richardson, eds.) pp. 357-393 (1980).

11. P.M. Harrison. Ferritin: An iron-storage molecule. Sem. Hematol. 14: 55-70 (1977).

12. E.C. Theil. Ferritin: Structure, function and regulation. In: Iron Binding Proteins Without Cofactors or Sulfur Clusters (E.C. Theil, G.L. Eichhorn, and L.G. Marzilli, eds.), pp. 1-38, Elsevier, New York (1983).

13. P.M. Harrison, T.G. Hoy, I.G. Macara, and R.J. Hoare. Ferritin Iron Uptake and Release. Structure-function relationships. Biochem. J. 143: 445-451 (1974).

14. C.F.A. Bryce and R.R. Crichton. The catalytic activity of horse spleen apoferritin. Preliminary kinetic studies and the effect of chemical modification. Biochem. J. 133: 301-309 (1973).

15. T. Jones, R. Spencer, and C. Walsh. Mechanism and kinetics of iron release from ferritin by dihydroflavins and dihydroflavin analogues. Biochemistry 17: 4011-4017 (1978).

16. F. Funk, J.P. Lenders, R.R. Crichton, and W. Schneider. Reductive mobilization of ferritin iron. Eur. J. Biochem. 152: 167-172 (1985).

17. G.D. Watt, R.B. Frankel and G.C. Papaefthymiou. Reduction of mammalian ferritin. Proceed. Natl. Acad. Sci. 82: 3640-3643 (1985).

18. C.E. Thomas, L.A. Morehouse, and S.D. Aust. Ferritin and superoxide-dependent lipid peroxidation. J. Biol. Chem. 260: 3275-3280 (1985).

19. P. Biemond, H.G. Van Eijk, A.J.G. Swaak, and J.F. Koster. Iron mobilization from ferritin by superoxide derived from stimulated polymorphonuclear leukocytes. J. Clin. Invest. 73: 1576-1579 (1984).

20. M. Saito, C.E. Thomas, and S.D. Aust. Paraquat and ferritin-dependent lipid peroxidation. J. Free Rad. Biol. Med. 1: 179-185 (1985).

21. J.S. Bus, S.D. Aust, and J.E. Gibson. Superoxide- and singlet oxygen-catalyzed lipid peroxidation as a possible mechanism for paraquat (methyl viologen) toxicity. Biochem. Biophys. Res. Comm. 58: 749-755 (1974).

22. H.M. Hassan, and I. Fridovich. Superoxide radical and the oxygen enhancement of the toxicity of paraquat in Escherichia Coli. J. Biol. Chem. 253: 8143-8148 (1978).

23. R.S. Simons, P.S. Jackett, M.E.W. Carrol, and D.B. Lowrie. Superoxide independence of paraquat toxicity in Escherichia coli. Toxicol. Appl. Pharmacol. 37: 271-280 (1976).

24. L.L. Smith, and M.S. Rose. Biochemical changes in lungs exposed to paraquat. In: Biochemical Mechanisms of Paraquat Toxicity (A.P. Autor, ed.), pp. 187-200, Academic Press, New York (1977).

25. A.P. Autor. Reduction of paraquat toxicity by superoxide dismutase. Life Sci. 14: 1309-1319 (1974).

26. C.E. Patterson, and M.L. Rhodes. The effect of superoxide dismutase on paraquat mortality in mice and rats. Toxicol. Appl. Pharmacol. 62: 65-72 (1982).

27. H.C. Sutton and C.C. Winterbourn. Chelated iron-catalyzed ·OH formation from paraquat radicals and H_2O_2: Mechanism of formate oxidation. Arch. Biochem. Biophys. 235: 106-115 (1984).

28. E.J. Land and A.J. Swallow. Electron transfer from pyridinyl radicals to cytochrome-c. C. Ber. Bunsenges. Phys. Chem. 79: 436-437 (1975).

29. R.F. Beers and I.W. Sizer. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. 195: 133-140 (1952).

30. J.M. McCord and I Fridovich. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J. Biol. Chem. 244: 6049-6055 (1969).

31. T.C. Pederson, J.A. Buege, and S.D. Aust. Microsomal electron transport. The role of reduced nicotinamide adenine dinucleotide phosphate-cytochrome c reductase in liver microsomal lipid peroxidation. J. Biol. Chem. 248: 7134-7141 (1973).

32. Y. Yasukochi and B.S.S. Masters. Some properties of a detergent-solubilized NADPH-cytochrome c (cytochrome P-450) reductase purified by biospecific affinity chromatography. J. Biol. Chem. 251: 5337-5344. (1976).

33. J.W. Halliday. Immunoassay of ferritin in plasma. In: Methods in Enzymology, Volume 84 (J. Langone and J. VanVunakis, eds.) pp. 148-171, Academic Press, New York (1982).

34. P.E. Brumby and V. Massey. Determination of nonheme iron, total iron, and copper. In: Methods In Enzymology, Volume 10 (R.W. Estabrook and M.E. Pullman, eds.) pp. 463-474, Academic Press, New York (1967).

35. P. Carter. Spectophometric determination of serum iron at the submicrogram level with a new reagent (Ferrozine). Anal. Biochem. 40: 450-458 (1971).

36. C.E. Thomas and S.D. Aust. Rat liver microsomal NADPH-dependent release of iron from ferritin and lipid peroxidation. J. Free Rad. Biol. Med. 1: 293-300 (1985).

37. M.G. Redinbaugh and R.B. Turley. Adaptation of the bicinchoninic acid protein assay for use with microtiter plates and sucrose gradient fractions. Anal. Biochem. 153: 267-271 (1986).

38. J.F. Collawn, Jr., D.G. Priest and W.W. Fish. A sensitive assay employing a solid-phase metal chelator to estimate rates of iron accumulation by ferritin. Anal. Biochem. 127: 105-111 (1982).

39. A. Ledwith. Electron transfer reactions of paraquat. In: Biochemical Mechanisms of Paraquat Toxicity (A.P. Autor, ed.), pp. 21-38, Academic Press, New York (1977).

40. R.P. Mason and J.L. Holtzman. The role of catalytic superoxide formation in the O_2 inhibition of nitroreductase. Biochem. Biophys. Res. Commun. 67: 1267-1274 (1975).

41. J.R. Mertz and E.C. Theil. Subunit dimers in sheep spleen apoferritin. The effect on iron storage. J. Biol. Chem. 258: 11719-11726 (1983).

42. J.A. Fee and J.S. Valentine. Chemical and physical properties of superoxide. In: Superoxide and Superoxide Dismutases (A.M. Michelson, J.M. McCord and I. Fridovich, eds.), pp. 19-60, Academic Press, New York (1977).

43. P.S. Ras and E. Hayon. Redox potentials of free radicals. IV. Superoxide and hydroperoxy radicals $\cdot O_2^-$ and $\cdot HO_2$. J. Phys. Chem. 79: 397-402 (1975).

44. R.R. Crichton. Studies on the structure of ferritin and apoferritin from horse spleen II. Chymotrypsin, subtilisin, cathepsin D and pepsin digestion of ferritin and apoferritin. Biochim. Biophys. Acta 229: 75-82 (1971).

45. R.R. Crichton and C.F.A. Bryce. Subunit interactions in horse spleen apoferritin. Dissociation by extremes of pH. Biochem. J. 133: 289-299 (1973).

46. G.C. Wood and R.R. Crichton. Optical rotary dispersion and circular dichroism studies on ferritin and apoferritin. Biochim. Biophys. Acta 229: 83-87 (1971).

47. H. Kieczka, H. Remmer, and H. Kappus. Influence of oxygen on the inhibition of liver microsomal activation of carbon tetrachloride by the catechol 2-hydroxy estradiol-17β. Biochem. Pharmacol. 30: 319-324 (1981).

48. H. Kieczka and H. Kappus. Oxygen dependence of CCl_4-induced lipid peroxidation in vitro and in vivo. Toxicol. Lett. 5: 191-196 (1980).

49. E. Cadenas, R. Brigelius and H. Sies. Paraquat-induced chemiluminescence of microsomal fractions. Biochem. Pharmacol. 32: 147-150 (1983).

50. M. Barry. Progress Report. Iron and the liver. Gut. 15: 324-334 (1974).

51. M.S. Rose, L.L. Smith and J. Wyatt. Evidence for energy-dependent accumulation of paraquat into rat lung. Nature (London) 252: 314-315 (1974).

52. R.R. Crichton. Ferritin. Struc. Bond. 17: 67-134 (1973).

53. G.W. Richter. The iron-loaded cell - The cytopathology of iron storage. Amer. J. Pathol. 91: 363-396 (1978).

54. J-C. Sibille, J-N. Octave, Y-J. Schneider, A. Trovet, and R.R. Crichton. Subcellular localization of transferrin protein and iron in the perfused rat liver. Effect of Triton WR 1339, digitonin and temperature. Eur. J. Biochem. 155: 74-55 (1986).

55. R. Kohen and M. Chevion. Paraquat toxicity is enhanced by iron and reduced by desferrioxamine in laboratory mice. Biochem. Pharmacol. 34: 1841-1843 (1985).

56. R. Kohen and M. Chevion. Transition metals potentiate paraquat toxicity. Free Rad. Res. Commun. 1: 79-88 (1985).

CHAPTER IV

RELEASE OF IRON FROM FERRITIN BY CARDIOTOXIC ANTHRACYCLINE ANTIBIOTICS

ABSTRACT

The use of the extremely effective anthracycline antitumor drugs, adriamycin and daunomycin, is limited by a severe, dose-dependent cardiomyopathy. Anthracycline induced toxicity has been proposed to involve iron-dependent oxidative damage to biological macromolecules yet little is known regarding the availability of physiologic iron. We now report that, in the presence of NADPH-cytochrome P450 reductase, these drugs undergo redox cycling to generate $O_2^{\cdot-}$ which mediates a slow, reductive release of iron from ferritin, the major intracellular iron storage protein. Anaerobically, the semiquinone free radical forms of adriamycin and daunomycin catalyze a very rapid, extensive release of iron from ferritin. In contrast, diaziquone, an aziridinyl quinone antitumorigenic agent which is less cardiotoxic, is unable to release iron from ferritin. Thus, the present studies suggest that the cardiomyopathy observed with the anthracyclines, and perhaps their antineoplastic activity as well, may be related to their ability to delocalize tissue iron, thereby contributing to the formation of strong oxidants capable of damaging critical cellular constituents.

INTRODUCTION

Adriamycin and daunomycin are members of a class of structurally related antibiotics collectively known as anthracyclines. They are widely used in chemotherapeutic treatment of cancer as their antineoplastic properties encompass a wide range of tumors including those associated with breast, lung, and soft tissue (1). Unfortunately, the clinical use of these very effective antineoplastics is severely limited by a dose-dependent cardiomyopathy leading to congestive heart failure (2). Total dosage of adriamycin must be limited to 550 mg/m^2 body surface as patients receiving a total dose of greater than 600 mg/m^2 had a 41% incidence of cardiomyopathy with approximately half of these cases being fatal (3). The mechanism(s) governing the cytotoxic and/or the antitumorigenic effects of the anthracyclines remain(s) to be elucidated, however present evidence suggests that reduction of the drugs to their corresponding semiquinone radicals by nuclear and microsomal flavoproteins (4,5) is prerequisite for their cytotoxicity. The resultant radical is then proposed to promote the oxidation of cellular constituents such as membrane unsaturated lipids and/or DNA via its ability to react with dioxygen to produce active oxygen species such as $O_2^{\cdot-}$ and H_2O_2 peroxide (6).

Recent evidence indicates that an interaction with iron is important in causing oxidative damage to both DNA and unsaturated lipids by these drugs (7). However, the significance of these findings is limited by a lack of knowledge concerning the availability of iron in vivo. The majority of intracellular iron is stored within ferritin, a large multi-subunit protein in which iron (up to 4500 atoms/molecule)

is stored as a ferric hydroxide core (8). Release of iron from ferritin appears to require reduction of the ferric iron and, in accordance, we have previously demonstrated the ability of $O_2^{\cdot-}$, generated by xanthine oxidase, to reductively release iron from ferritin (9). The propensity of the anthracycline antibiotics to undergo cyclic reduction and autoxidation to generate $O_2^{\cdot-}$ led us to investigate their ability to catalyze the release of iron from ferritin.

MATERIALS AND METHODS

Materials

Bathophenanthroline sulfonate, 4-7-diphenyl-1,10-phenanthroline, NADPH (Type III), cytochrome c (Type IV), Sephadex G-25, xanthine, and ortho- phenanthroline were purchased from Sigma Chemical Company (St. Louis, MO). Glucose and ferric chloride were obtained from Baker Chemical Company (Phillipsburg, NJ) and thioglycolate from Fisher Chemical Company (Fairlawn, NJ). EDTA was a product of Mallinckrodt Chemical Company (Paris, KY) while Ultragel AcA 44 was purchased from LKB (Bromma, Sweden), Chelex 100 from Bio-Rad Laboratories (Richmond, CA) and Imferon from Merrell Dow (Cincinnati, OH). Diaziquone was a generous gift of the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD) while adriamycin was kindly supplied by Adria Laboratories (Columbus, OH).

Enzymes

Glucose oxidase (EC 1.1.3.4), xanthine oxidase (EC 1.2.3.2), and bovine erythrocyte SOD (EC 1.15.1.1) were obtained from Sigma and catalase (EC 1.11.1.6) was purchased from Millipore (Freehold, NJ). Glucose oxidase contained contaminating iron (2.7 nmol Fe^{3+}/Unit of activity) and was therefore incubated with 10 mM EDTA for one hour on ice and chromatographed over a Sephadex G-25 column equilibrated with 0.3 M NaCl, pH 7.0 to remove adventitious iron. Catalase was also chromatographed on Sephadex G-25 and was assayed as per Beers and Sizer (10) and xanthine oxidase was similarly treated to remove ammonium sulfate and activity measured by aerobic reduction of cytochrome c (11). The activity of SOD was also measured by the

method of McCord and Fridovich (11). Protease solubilized NADPH-cytochrome P450 reductase (EC 1.6.2.4) was purified from microsomes isolated from the livers of male Sprague-Dawley rats (250-300 g), previously pretreated with 0.1% phenobarbital in their drinking water for 10 days, as previously described (12) with the modification that enzyme eluting from the Sephadex G-100 column was subjected to affinity chromatography on 2',5'-ADP-Sepharose using buffers containing no detergent. The final preparation of NADPH-cytochrome P450 reductase was chromatographed on a Sephadex G-25 column with 0.3 M NaCl, pH 7.0 just prior to use, assayed, and activity defined as 1 μmol cytochrome c reduced/min/ml.

Preparation of Ferritin and Iron Release Assays

Ferritin was purified from the livers of rats given an intraperitoneal injection of Imferon (iron-dextran) as described by Halliday (13) with modification (9). In addition, Ultragel AcA 44 was substituted for Sepharose 6B. Following purification the ferritin was incubated on ice in 10 mM EDTA for one hour and chromatographed on Sephadex G-25 to remove any adventitious iron. The total iron content of ferritin was determined essentially as described by Brumby and Massey (14) with minor modification (9) and found to contain 3.12 μM Fe/μg protein.

Iron release from ferritin was determined according to Thomas et al. (9) with modification. Ortho-phenanthroline was used as the ferrous iron chelator and the increase in absorbance at 510 nm was continuously monitored in a Cary 219 dual beam recording spectrophotometer. A reference cuvette lacking the chelator but containing the antitumorigenic drugs was used as a reagent blank. To

achieve anaerobiosis all solutions were purged with argon, and glucose and glucose oxidase were added to the incubation mixtures to remove any residual oxygen. To prevent ferrous iron oxidation by H_2O_2, catalase was also included. Incubation mixtures (1 ml final volume) were constituted in 50 mM NaCl, pH 7.0 and reactions were started by the addition of NADPH (0.5 mM) with other chemicals at concentrations specified in Table and Figure legends. The amount of iron released from ferritin was calculated using E = 12.11 mM^{-1} cm^{-1} for the ferrous-ortho-phenanthroline complex. Measurement of the stimulation of NADPH oxidation by the quinones was also monitored under anaerobic conditions at 340 nm. Incubations (1 ml final volume) contained all reagents at the same concentrations as indicated for the anaerobic iron release assays except that NADPH was included at 0.2 mM.

ESR Measurements

Detection of the semiquinone free radicals of adriamycin, daunomycin, and diaziquone was done using a Varian Century 112 ESR spectrometer at room temperature under anaerobic conditions. Spectrometer settings for incubations containing adriamycin were 3327.0 G magnetic field, 15 mW microwave power, 9.4215 GHz frequency, 1000 KHz modulation frequency, 0.63 modulation amplitude, 0.5 sec time constant, $4x10^4$ gain and a 100 G scan over 4 minutes. The conditions which differed for daunomycin were that the magnetic field was 3329.2 G and the frequency was 9.4274 GHz. For detection of the diaziquone radical the spectrometer settings which differed from that of the anthracyclines were: 3320.0 G magnetic field, 9.4294 GHz frequency, 1.25 modulation amplitude, 0.25 sec time constant, and a 16 min time scan.

RESULTS

Aerobic and Anaerobic Release of Iron from Ferritin

As seen in Table 1, adriamycin and daunomycin, in the presence of NADPH and NADPH-cytochrome P450 reductase, were capable of releasing iron from ferritin. However, SOD, a scavenger of $O_2^{\cdot-}$, caused only 48% and 31% inhibition of adriamycin and daunomycin-dependent iron release, respectively, whereas SOD essentially completely inhibited the release of iron from ferritin by the $O_2^{\cdot-}$ generator xanthine oxidase.

Table 1. Effect of SOD on Aerobic Release of Iron from Ferritin by Xanthine Oxidase and the Redox Cycling of Antitumorigenic Agents.

System	(nmol Fe^{2+}/min) -SOD	+SOD
Xanthine oxidase	0.66	0.04
NADPH-cytochrome P450 reductase	0.11	0.06
Plus: Adriamycin	0.50	0.26
Daunomycin	0.52	0.36
Diaziquone	0.12	0.04

Reaction mixtures (1 ml final volume) contained xanthine oxidase (0.025 U) and xanthine (0.33 mM) or NADPH-cytochrome P450 reductase (0.10 U) and NADPH (0.50 mM), rat liver ferritin (1000 nmol Fe^{3+}), catalase (500 U), ortho-phenanthroline (1 mM) and, where indicated, SOD (500 U), adriamycin (0.40 mM), daunomycin (0.40 mM), or diaziquone (0.40 mM) in 50 mM NaCl, pH 7.0. Reactions were continuously monitored at 510 nm for formation of the phenanthroline-ferrous complex.

The semiquinone radicals of several antitumorigenic compounds including adriamycin have been shown to reduce ferric iron under limiting oxygen concentrations (15) thus it was conceivable that the SOD-insensitive rate of iron release from ferritin was due to direct reduction of ferritin iron by the semiquinone radicals. Results shown in Table 2 clearly demonstrate that under anaerobic conditions, where the reaction of the semiquinone radical with oxygen is prevented, a rapid rate of iron release from ferritin was observed.

Table 2. Anaerobic Release of Iron from Ferritin and NADPH Oxidation by Antitumorigenic Agents.

System	iron released (nmol/min)	NADPH oxidation (nmol/min)	NADPH oxidized / iron released
NADPH-cytochrome P450 reductase	0.00	0.12	----
Plus: Adriamycin	3.05	11.57	3.79
Daunomycin	2.11	8.36	3.96
Diaziquone	0.00	6.11	----

Reaction mixtures (1 ml final volume) contained NADPH-cytochrome P450 reductase (0.10 U), rat liver ferritin (25 nmol Fe^{3+}), catalase (500 U), ortho- phenanthroline (1 mM) and, where indicated, adriamycin (0.25 mM), daunomycin (0.25 mM), or diaziquone (0.25 mM). Glucose (5 mM) and glucose oxidase (10 U) were included to assure anaerobiosis. Reactions were initiated by the addition of NADPH (0.50 mM) and continuously monitored at 510 nm. The stimulation of NADPH oxidation by the antitumorigenic agents was also monitored anaerobically at 340 nm. Incubations were identical to those for measurements of anaerobic iron release except that NADPH was 0.20 mM.

These data suggest that the semiquinone free radicals of adriamycin and daunomycin rapidly release iron from ferritin. The ratio of the rate of NADPH oxidation to the rate of iron release was nearly four for both compounds indicating that the process was not tightly coupled yet still resulted in a rate of iron release which was much greater than that observed aerobically (Table 1).

The total amount of iron released increased as the concentration of ferritin increased (Figure 1), although at all ferritin concentrations, a maximum of 50-60% iron release was achieved in 15 minutes. Similar iron release kinetics and the resistance of a portion of the iron to be released has been reported by others (16-18) and is thought to be related to heterogeneity of the iron core. However, this is still a much greater percentage of iron release than was observed in the xanthine oxidase system where approximately 5% of the iron within ferritin was released over 15 minutes (9). Similarly, iron release was a function of the adriamycin concentration (Figure 2) and the activity of NADPH-cytochrome P450 reductase (Figure 3).

If the cardiotoxicity resulting from anthracycline therapy is indeed related to the release of iron from ferritin, it is important to compare these compounds to one which is relatively non-toxic. We chose to study diaziquone (also known as AZQ), an aziridinyl quinone which is also reduced by NADPH-cytochrome P450 reductase to its corresponding anion radical (19), but appears to be better tolerated clinically (20). As shown in Tables 1 and 2, diaziquone was unable to mediate the release of iron from ferritin either aerobically or anaerobically. The diaziquone free radical autoxidizes much more slowly than the anthracyclines (21,22), perhaps explaining its

Figure 1. Effect of Varying Ferritin Concentration on the Anaerobic Release of Iron from Ferritin by Adriamycin. Reaction mixtures (1 ml final volume) contained NADPH-cytochrome P450 reductase (0.1 U), catalase (500 U), glucose (5 mM), glucose oxidase (10 U), ortho-phenanthroline (1 mM), adriamycin (0.2 mM), and varying amounts of rat liver ferritin as indicated in 50 mM NaCl, pH 7.0. Reactions were initiated by the addition of NADPH (0.5 mM) and continuously monitored at 510 nm. 0 = no additions, Δ = 25 μM Fe^{3+}, ● = 50 μM Fe^{3+}, □ = 75 μM Fe^{3+}, ▲ = 100 μM Fe^{3+} as ferritin.

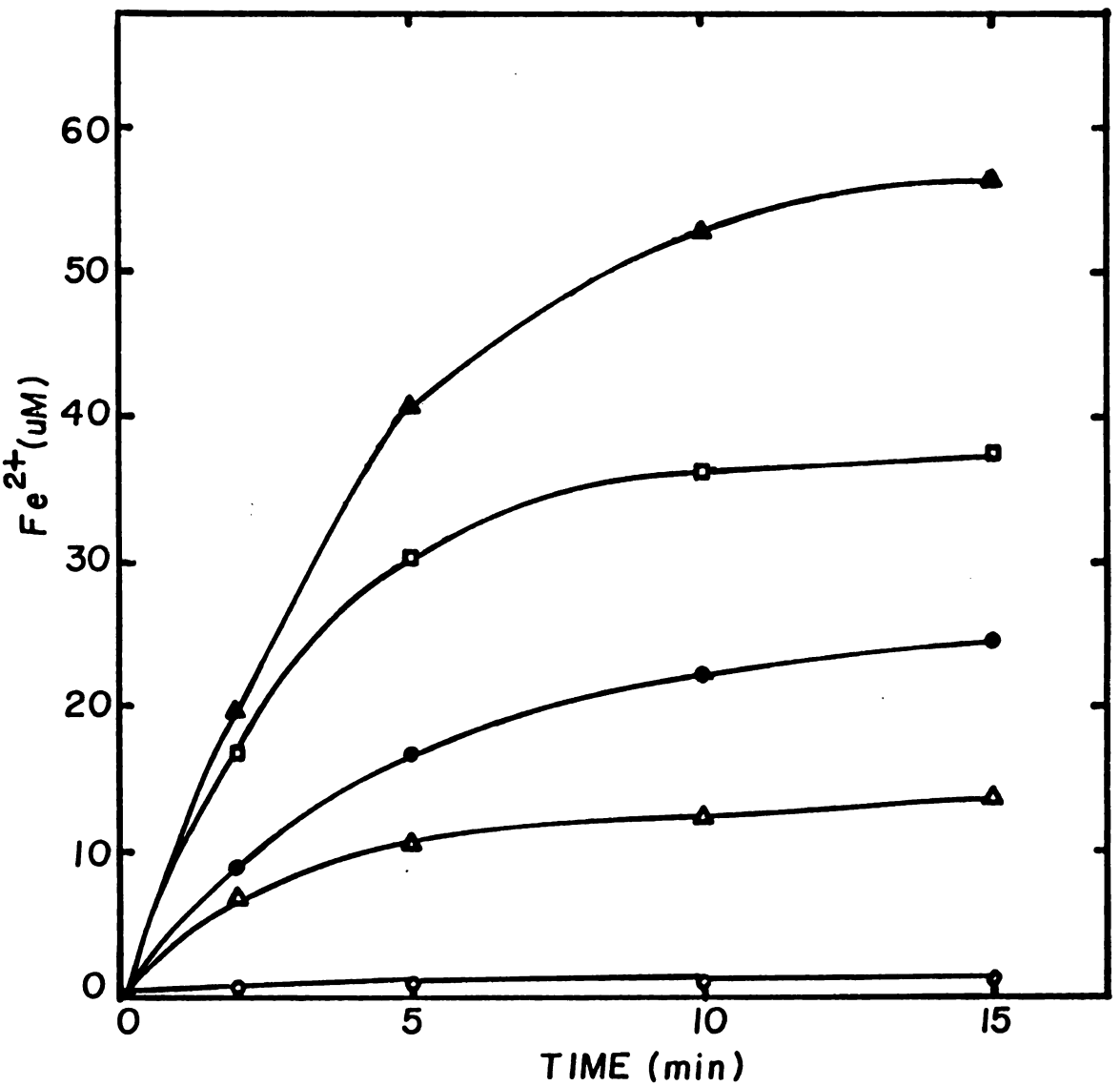

60
50
40
30
20
10
0
Fe^{2+}(uM)
0
5
10
15
TIME (min)

Figure 2. Effect of Varying Adriamycin Concentration on the Anaerobic Release of Iron from Ferritin by Adriamycin. Reaction mixtures (1 ml final volume) contained NADPH-cytochrome P450 reductase (0.1 U), catalase (500 U), glucose (5 mM), glucose oxidase (10 U), ortho-phenanthroline (1 mM), rat liver ferritin (25 μM Fe^{3+}), and varying concentrations of adriamycin as indicated in 50 mM NaCl, pH 7.0. Reactions were initiated by the addition of NADPH (0.5 mM) and continuously monitored at 510 nm. 0 = no adriamycin, Δ = .025 mM, ■ = 0.5 mM, ● = .1 mM, and □ = .2 mM adriamycin.

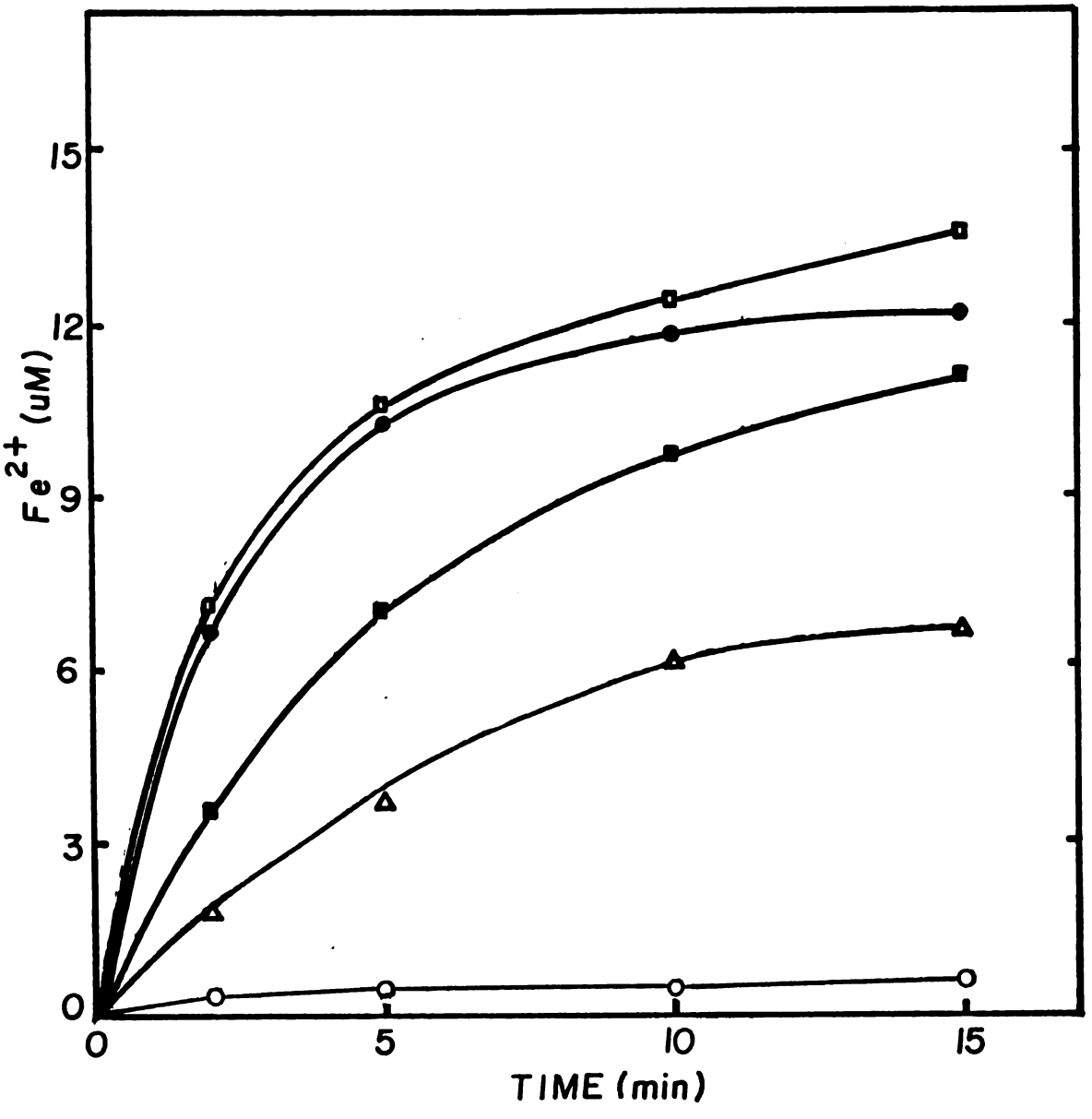
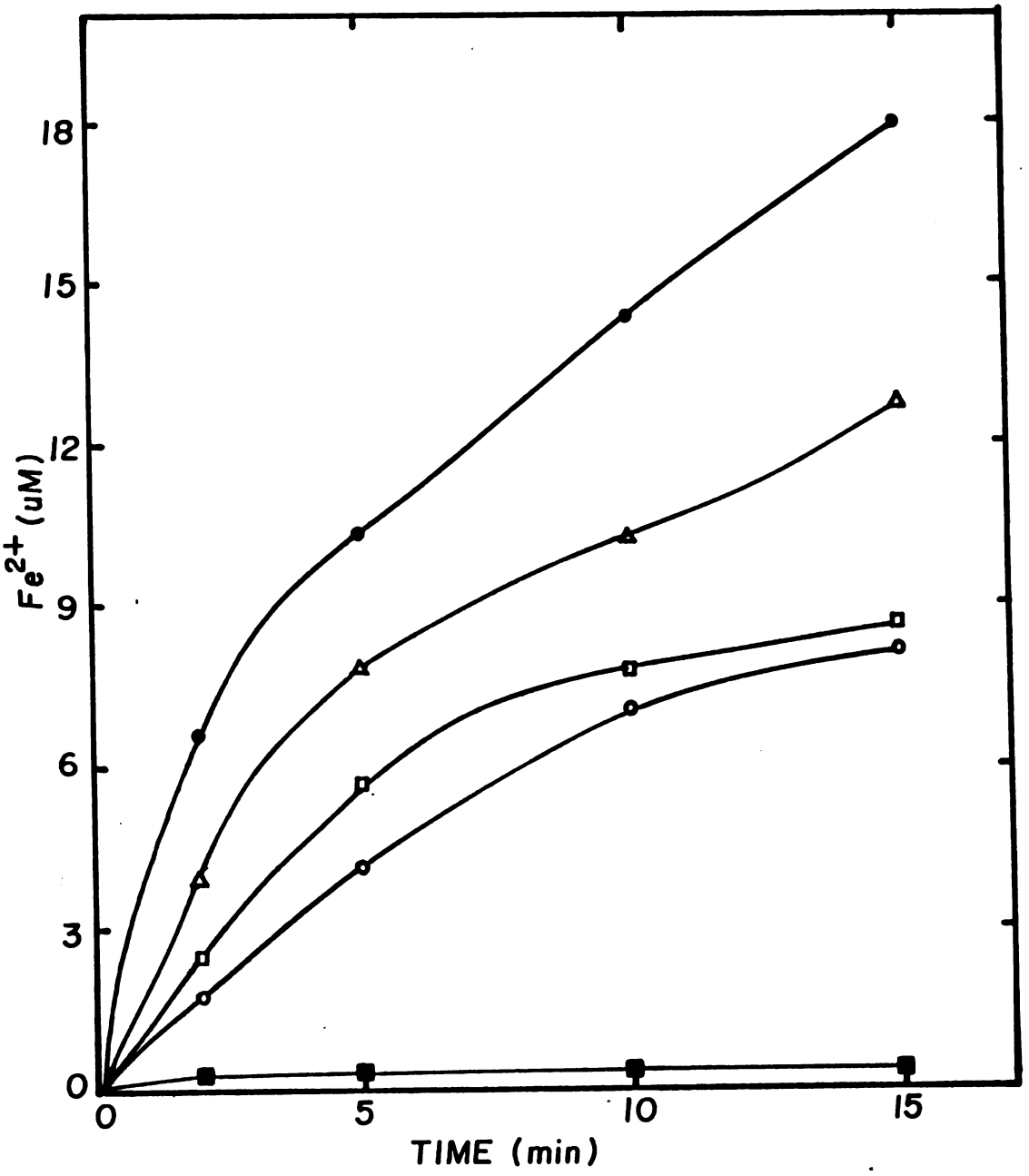

15
12
9
6
3
0
Fe^{2+} (uM)
0
5
10
15
TIME (min)

Figure 3. Effect of Varying NADPH-Cytochrome P450 Reductase Activity on the Anaerobic Release of Iron from Ferritin by Adriamycin. Reaction mixtures (1 ml final volume) contained adriamycin (0.2 mM), catalase (500 U), glucose (5 mM), glucose oxidase (10 U), ortho-phenanthroline (1 mM), rat liver ferritin (25 μM Fe^{3+}), and varying activities of NADPH-cytochrome P450 reductase as indicated in 50 mM NaCl, pH 7.0. Reactions were initiated by the addition of NADPH (0.5 mM) and continuously monitored at 510 nm. ■ = no enzyme, 0 = .0125 units, □ = .025 units, △ = .05 units, and ● = .10 units of enzyme activity.


18
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Fe^{2+} (uM)
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TIME (min)

inability to stimulate iron release from ferritin aerobically. Anaerobically, NADPH oxidation was stimulated by diaziquone, suggesting that radical formation was occurring, but the radical was apparently unable to mediate the release of iron from ferritin (Table 2).

Effect of Ferritin on ESR Detection of Free Radicals

To confirm and extend these results, ESR spectroscopy was used to detect the corresponding free radicals of the three drugs, and to study their interaction with ferritin. As shown in Figures 4, 5 and 6, spectra characteristic of the semiquinone radicals of adriamycin, daunomycin, and diaziquone were obtained when the compounds were incubated with NADPH-cytochrome P450 reductase under anaerobic conditions. The addition of 32 μg of ferritin (100 μM Fe^{3+}) resulted in a marked diminution of the free radical signals of the anthracyclines (Figures 4 and 5). However, ferritin had essentially no effect on the free radical signal of diaziquone (Figure 6). Even at higher ferritin concentrations the signal was not diminished (results not shown). These results are indicative of electron transfer from the semiquinone radicals of adriamycin and daunomycin to ferritin with a concomitant loss of the free radical signals and a release of ferrous iron.

As the reactions were scanned over time a gradual reappearance of the free radical signals of adriamycin and daunomycin was observed as the iron was released, allowing a detectable accumulation of the radicals. Electron transfer may be directly to the ferric iron core as has been proposed (17), or may occur at oxido-reduction sites on the interior of the channels leading to the central core (23). This

question has yet to be resolved for a number of reductants capable of releasing iron from ferritin although the most recent evidence indicates at least partial penetration of the shell by the reductant is required (16). Irrespective of mechanism, ferritin did not quench the free radical signal attributed to the aziridinyl semiquinone, thereby confirming the inability of diaziquone to release iron from ferritin.

Figure 4. Repetitive Scan of the Effect of Ferritin on Adriamycin Semiquinone Radical Detection by ESR. Reaction mixtures (1 ml final volume) contained NADPH-cytochrome P450 reductase (0.1 U), catalase (500 U), adriamycin (1 mM), glucose (5 mM), glucose oxidase (10 U), and rat liver ferritin (100 μM Fe^{3+}). Reactions were initiated by the addition of NADPH (0.5 mM) and scanned repetitively with ESR conditions as outlined in Materials and Methods.

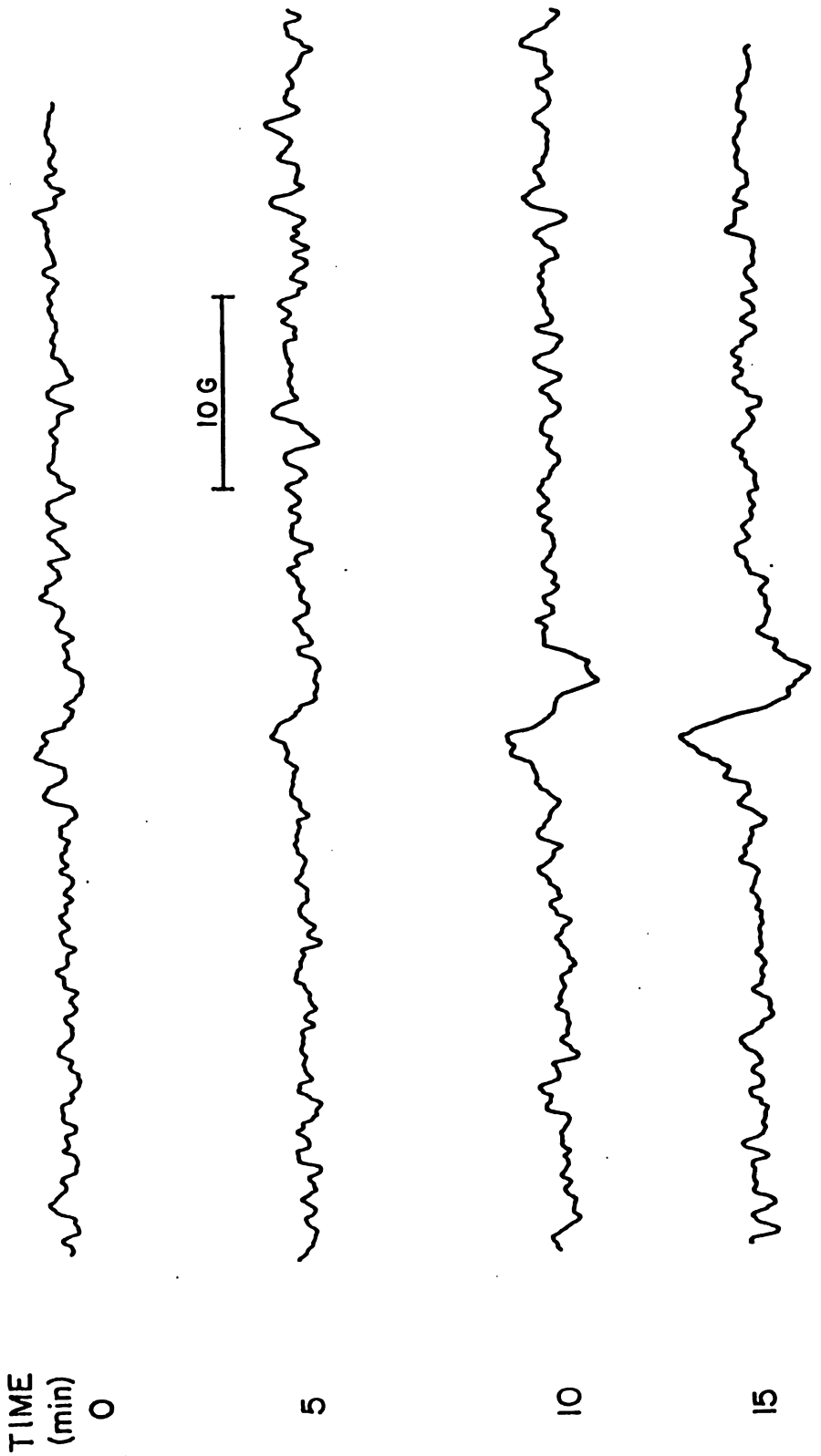
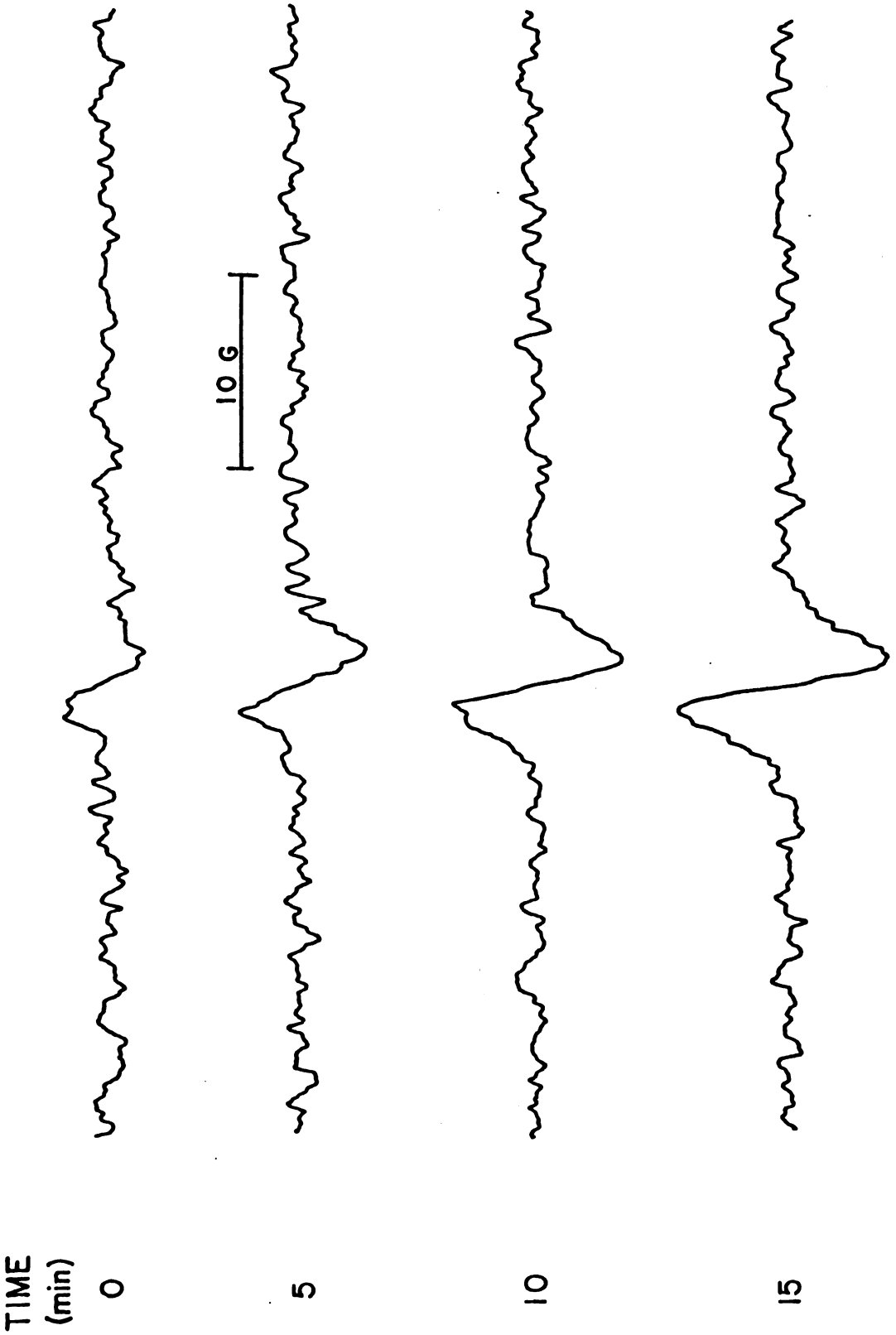

TIME
(min)
0
5
10
15
10 G

Figure 5. Repetitive Scan of the Effect of Ferritin on Daunomycin Semiquinone Radical Detection by ESR. Reaction mixtures (1 ml final volume) contained NADPH-cytochrome P450 reductase (0.1 U), catalase (500 U), daunomycin (1 mM), glucose (5 mM), glucose oxidase (10 U), and rat liver ferritin (100 μM Fe^{3+}). Reactions were initiated by the addition of NADPH (0.5 mM) and scanned repetitively with ESR conditions as outlined in Materials and Methods.


TIME
(min)
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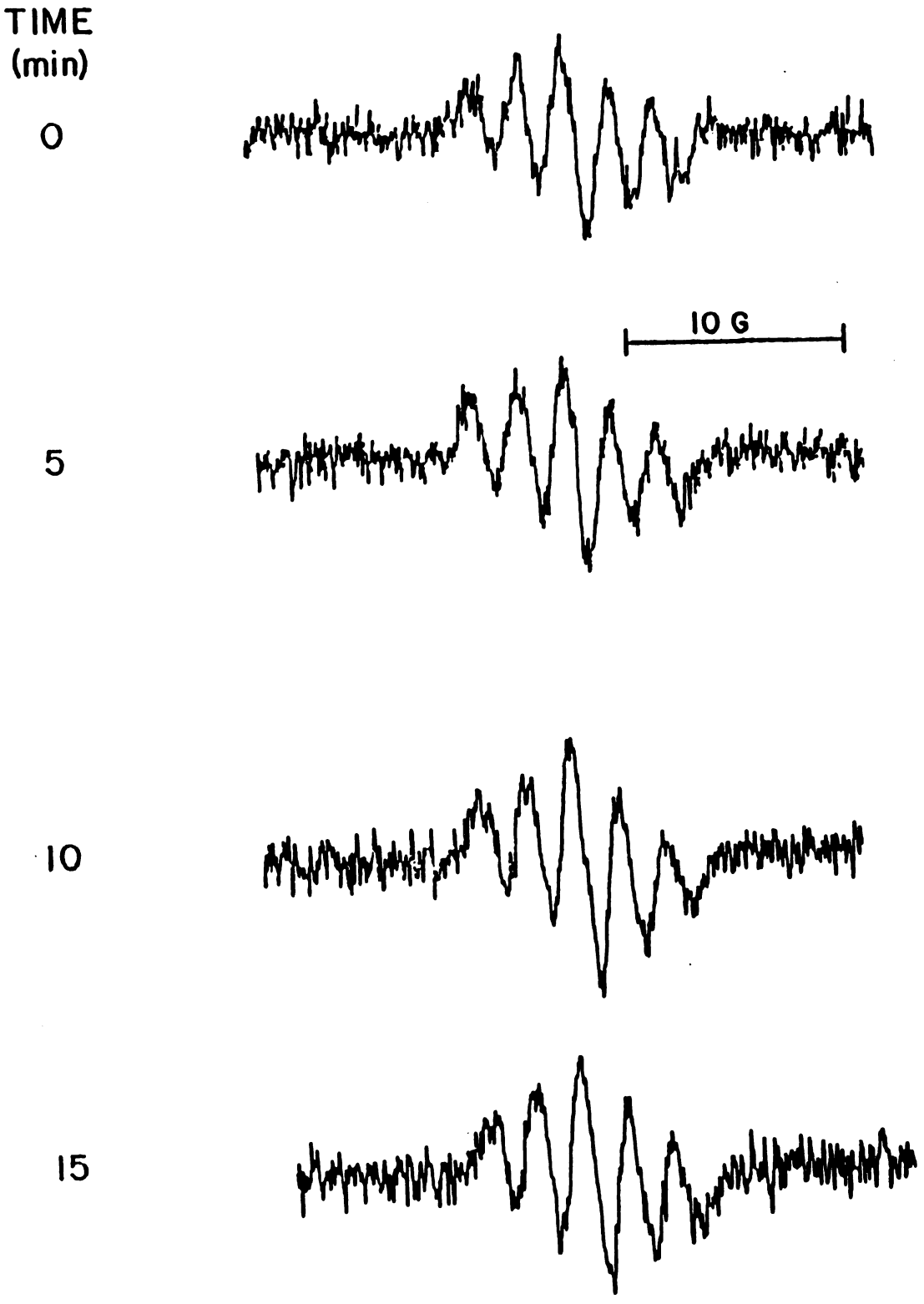
10 G

Figure 6. Repetitive Scan of the Effect of Ferritin on Diaziquone Semiquinone Radical Detection by ESR. Reaction mixtures (1 ml final volume) contained NADPH-cytochrome P450 reductase (0.2 U), catalase (500 U), diaziquone (1 mM), glucose (5 mM), glucose oxidase (10 U), and rat liver ferritin (100 μM Fe^{3+}). Reactions were initiated by the addition of NADPH (0.5 mM) and scanned repetitively with ESR


TIME
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DISCUSSION

The demonstration of the release of iron from its storage protein by the anthracycline antibiotics may be extremely significant with respect to their toxicity as there exists an ever increasing body of evidence which suggests that complexation with iron greatly potentiates the cytotoxicity of adriamycin. Detailed studies have demonstrated that adriamycin and daunomycin bind ferric iron at a ratio of 3:1 with an overall association constant of 10^{33} (24,25). The adriamycin-iron complex possesses a unique chemistry in that the adriamycin is capable of reducing its bound iron (26). Subsequent autoxidation of the ferrous iron apparently produces active oxygen species (27) which may promote the oxidation of biological macromolecules. Accordingly, adriamycin-iron complexes have been demonstrated to initiate lipid peroxidation via self-reduction (26) or in the presence of reducing systems such as GSH or NADPH-cytochrome P450 reductase (24,28). Reduction of adriamycin by xanthine oxidase or ferredoxin reductase has also been shown to damage DNA in an iron-dependent fashion (29). Similarly, the adriamycin-iron complex was capable of cleaving DNA in the presence of H_2O_2 (30).

Thus, if oxidative stress is in fact responsible for the toxicity of the anthracyclines it appears that, irrespective of mechanism, iron is intimately involved. In agreement, the *in vivo* cardiotoxicity of adriamycin has been partially ameliorated by free radical scavengers and iron chelators (31,32). Therefore, identification of physiologic sources of iron is critical to an understanding of anthracycline toxicity. Demant (33) has recently demonstrated *in vitro* a transfer

of iron from ferritin to adriamycin, dependent solely upon the high affinity of adriamycin for iron, however, only 2% of the iron in ferritin was released over 6 hours. Our present work demonstrates that reduction of the anthracyclines by NADPH-cytochrome P450 reductase results in a much more efficient release of iron from ferritin via two mechanisms. Redox cycling of the reduced drug generates $O_2^{\cdot-}$ which catalyzes a slow rate of iron release from ferritin. Alternatively, the semiquinone free radicals of adriamycin and daunomycin rapidly release much of the iron via reduction of the ferric hydroxide core.

Heart tissue contains 30-60 μg of ferritin protein per gram (34). Ferritin generally averages 20% iron loading (8), therefore heart tissue may contain up to 120 nmol iron per gram. In addition to containing significant amounts of ferritin, cardiac cells also contain only low amounts of catalase and SOD (35), that are important antioxidant defense enzymes. The combination of iron availability (which would promote the formation of damaging anthracycline-iron complexes and subsequent oxygen radical generation) and low levels of protective enzymes could perhaps explain the selective sensitivity of the heart to anthracycline induced toxicity. Reduction of the anthracyclines by nuclear and microsomal flavoproteins, and continued redox cycling, would be expected to produce increasingly hypoxic conditions within tissue, eventually mitigating conditions that favor the rapid release of iron from ferritin. It is noteworthy that many tumor cells contain high levels of ferritin (36) and that adriamycin has been shown to be much more cytotoxic to hypoxic tumor cells (37), conditions which this study suggests would favor the release of iron

from ferritin. Thus, it is possible that the selectivity of the anthracyclines towards neoplastic cells may also be related to an interaction between ferritin and the drugs.

The inability of diaziquone, which appears to be well tolerated in clinical studies (20), to release iron from ferritin may suggest a potentially important means of diminishing the cardiotoxicity of antitumor drugs. These studies do not allow us to distinguish whether the aziridinyl structure is unable to gain access to the iron core, or to oxido-reduction sites in the channels, or that the redox potential of its radical (-168 mV) (22) is not sufficient to reduce ferritin iron (-230 mV at pH 7.0) (38). However, these possibilities suggest that altering the redox potential or sterically hindering access to the inner core of ferritin to prevent iron release may be a potentially effective means of diminishing cardiotoxicity. Alternatively, administration of appropriate iron chelators such as desferrioxamine may help to ameliorate anthracycline induced toxicity. Alteration of structure to prevent iron binding also appears to significantly lessen the ability of the compounds to potentiate oxidative damage (7) and correspondingly, to lessen cardiac toxicity. We suggest that the delocalization of tissue iron may be a common feature contributing to anthracycline mediated toxicities. These findings may aid in designing antidotes to diminish the cardiotoxicity of existing drugs and in the development of new, less toxic chemotherapeutic agents.

LIST OF REFERENCES

1. W.B. Pratt and R.W Ruddon. The antibiotics. In: The Anticancer Drugs, pp. 148-194. Oxford University Press, New York (1979).

2. L. Lenaz and J.A. Page. Cardiotoxicity of adriamycin and related anthracyclines. Cancer Treat. Rev. 3: 111-120 (1976).

3. R.A. Minow, R.S. Benjamin and J.A. Gottlieb. Adriamycin (NSC-123127) Cardiomyopathy - An overview with determination of risk factors. Cancer Chemother. Rep. 6: 195-210 (1975).

4. N.R. Bachur, S.L. Gordon, M.V. Gee and H. Kon. NADPH cytochrome P-450 reductase activation of quinone anticancer agents to free radicals. Proceed. Natl. Acad. Sci. 76: 954-957 (1979).

5. N.R. Bachur, M.V. Gee and R.D. Friedman. Nuclear catalyzed antibiotic free radical formation. Cancer Res. 42: 1078-1081 (1982).

6. R.D. Olson, R.C. Boerth, J.G. Gerber and S. Nies. Mechanism of adriamycin cardiotoxicity: evidence for oxidative stress. Life Sci. 29: 1393-1401 (1981).

7. J. Muindi, B.K. Sinha, L. Gianni and C. Myers. Thiol-dependent DNA damage produced by anthracycline-iron complexes. The structure activity relationships and molecular mechanisms. Molec. Pharmacol. 27: 356-365 (1985).

8. P.M. Harrison. Ferritin: An iron-storage molecule. Sem. Hematol. 14: 55-70 (1977).

9. C.E. Thomas, L.A. Morehouse and S.D. Aust. Ferritin and superoxide-dependent lipid peroxidation. J. Biol. Chem. 260: 3275-3280 (1985).

10. R.F. Beers, Jr. and I.W. Sizer. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. 195: 133-140 (1952).

11. J.M. McCord and I. Fridovich. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J. Biol. Chem. 244: 6049-6055 (1969).

12. T.C. Pederson, J.A. Buege and S.D. Aust. Microsomal electron transport. The role of reduced nicotinamide adenine dinucleotide phosphate-cytochrome c reductase in liver microsomal lipid peroxidation. J. Biol. Chem. 248: 7134-7141 (1973).

13. J.W. Halliday. Immunoassay of ferritin in plasma. In: Methods in Enzymology, Volume 84 (J. Langone and J. VanVunakis, eds.) pp. 148-171, Academic Press, New York (1982).

14. P.E. Brumby and V. Massey. Determination of nonheme iron, total iron, and copper. In: Methods in Enzymology, Volume 10 (R.W. Estabrook and M.E. Pullman, eds.) pp. 463-474, Academic Press, New York (1967).

15. J. Butler, B.M. Hoey and A.J. Swallow. Reactions of the semiquinone free radicals of anti-tumor agents with oxygen and iron complexes. FEBS Lett. 182: 95-98 (1985).

16. F. Funk, J.P. Lenders, R.R. Crichton and W. Schneider. Reductive mobilization of ferritin iron. Eur. J. Biochem. 152: 167-172 (1985).

17. T. Jones, R. Spencer and C. Walsh. Mechanism and kinetics of iron release from ferritin by dihydroflavins and dihydroflavin analogues. Biochemistry 17: 4011-4017 (1978).

18. T.G. Hoy, P.M. Harrison, M. Shabbir and J.G. Macara. The release of iron from horse spleen ferritin to 1,10-phenanthroline. Biochem. J. 137: 67-70 (1974).

19. P.L. Gutierrez and N.R. Bachur. Free radicals in quinone containing antitumor agents. The nature of the diaziquone (3,6-diaziridinyl-2,5-bis (carboethoxyamino)-1,4-benzoquinone) free radical. Biochim. Biophys. Acta 758: 37-41 (1983).

20. G.A. Curt, J.A. Kelley, C.V. Kufta, B.H. Smith, P.L. Kornblith, R.C. Young and J.M. Collins. Phase II and pharmacokinetic study of aziridnylbenzoquinone [2,5-diaziridinyl-3,6-bis (carboethoxyamino)-1,4-benzoquinone, diaziquone. NSC 182986] in high grade gliomas. Cancer Res. 43: 6102-6105 (1983).

21. P.L. Gutierrez, M.J. Egorin, B.M. Fox, R. Friedman and N.R. Bachur. Cellular activation of diaziquone [2,5-diaziridinyl-3,6-bis (carboethoxyamino)-1,4-benzoquinone] to its free radical species. Biochem. Pharmacol. 34: 1449-1455.

22. B.A. Svingen and G. Powis. Pulse radiolysis studies of antitumor quinones: radical lifetimes, reactivity with oxygen, and one-electron reduction potentials. Arch. Biochem. Biophys. 209: 119-126 (1981).

23. C.F.A. Bryce and R.R. Crichton. The catalytic activity of horse spleen apoferritin. Preliminary kinetic studies and the effect of chemical modification. Biochem. J. 133: 301-309 (1973).

24. P.M. May, G.N. Williams and D.R. Williams. Solution chemistry studies of adriamycin-iron complexes present in vivo. Eur. J. Cancer 16: 1275-1276 (1980).

25. C.E. Myers, L. Gianni, C.B. Simone, R. Klecker and R. Greene. Oxidative destruction of erythrocyte ghost membranes catalyzed by the doxorubicin-iron complex. Biochem. 21: 1707-1713 (1982).

26. K. Sugioka and M. Nakano. Mechanism of phospholipid peroxidation induced by ferric ion-ADP-adriamycin co-ordination complex. Biochim. Biophys. Acta 713: 333-343 (1982).

27. S.D. Aust, L.A. Morehouse and C.E. Thomas. Role of metals in oxygen radical reactions. J. Free Rad. Biol. Med. 1: 3-25 (1985).

28. E.G. Mimnaugh, T.E. Gram and M.A. Trush. Stimulation of mouse heart and liver microsomal lipid peroxidation by anthracycline anticancer drugs: Characterization and effects of reactive oxygen scavengers. J. Pharmacol. Exper. Ther. 226: 806-813 (1983).

29. D.A. Rowley and B. Halliwell. DNA damage by superoxide-generating systems in relation to the mechanism of action of the anti-tumor antibiotic adriamycin. Biochim. Biophys. Acta 761: 86-93 (1983).

30. H. Eliot, L. Gianni and C. Myers. Oxidative destruction of DNA by the adriamycin iron complex. Biochem. 23: 928-936 (1984).

31. C.E. Myers, W.P. McGuire, R.H. Liss, I. Ifrim, K. Grotzinger and R.O. Young. Adriamycin: The role of lipid peroxidation in cardiac toxicity and tumor response. Science 197: 165-167 (1977).

32. E.H. Herman, A.M. El-Hage, V.J. Ferrans and D.T. Witiak. Reduction by ICRF-187 of acute daunorubicin toxicity in Syrian golden hamsters. Res. Comm. Chem. Pathol. Pharmacol. 40: 217-231 (1983).

33. E.J.F. Demant. Transfer of ferritin-bound iron to adriamycin. FEBS Lett. 176: 97-100 (1984).

34. A. Bezkorovainy. Chemistry and biology of iron storage. In: Biochemistry of Nonheme Iron (E. Frieden, ed.) pp. 207-269, Plenum Press, New York (1980).

35. J.H. Doroshow, G.Y. Locker and C.E. Myers. Enzymatic defenses of the mouse heart against reductive oxygen metabolites. Alterations produced by doxorubicin. J. Clin. Invest. 65: 128-135 (1980).

36. C. Cohen, G. Shulman and L.R. Budgeon. Immunohistochemical ferritin in testicular seminoma. Cancer 54: 2190-2194 (1984).

37. B.A. Teicher, J.S. Lazo and A.C. Sartorelli. Classification of anti-neoplastic agents by their selective toxicities toward oxygenated and hypoxic tumor cells. Cancer Res. 41: 73-81 (1981).

38. G.D. Watt, R.B. Frankel and G.C. Papaefthymiou. Reduction of mammalian ferritin. Proceed. Natl. Acad. Sci. 82: 3640-3643 (1985).

CHAPTER V

RAT LIVER MICROSOMAL NADPH-DEPENDENT RELEASE OF IRON FROM FERRITIN AND LIPID PEROXIDATION

ABSTRACT

Microsomes prepared by the usual method of differential centrifugation were found to contain ferritin, SOD, and catalase which could be separated from microsomes by chromatography on Sepharose CL-2B. Addition of purified rat liver ferritin to chromatographed microsomes resulted in a significant stimulation of NADPH-dependent lipid peroxidation which was inhibited by exogenously added SOD. Iron release from ferritin by these microsomes was also inhibited by SOD. Ferritin did not promote NADPH-dependent microsomal lipid peroxidation when added to microsomes isolated in the usual manner, presumably due to the endogenous SOD present in the microsomes. Accordingly, only very low rates of iron release from ferritin were observed with these microsomes. Paraquat,which generates $O_2^{\cdot-}$ via redox cycling, greatly stimulated iron release from ferritin and lipid peroxidation in chromatographed microsomes. Paraquat had no effect on iron release from ferritin or lipid peroxidation in microsomes which were not chromatographed unless they were first treated with CN^- to inhibit endogenous SOD. These studies indicate that the majority of microsomal iron is contained within ferritin and that following release by $O_2^{\cdot-}$ this iron serves to promote the peroxidation of microsomal lipids.

INTRODUCTION

The ability of iron to promote microsomal lipid peroxidation was first reported by Hochstein et al. (1) and numerous studies have since confirmed the integral role transition metals play in the peroxidative process (2-4). Most in vitro studies have employed low molecular weight iron complexes such as ADP-Fe^{3+} because they are envisioned to exist within the cell. Some evidence for such complexes in reticulocytes has been presented (5,6), however an analagous iron chelate has not been conclusively identified in other tissues (7).

The majority of cellular iron is stored within ferritin as a ferric hydroxide core complexed with phosphate (8). Some time ago it was proposed that much of the non-heme iron present in microsomes may be ferritin but it was suggested that this iron was unavailable for peroxidation (9). Ferritin was later identified as co-sedimenting with the microsomal fraction (10) and others (11) further demonstrated that a portion of the ferritin was tightly associated with the microsomal membranes. However, it is unlikely that this ferritin iron is available to generate an oxidizing species as it is surrounded by the spherical protein shell. Mobilization of iron from ferritin appears to require reduction and is enhanced by chelators (12), conditions that can also promote lipid peroxidation. Accordingly, Wills (13) and Gutteridge (14) have demonstrated that ferritin iron is released in an ascorbate-dependent lipid peroxidation system.

Recently, Rowley and Sweeney (15) have demonstrated that NADPH-cytochrome P450 reductase (cytochrome c reductase) is capable of releasing ferritin iron in the presence of FMN under anaerobic conditions. Similar results have previously been reported for mitochondria that also appear to contain distinct binding sites for ferritin (16,17). These reports indicate that ferritin may not serve only as an iron storage protein but may play a more dynamic role in the cellular metabolism of iron. We have recently demonstrated that $O_2^{\cdot-}$ can release iron from ferritin (18,19) in agreement with others (20). It has also been shown that microsomes generate small amounts of $O_2^{\cdot-}$ (20-22) and that SOD inhibits microsomal lipid peroxidation (21). These results, in conjunction with the apparent association of ferritin with microsomal membranes, have led us to investigate whether iron can be released from ferritin by microsomes aerobically and subsequently promote the peroxidation of microsomal lipids.

MATERIALS AND METHODS

Materials

NADPH, ADP, cytochrome c (Type VI), 2-TBA, 4,7-diphenyl-1,10-phenanthroline, bathophenanthroline sulfonate, paraquat, ortho-phenylenediamine, para-nitrophenyl-N-acetyl-β-D-glucosaminide, and butylated hydroxytoluene were from Sigma Chemical Company (St. Louis, MO). Thioglycolate and sodium hydrosulfite were purchased from Fischer (Fairlawn, NJ), sodium cyanide from Baker Chemical Company (Phillipsburg, NJ) and H_2O_2 from Mallinckrodt Chemical Works (Paris, KY). Imferon was a gift from Merrell Dow Pharmaceuticals (Cincinnati, OH). All buffers and reagents were passed through Chelex 100 (Bio-Rad Laboratories, Richmond, CA) ion exchange resin to free them of contaminating transition metals.

Enzymes

Bovine erythrocyte SOD (EC 1.15.1) was obtained from Sigma Chemical Company and catalase (EC 1.11.1.6) from Millipore (Freehold, NJ). Catalase was chromatographed on Sephadex G-25 (Pharmacia Fine Chemicals, Piscataway, NJ) prior to use to remove the antioxidant thymol. Superoxide dismutase activity was measured by a modified method of McCord and Fridovich (23) using acetylated cytochrome c prepared as per Morehouse et al. (22). Catalase was assayed according to Beers and Sizer (24) while NADPH-cytochrome P450 reductase activity was determined by cytochrome c reduction (25) and cytochrome P450 from its carbon monoxide difference spectrum (26). Hexosaminidase activity was assayed as described by Horvat et al. (27).

Preparation of Antibodies and Ferritin Quantitation

Ferritin was purified from the livers of rats given an intraperitoneal injection of Imferon (iron-dextran) as described by Halliday (28) with modification (18). The purified protein was mixed 1:1 with Freund's adjuvant and injected subcutaneously (1 ml total volume containing 1 mg protein) into the backs of rabbits. Following three biweekly injections the animals were bled from the marginal ear vein. The serum obtained was precipitated with ammonium sulfate and chromatographed on DEAE-cellulose to obtain a purified IgG fraction. Purity was determined by polyacrylamide gel electrophoresis.

Ferritin in microsomes was quantitated using a modification of an ELISA previously described (29). Microtiter plates (Dynatech 96 well, Alexandria, VA) were coated with 5 µg (200 µl total volume) of purified rat liver ferritin in 50 mM sodium bicarbonate buffer, pH 9.6, at 4°C for 15 hours. The plates were rinsed with water and coated with 0.1% gelatin in PBS, pH 7.5. After incubation at 37°C for 30 min the plates were rinsed with water and 3 µg of anti-ferritin IgG plus microsomes (various dilutions containing 1.15 - 5 µg protein), or purified ferritin (0-50 ng) for the standard curve, in 0.2% gelatin in PBS containing 0.2% Tween 20 (total volume 100 µl). The plates were then carefully washed with water after a one hour incubation at 37°C and 50 µl of a 1:2000 dilution of goat, anti-rabbit horseradish peroxidase coupled IgG (Cappel Laboratories, Cochranville, PA) was added in PBS containing 1% gelatin and 0.1% Tween 20 and allowed to react for 30 min at 37°C. The plates were again thoroughly rinsed and substrate, 2.2 mM ortho-phenylenediamine in 0.1 M citrate, pH 5.0,

containing 2.6 mM H_2O_2 (total volume 100 µl), was added. The reaction was stopped after 10 min by the addition of 50 µl of 4 N H_2SO_4 and the absorbance recorded at 490 nm.

Preparation of Microsomes

Male Sprague-Dawley rats (250-275 g) were obtained from Charles River (Boston, MA) from which liver microsomes were isolated as per Pederson and Aust (30) with the exception that an additional centrifugation at 25,000 x g prior to ultracentrifugation was included to ensure removal of lysosomes. The pellet obtained after centrifugation at 105,000 x g was rehomogenized in 0.02 M Tris-HCl/0.15 M KCl pH 7.4 and applied to a Sepharose CL-2B column (2.5 x 25 cm) equilibrated in the same buffer (31). Microsomes, which eluted in the void volume, were pooled and centrifuged again at 105,000 x g and resuspended in 50 mM NaCl containing 50% glycerol. All solutions utilized were thoroughly purged with argon and all steps performed at 4°C to minimize autoxidation of unsaturated lipids.

Lipid Peroxidation Assays

NADPH-dependent peroxidation of microsomes was performed by incubating microsomes (0.5 mg/ml) with NADPH and other additions as specified in the figure legends. Reaction mixtures were constituted in 50 mM NaCl, pH 7.0, and incubated at 37°C in a Dubnoff metabolic shaker under an air atmosphere. Although unbuffered, incubations remained at pH 7.0 throughout the course of the experiments. Peroxidation was monitored by taking aliquots from the incubations at 0, 8, 16, and 24 min to measure the rate of MDA formation using the TBA test (32). Rates shown are those calculated at 16 min.

Assays for Total Iron and Ferritin Iron Release

Total iron was determined by the method of Brumby and Massey (33). The release of iron from ferritin was measured according to Ulvik and Romslo (16) with modification. Microsomes (2 mg/ml) were incubated in an open cuvette in 50 mM NaCl, pH 7.0, containing 175 μM bathophenanthroline sulfonate and catalase (1000 U/ml) to prevent ferrous iron oxidation (18), with other additions as indicated in the figure legends. The formation of the ferrous-bathophenanthroline complex was determined by continuously monitoring the difference in absorbance between 530 and 560 nm using the dual wavelength, non-scan mode of an Aminco DW-2 UV/VIS spectrophotometer (16). Reactions were started by the addition of NADPH and the amount of iron released from ferritin was determined from a standard curve using ferric chloride reduced with 0.1 ml of 10% thioglycolate.

RESULTS

Chromatography of Microsomes and Activities of Associated Enzymes

Protein elution profiles obtained from a Sepharose CL-2B column following chromatography of microsomes and purified rat liver ferritin, which were run separately, are shown in Figure 1A. When microsomes were chromatographed a readily visible yellow peak trailed the microsomal fraction (identified by NADPH-cytochrome P450 reductase activity) and was found to elute at essentially the same volume as did purified ferritin. This fraction was found to react with anti-ferritin antibody using Ouchterlony double diffusion analysis (Figure 1B). The ferritin content of the microsomes, determined using an indirect competitive ELISA, was 6.97 μg of ferritin per mg of microsomal protein, however following chromatography little ferritin could be detected (Figure 1B and Table 1). This suggests that ferritin was effectively removed by chromatography as the ELISA was able to detect very low levels of ferritin as indicated in Figure 2. In agreement, the total iron concentration of the microsomes was greatly decreased by chromatography. Microsomes contained low hexosaminidase activity (3.5 nmol p-nitrophenol/min/mg protein) therefore the ferritin was unlikely a result of lysosomal contamination.

The activities of NADPH-cytochrome P450 reductase, cytochrome P450, SOD, and catalase were measured before and after chromatography to assess the effects of chromatography on the microsomes. Table 1 demonstrates that the specific activity of NADPH-cytochrome P450 reductase and the specific content of cytochrome P450 are increased by chromatography, a likely result of the removal of loosely associated

Figure 1A. Protein Elution Profile for the Chromatography of Microsomes and Purified Rat Liver Ferritin on Sepharose CL-2B. Microsomes (approximately 400 mg) or purified rat liver ferritin in 0.02 M Tris-HCl/0.15 M KCl, pH 7.4 were separately chromatographed on Sepharose CL-2B (2.5 x 25 cm column) as outlined in Materials and Methods. Fractions were analyzed by absorbance at 280 nm and microsomes identified by NADPH-cytochrome P450 reductase activity and ferritin by iron analysis. ■ = microsomes, O purified rat liver ferritin.

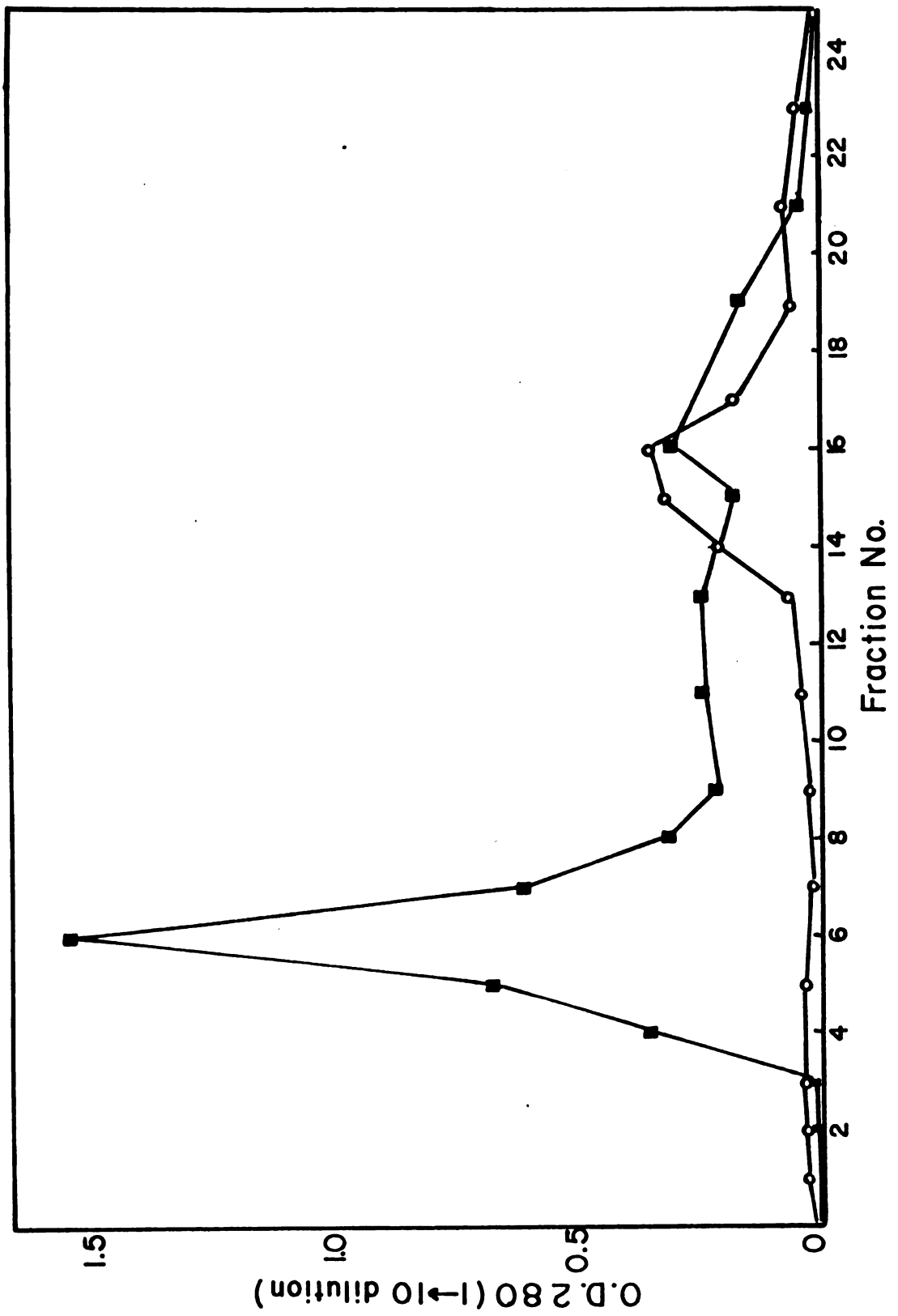

O.D. 280 (1→10 dilution)
0
0.5
1.0
1.5
2
4
6
8
10
12
14
16
18
20
22
24
Fraction No.

Figure 1B. Ouchterlony Double Diffusion Analysis of Microsomes. The center well contained anti-ferritin IgG (0.3 mg). Other wells contained: A, microsomes (0.5 mg); B, microsomes (2.5 mg); C, chromatographed microsomes (0.75 mg); D, yellow peak from Sepharose CL-2B column (fractions 14-18 0.08 mg); E, purified rat liver ferritin (0.08 mg); F, pre-immune IgG (0.3 mg).

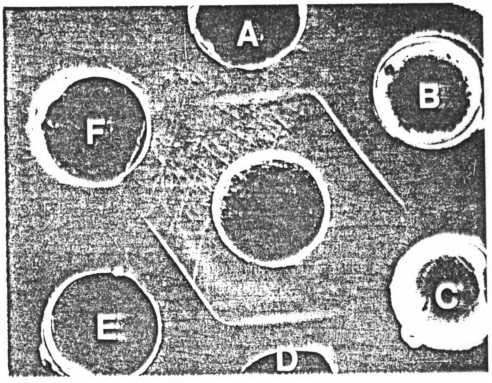

A
B
F
C
E
D

Figure 2. ELISA Standard Curve. The standard curve was obtained using purified rat liver ferritin (0-50 ng) which was assayed by the indirect, competitive ELISA described in Materials and Methods.

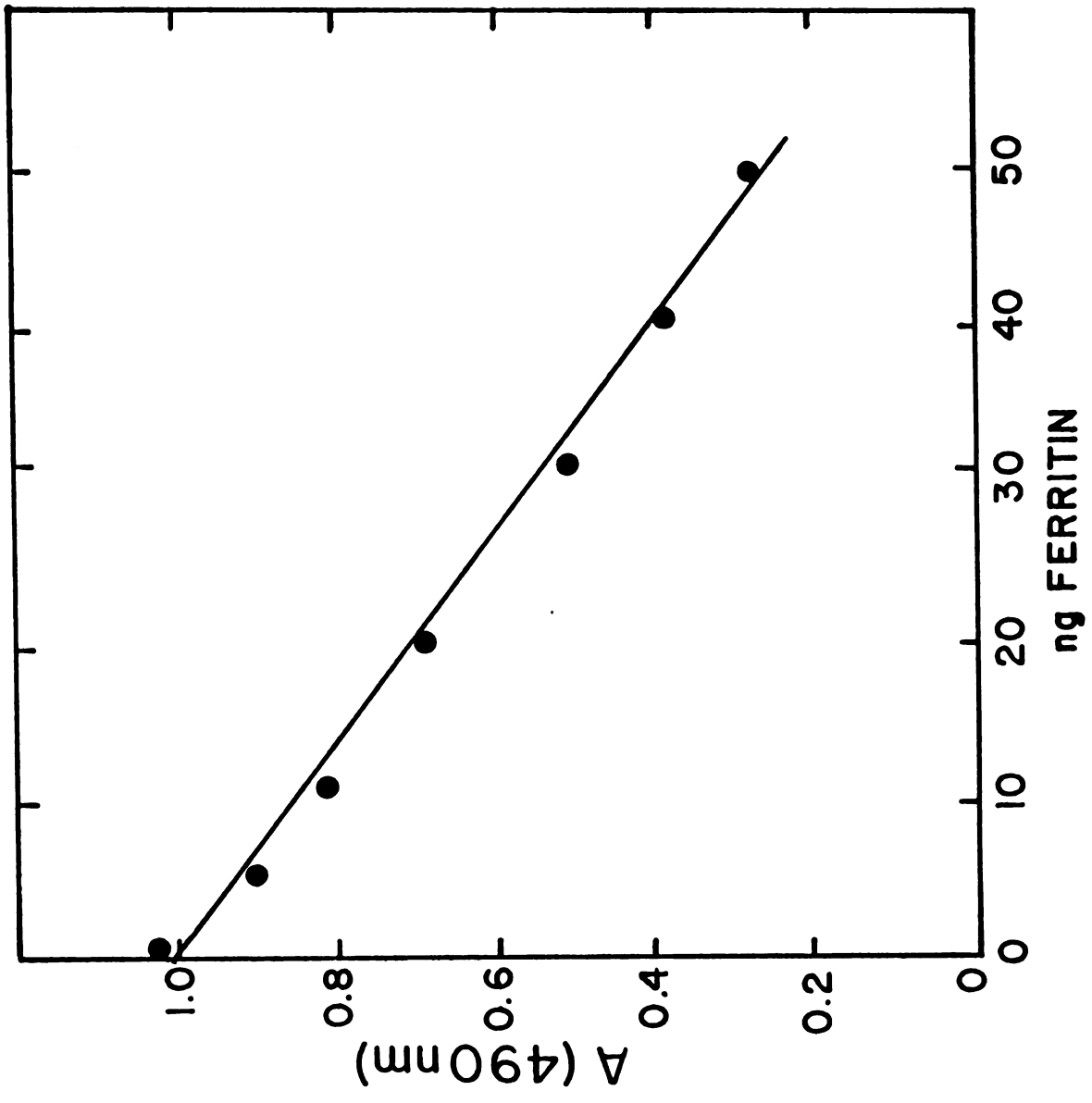

A (490nm)
1.0
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
ng FERRITIN

Table 1. Effect of Sepharose CL-2B Chromatography on Ferritin, Total Iron, and Enzymes Associated with Microsomes[a]

	Cytochrome P450 Reductase	Cytochrome P450	Catalase	SOD	Total Fe	Ferritin
	U/mg	nmol/mg	U/mg	U/mg	nmol/mg	µg/mg
Microsomes	0.09±0.01	0.62±0.16	153.7±39.8	5.6±1.7	18.6±5.0	6.97±2.50
Chromatographed Microsomes	0.16±0.03	0.93±0.06	12.3±7.1	1.5±0.1	3.6±3.0	0.40±0.19

Microsomes were prepared by differential centrifugation and/or chromatography on Sepharose CL-2B as outlined in Materials and Methods. Ferritin, total iron, and associated enzymes were assayed as described in Materials and Methods.

[a] Mean ± S.D. where n = three separate microsomal preparations (6-10 animals each). For total Fe, n = 7.

protein from microsomes. Catalase activity in microsomes was quite high, in agreement with others (34,35), however, much of that activity was removed during chromatography. Superoxide dismutase activity, measured as the inhibition of xanthine oxidase-dependent reduction of cytochrome c (23), was also detected in microsomes. Acetylated cytochrome c was used as it is not directly reduced by NADPH-cytochrome P450 reductase and is therefore more specific for $O_2^{\cdot-}$-dependent reduction. Again most of the SOD activity was removed by the chromatography step. These results were also confirmed by subjecting microsomes and chromatographed microsomes to polyacrylamide gel electrophoresis under nondenaturing conditions and staining the gel for SOD activity using the procedure reported by Beauchamp and Fridovich (36) (data not shown).

Ferritin Iron Release

Since it was previously demonstrated that $O_2^{\cdot-}$ could release iron from ferritin (18-20) and since microsomes are reported to generate $O_2^{\cdot-}$, it was of interest to determine first whether microsomes could release ferritin iron and secondly, whether $O_2^{\cdot-}$ production was necessary for iron release. Because microsomes contain SOD these experiments were conducted with chromatographed microsomes. As shown in Figure 3 the rate of NADPH-dependent iron release from rat liver ferritin was 0.14 nmol Fe^{2+} released per min and could be increased about 7-fold by the addition of 0.5 mM paraquat. Figure 4 demonstrates that rates of iron release were essentially linear with respect to microsomal protein at concentrations greater than 0.25 mg protein per ml. In the absence of microsomes less than 0.03 nmol Fe^{2+} per min was released from ferritin. Iron release was also linear with

Figure 3. Effect of Varying Paraquat Concentration on NADPH-Dependent Iron Release from Ferritin. Incubations (1 ml) contained chromatographed microsomes (2 mg), ferritin (1 mM Fe^{3+}), NADPH (0.5 mM), catalase (1000 U/ml), bathophenanthroline sulfonate (175 μM), and paraquat at the concentrations indicated in 50 mM NaCl, pH 7.0. Formation of the ferrous-bathophenanthroline complex was continuously monitored at 530 nm as described under Materials and Methods.

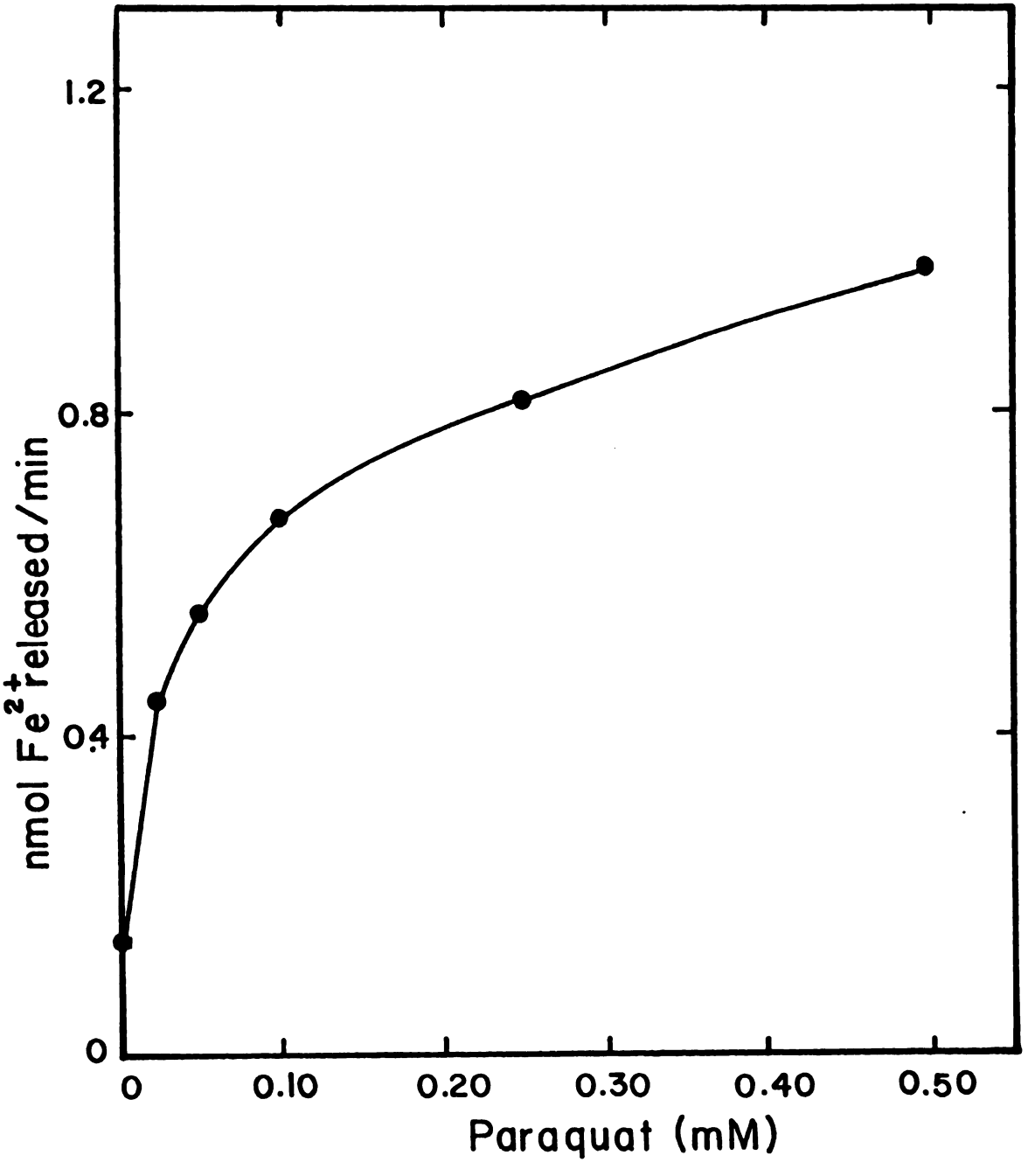
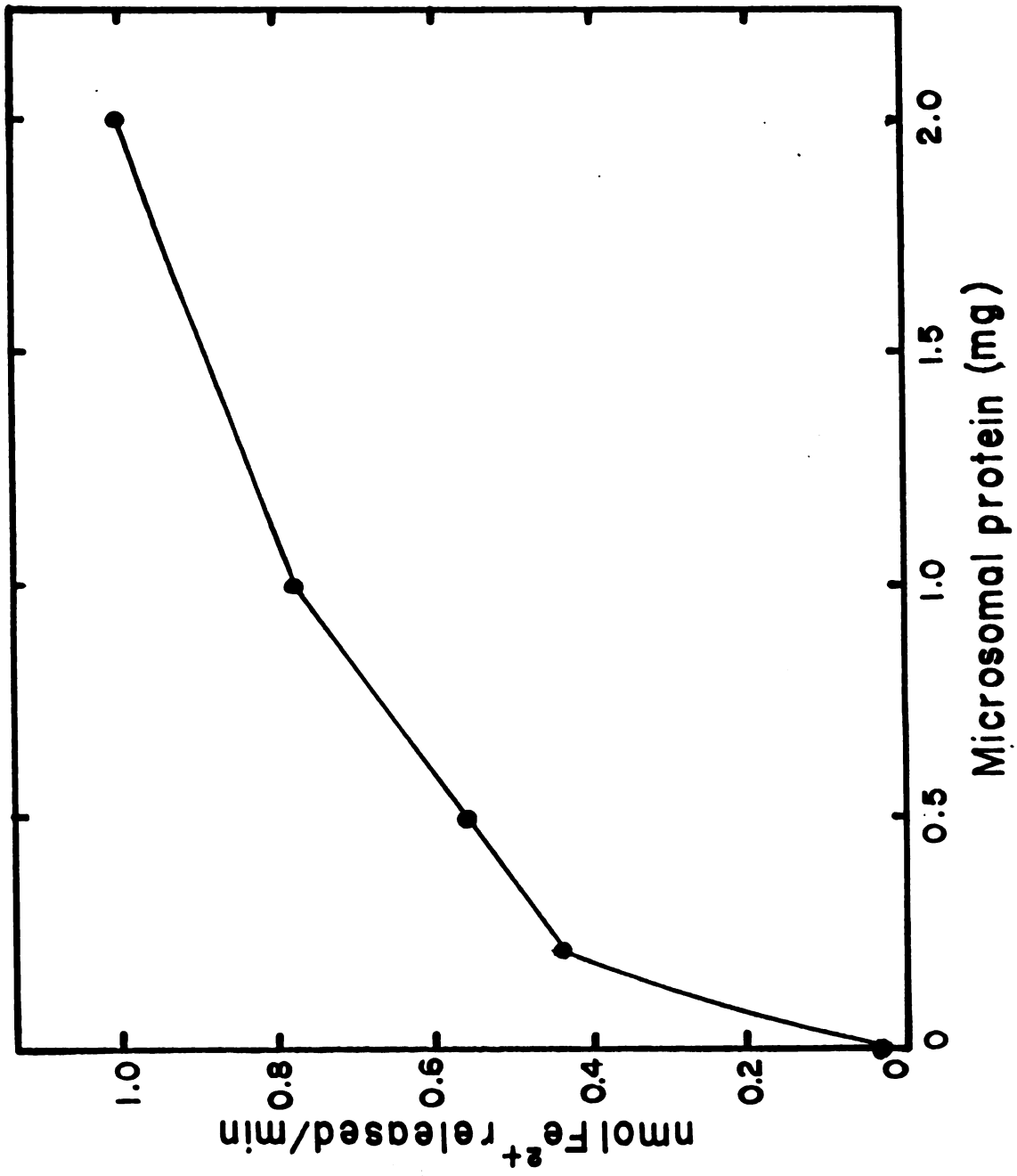

1.2
0.8
0.4
0
nmol Fe^{2+} released/min
0
0.10
0.20
0.30
0.40
0.50
Paraquat (mM)

Figure 4. Effect of Varying Microsomal Protein Concentration on NADPH-Dependent Iron Release from Ferritin. Incubations (1 ml) contained chromatographed microsomes as indicated, ferritin (1 mM Fe^{3+}), NADPH (0.5 mM), catalase (1000 U/ml), paraquat (0.5 mM) and bathophenanthroline sulfonate (175 μM) in 50 mM NaCl, pH 7.0. Reactions were continuously monitored for formation of the ferrous-bathophenanthroline complex as outlined under Materials and Methods.


nmol Fe^{2+} released/min
0
0.2
0.4
0.6
0.8
1.0
0
0.5
1.0
1.5
2.0
Microsomal protein (mg)

respect to ferritin concentration over the range tested (Figure 5). Paraquat was included in both of these experiments to ensure that $O_2^{\cdot-}$ was not rate limiting. In the absence of ferritin a small amount of iron release was observed, however this rate subsided within 5 min. This iron release probably represents the mobilization of some as yet unidentified non-heme, non-ferritin iron from microsomes.

A comparison of iron release from ferritin by microsomes and chromatographed microsomes was also made (Table 2). Rates of iron mobilized are normalized to 1 unit of NADPH-cytochrome P450 reductase activity to account for the different specific activities of the enzyme in the two microsomal preparations. With microsomes, the rate of iron release was only slightly increased upon the addition of purified ferritin and addition of paraquat or SOD had little effect. When these microsomes were treated with 2 mM CN^-, which is known to inhibit the Cu, Zn-SOD, a 50% stimulation in the rate of iron release was observed upon the addition of ferritin. Accordingly, in the presence of CN^-, paraquat now greatly stimulated iron release from ferritin. When ferritin was added to chromatographed microsomes, which are essentially free of SOD activity, iron release was increased over 2-fold, which could be inhibited by the addition of exogenous SOD. Paraquat greatly stimulated iron release irrespective of the presence of CN^-. In the presence of CN^-, paraquat, and ferritin, rates of iron release were comparable for the two microsomal preparations.

NADPH-Dependent Lipid Peroxidation Using Purified Ferritin

The demonstration that microsomes could catalyze the NADPH-dependent release of iron from ferritin suggested that ferritin may provide a source of iron for promotion of lipid peroxidation. In

Figure 5. Effect of Varying Ferritin Concentration on NADPH-Dependent Iron Release from Ferritin. Incubations (1 ml) contained chromatographed microsomes (2 mg), NADPH (0.5 mM), paraquat (0.5 mM), catalase (1000 U/ml), bathophenanthroline sulfonate (175 μM), and varying concentrations of ferritin in 50 mM NaCl, pH 7.0. Formation of the ferrous-bathophenanthroline complex was continuously monitored as described under Materials and Methods.

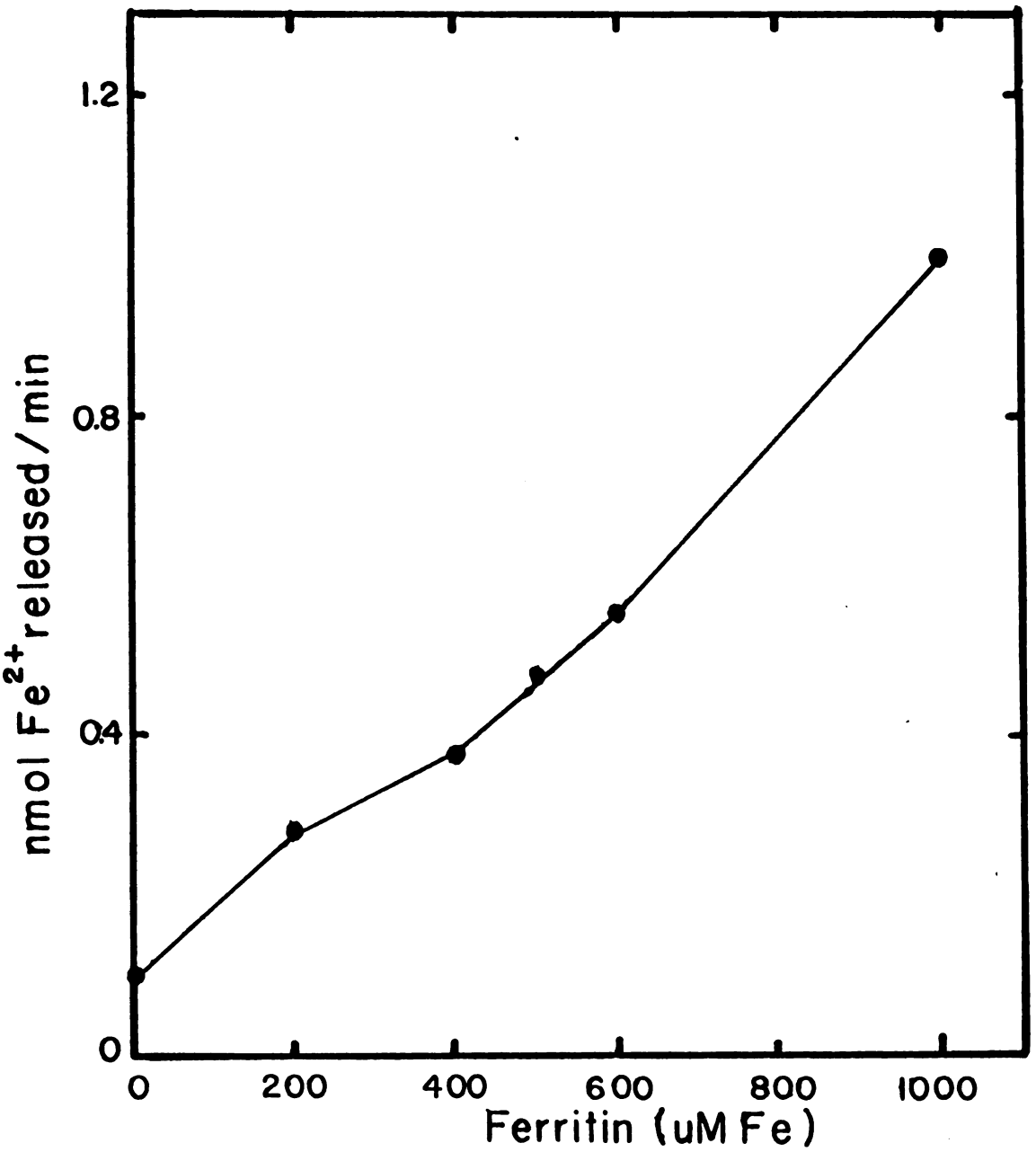

1.2
0.8
0.4
0
nmol Fe^{2+} released / min
0
200
400
600
800
1000
Ferritin (uM Fe)

Table 2. Effect of SOD, CN^-, and Paraquat on Ferritin Iron Release by Microsomes

	nmol Fe^{2+} released/min/U reductase	
	Microsomes	Chromatographed Microsomes
- NADPH	0.00	0.00
- Ferritin	0.78	0.42
Complete	0.88	0.91
+ SOD	0.88	0.62
+ CN^-	1.28	0.83
+ paraquat	1.17	3.75
+ paraquat, + SOD	1.17	0.67
+ paraquat, + CN^-	3.89	3.67

Incubations (1 ml) contained microsomes (2 mg for both preparations), catalase (1000 U/ml) and bathophenanthroline sulfonate (175 μM) in 50 mM NaCl, pH 7.0. Where indicated additions were as follows: NADPH (0.5 mM), ferritin (1 mM Fe^{3+}), SOD (100 U/ml), CN^- (2 mM), and paraquat (0.5 mM). Formation of the ferrous-bathophenanthroline complex was determined by continuously monitoring the difference in absorbance between 530 and 560 nm using the dual wavelength, non-scan mode of an Aminco DW-2 UV/VIS spectrophotometer (Results are the averages of two separate experiments).

the presence of NADPH, microsomes isolated by conventional differential centrifugation were found to exhibit greater rates of MDA formation than chromatographed microsomes (Table 3).

Table 3. Effect of Catalase, SOD and Paraquat on NADPH-Dependent Microsomal Lipid Peroxidation.

	nmol MDA/min/U reductase			
	No Additions	+ Paraquat	+ Catalase	+ SOD
Microsomes	3.75	3.47	4.03	3.75
+ Ferritin	3.75	2.67	4.25	4.25
Chromatographed Microsomes	1.37	1.56	1.37	1.37
+ Ferritin	4.38	5.74	4.30	1.75

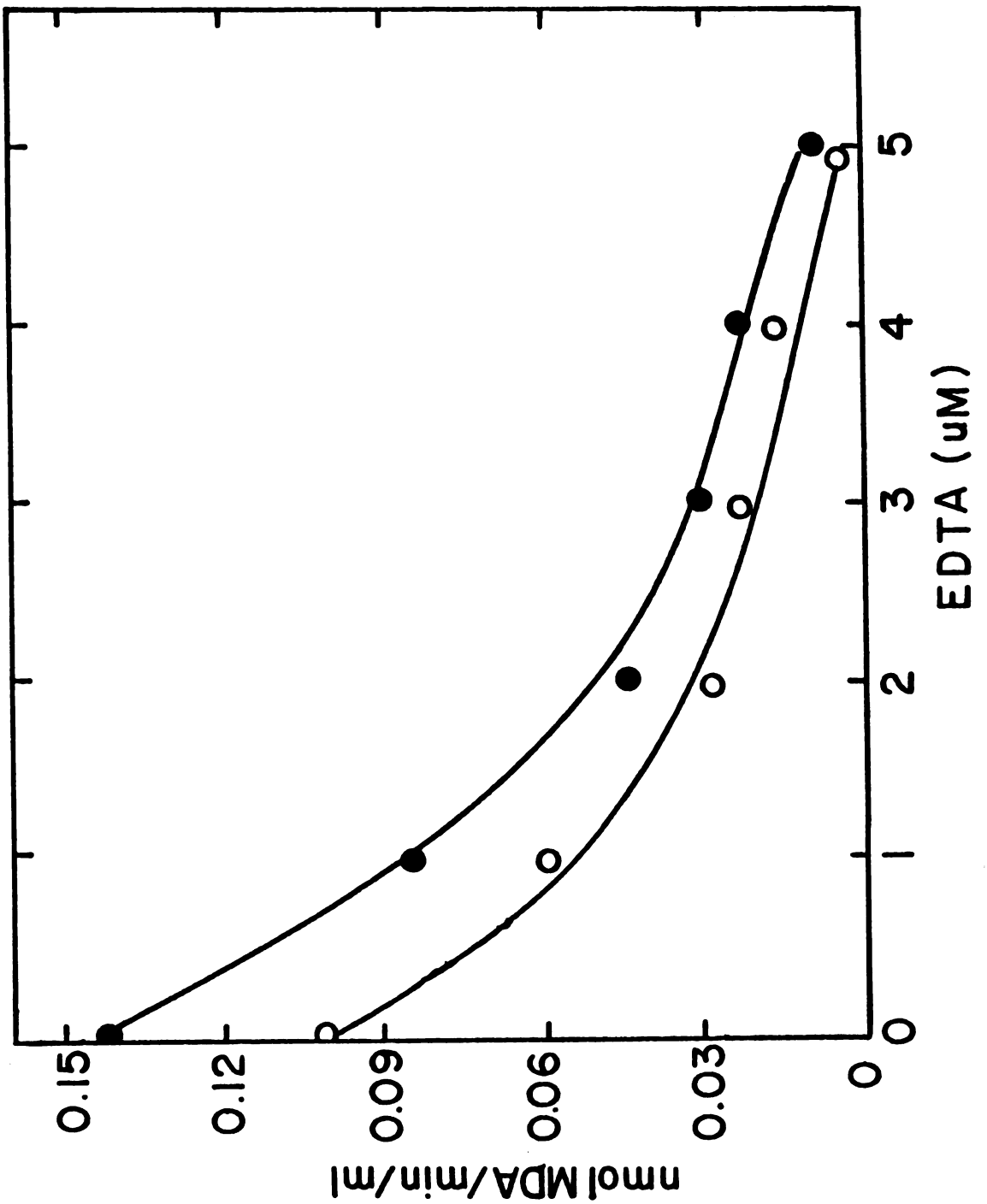
Reaction mixtures (2.5 ml, final volume) contained microsomes (0.5 mg/ml) and ADP (100 μM) in 50 mM NaCl, pH 7.0. Where indicated additions were as follows: ferritin (200 μM Fe^{3+}), paraquat (0.5 mM), catalase (100 U/ml) and SOD (100 U/ml). Reactions were initiated by the addition of NADPH (0.5 mM) and aliquots from the reaction mixtures were assayed as described in Materials and Methods to determine the rate of MDA formation. (Results are the averages of two separate experiments).

Again, rates are expressed per unit of NADPH-cytochrome P450 reductase activity to permit direct comparisons. However, upon the addition of purified rat liver ferritin to lipid peroxidation incubations an increase in MDA formation was noted only in chromatographed microsomes. The addition of exogenous SOD prevented the ferritin-dependent increase in peroxidation of chromatographed microsomes but had no effect on the rate observed in the absence of ferritin for either

microsomal preparation, or the rate observed with microsomes plus ferritin. The inclusion of paraquat resulted in a marked stimulation of MDA formation in chromatographed microsomes but only when ferritin was included. The addition of CN^- or DDC to inhibit endogenous SOD activity in microsomes resulted in a non-specific inhibition of NADPH-dependent lipid peroxidation perhaps either by inhibition of microsomal monoxygenase activity (38) or by free radical quenching (39). Therefore, lipid peroxidation experiments analagous to the iron release studies with microsomes (Table 2) are not shown.

It was of interest to determine if a difference in the amount of iron available for the formation of an initiator was responsible for the greater rates of lipid peroxidation in microsomes than chromatographed microsomes. To assess this, NADPH-dependent lipid peroxidation incubations were titrated with EDTA, which will inhibit lipid peroxidation when it is in excess of iron (37). As shown in Figure 6, peroxidation in microsomes was significantly inhibited by approximately 4 μM EDTA. Lipid peroxidation in chromatographed microsomes was similarly inhibited by approximately 2 μM EDTA.

Figure 6. EDTA Titration of NADPH-Dependent Microsomal Lipid Peroxidation. Reaction mixtures (2.5 ml, final volume) contained microsomes (1.0 mg/ml) and varying amounts of EDTA in 50 mM NaCl, pH 7.0. Reactions were initiated by the addition of NADPH (0.5 mM) and aliquots from the reaction mixtures were assayed as described in Materials and Methods.


nmol MDA/min/ml
0.15
0.12
0.09
0.06
0.03
0
0
1
2
3
4
5
EDTA (uM)

DISCUSSION

While a requirement for transition metals including iron for promotion of lipid peroxidation has been demonstrated *in vitro* (1-4) the nature of the iron complex(es) able to catalyze redox reactions *in vivo* remains unknown. Most *in vitro* microsomal lipid peroxidation studies have utilized exogenously-added low molecular weight ferric iron chelates as the iron source. However, our results have confirmed original studies (9) demonstrating that microsomes contain measurable quantities of endogenous iron which can promote lipid peroxidation. Extreme care was taken to remove metals from all solutions thus it appears that this iron is associated with the microsomal fraction and is not due to the contaminating iron often found in buffers, etc.

The present study has also confirmed previous reports (10,11) that much of the endogenous iron in microsomes isolated by the usual method of differential centrifugation is ferritin. Microsomes prepared by differential centrifugation were found to contain 6.97 μg of ferritin per mg of protein. Ferritin (M_r = 440 kdaltons) can contain up to 4500 atoms of iron per molecule but generally averages 20% loading (8). Thus, using these estimates, the iron content of ferritin is 2.04 nmol Fe per μg of ferritin. Therefore the iron in ferritin accounts for 14.2 nmol Fe per mg of microsomal protein or, subtracting 1.5 mg of the total iron per mg protein (Table 1) for cytochromes P450 and b_5, 83% (14.2/17.1) of the non-heme iron in microsomes. As microsomes have been shown to produce $O_2^{\cdot}$ (21,22), and it has recently been demonstrated that $O_2^{\cdot}$ can reductively release iron from ferritin (18-20), the possibility existed that the

endogenous iron promoting microsomal lipid peroxidation may have originated from ferritin. To demonstrate this it was necessary to first remove endogenous ferritin from microsomes. Treatment of microsomes with calcium or antibody precipitation has been shown to remove a portion of the ferritin (10), however, we found that chromatography of microsomes on Sepharose CL-2B was a more rapid, effective means for separating ferritin from the microsomes.

The addition of purified rat liver ferritin to chromatographed microsomes resulted in a significant stimulation of lipid peroxidation which could be completely reversed by exogenous SOD. In agreement, when paraquat was included to increase $O_2^{\cdot-}$ production rates of MDA formation in the presence of ferritin were greatly enhanced. Iron release studies confirmed that chromatographed microsomes can catalyze NADPH-dependent release of ferritin iron under aerobic conditions. Iron release from ferritin was stimulated by paraquat and inhibited by SOD, further indicating that release is $O_2^{\cdot-}$-dependent, in agreement with the lipid peroxidation data.

When purified ferritin was added to microsomes isolated by differential centrifugation there was no stimulation of NADPH-dependent lipid peroxidation. These results, in conjunction with the inhibition of ferritin-dependent lipid peroxidation by SOD in chromatographed microsomes suggested that microsomes prepared using differential centrifugation may contain SOD. Assay of microsomes for SOD activity by inhibition of acetylated cytochrome c reduction or by activity staining in polyacrylamide gels (results not shown) revealed that this was indeed the case. The SOD present was apparently the

cytosolic Cu, Zn form as its activity was inhibitable by CN^-. Following chromatography, microsomes were essentially free of endogenous SOD activity when measured with either method.

Again, iron release experiments confirmed the results obtained in the lipid peroxidation experiments. When ferritin was added to the microsomes essentially no increase in iron release was observed. However, when the microsomes were pretreated with CN^- iron release from ferritin was observed and paraquat also greatly stimulated iron release. When both microsomal preparations were treated with CN^-, and paraquat was included, rates of iron release from ferritin were virtually identical.

Interestingly, in the absence of added ferritin, microsomes exhibited greater rates of peroxidation than chromatographed microsomes. To ensure that the mechanism of iron release from endogenous ferritin was the same as that for exogenously added, purified ferritin, the partially pure ferritin fraction from the Sepharose CL-2B column (fractions 14-18, Figure 1A) was collected. Addition of this fraction (200 μM Fe^{3+}) to peroxidation experiments using chromatographed microsomes gave results similar to those in Table 3 in the presence and absence of paraquat (not shown), indicating that $O_2^{\cdot-}$ was also required to release the iron from the endogenous ferritin associated with microsomes. Thus, since it appeared that iron release from endogenous ferritin was unlikely to account for the higher rates of peroxidation in microsomes (due to the presence of SOD), another form of iron in these microsomes must be responsible for promoting lipid peroxidation. These results are in agreement with Montgomery et al. (10) who postulated that microsomes contain a small, non-heme iron pool which is not ferritin.

Attempts to further identify and quantitate this non-ferritin iron pool were not successful. However, by titrating lipid peroxidation incubations with EDTA, the size of the iron pool was estimated to be 3-4 nmol iron per mg protein in microsomes and 1.5-2.0 nmol per mg protein following chromatography. These values agree very well with the 17% of the non-heme iron not accounted for by ferritin in microsomes. Lipid peroxidation dependent upon this non-heme, non-ferritin iron is SOD-insensitive, suggesting that it may be directly reduced by microsomes, as is ADP-Fe^{3+} (22). When iron release experiments were conducted in the absence of purified ferritin only very low rates were observed which were linear for only a short period of time. This rate is likely to be reduction and release of the non-heme, non-ferritin iron and, in agreement with the lipid peroxidation data, SOD did not inhibit this iron reduction and/or release.

Chromatography of microsomes also resulted in a significant decrease in catalase activity. Catalase has been shown to stimulate lipid peroxidation dependent upon low concentrations of iron by preventing H_2O_2 induced oxidation of ferrous iron (18). Therefore, it was necessary to determine if the differences in rates of lipid peroxidation between microsomal preparations was due to the differences in catalase activity. The addition of exogenous catalase was found to have little effect in both microsomal preparations, irrespective of the presence of ferritin. Previous work had demonstrated that 10 units of catalase activity greatly stimulated liposomal peroxidation (18,19), thus the catalase activity remaining in microsomes is likely to be sufficient to prevent H_2O_2 accumulation.

These results suggest that the differences in the rates of lipid peroxidation are not a result of the differences in catalase activity but are attributable to the non-heme, non-ferritin iron pool.

The results presented demonstrate that microsomes are capable of releasing iron from ferritin and that ferritin is present in microsomes prepared by the usual differential centrifugation technique. While it is known that the microsomes generate only low amounts of $O_2^{\cdot-}$ it is apparently produced in quantities sufficient to release ferritin iron. It should be noted that similar results are obtained if microsomes are washed with 10 mM EDTA, however the EDTA must then be judiciously removed prior to use of the microsomes in lipid peroxidation studies. The data indicate that microsomes also contain a small amount of non-heme iron which does not require $O_2^{\cdot-}$ for reduction. Our recent work indicates that both ferritin and the unidentified, non-heme iron pool are increased in microsomes following iron-loading of rats (unpublished work). It will be of interest to determine whether ferritin is associated with the endoplasmic reticulum <u>in vivo</u> to further assess its potential to serve as a source of iron for promotion of lipid peroxidation.

LIST OF REFERENCES

1. P. Hochstein, K. Nordenbrand and L. Ernster. Evidence for the involvement of iron in the ADP-activated peroxidation of lipids in microsomes and membranes. Biochem. Biophys. Res. Comm. 14: 323-328 (1964).

2. D.J. Kornburst and R.D. Mavis. Microsomal lipid peroxidation. 1. Characterization of the role of iron and NADPH. Mol. Pharmacol. 17: 400-407 (1980).

3. S.D. Aust, L.A. Morehouse and C.E. Thomas. Role of metals in oxygen radical reactions. J. Free Rad. Biol. Med. 1: 3-25 (1985).

4. E.D. Wills. Mechanisms of lipid peroxide formation in tissues. Role of metals and hematin proteins in the catalysis of the oxidation of unsaturated fatty acids. Biochim. Biophys. Acta 98: 238-251 (1965).

5. G.R. Bartlett. Phosphate compounds in rat erythrocytes and reticulocytes. Biochem. Biophys. Res. Comm. 70: 1055-1062 (1976).

6. S. Pollack, T. Campana and J. Weaver. Low molecular weight iron in guinea pig reticulocytes. Amer. J. Hematol. 19: 75-84 (1985).

7. A. Jacobs. Ferritin: an interim review. In: Current Topics in Hematology, Volume V (S. Piomelli and S. Yachnin, eds.) pp. 25-62, Alan R. Liss, New York (1985).

8. P.M. Harrison. Ferritin: an iron-storage molecule. Sem. Hematol. 14: 55-70 (1977).

9. E.D. Wills. Lipid peroxide formation in microsomes. The role of non-heme iron. Biochem. J. 113: 325-332 (1965).

10. M.R. Montgomery, C. Clark and J.L. Holtzman. Iron species of hepatic microsomes from control and phenobarbital-treated rats. Arch. Biochem. Biophys. 160: 113-118 (1974).

11. K.S. Sargent and H.N. Munro. Association of ferritin with liver cell membrane fractions. Exp. Cell Res. 93: 15-22 (1975).

12. E.C. Theil. Ferritin: structure, function, and regulation. In: Iron Binding Proteins Without Cofactors or Sulfur Clusters (E.C. Theil, G.L. Eichorn and L.G. Marzilli, eds.) pp. 1-38, Elsevier, New York (1983).

13. E.D. Wills. Mechanisms of lipid peroxide formation in animal tissues. Biochem. J. 99: 667-676 (1966).

14. J.M.C. Gutteridge, B. Halliwell, A. Treffry, P.M. Harrison and D. Blake. Effect of ferritin-containing fractions with different iron loading on lipid peroxidation. Biochem. J. 209: 557-560 (1983).

15. B. Rowley and G.D. Sweeney. Release of ferrous iron from ferritin by liver microsomes: a possible role in the toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin. Can. J. Biochem. and Cell Biol. 62: 1293-1300 (1984).

16. R. Ulvik and I. Romslo. Studies on the mobilization of iron from ferritin by isolated rat liver mitochondria. Biochim. Biophys. Acta 588: 256-271 (1979).

17. R.J. Ulvik. Relevance of ferritin-binding sites on isolated mitochondria to the mobilization of iron from ferritin. Biochim. Biophys. Acta 715: 42-51 (1982).

18. C.E. Thomas, L.A. Morehouse and S.D. Aust. Ferritin and superoxide-dependent lipid peroxidation. J. Biol. Chem. 260: 3275-3280 (1985).

19. M. Saito, C.E. Thomas and S.D. Aust. Paraquat and ferritin-dependent lipid peroxidation. J. Free Rad. Biol. Med. 1: 179-185 (1985).

20. P. Biemond, H.G. VanEijk, A.J.G. Swaak and J.F. Koster. Iron mobilization from ferritin by superoxide derived from stimulated polymorphonuclear leukocytes. J. Clin. Invest. 73: 1576-1579 (1984).

21. R.L. Fong, P.B. McCay, J.L. Poyer, B.B. Keele and H. Misra. Evidence that peroxidation of lysosomal membranes is initiated by hydroxyl free radicals produced during flavin enzyme activity. J. Biol. Chem. 248: 7792-7797 (1973).

22. L.A. Morehouse, C.E. Thomas and S.D. Aust. Superoxide generation by NADPH-cytochrome P450 reductase: the effect of iron chelators and the role of superoxide in microsomal lipid peroxidation. Arch. Biochem. Biophys. 232: 366-377 (1984).

23. J.M. McCord and I. Fridovich. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J. Biol. Chem. 244: 6049-6055 (1969).

24. R.F. Beers, Jr. and I.W. Sizer. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. 195: 133-140 (1952).

25. T.C. Pederson, J.A. Buege and S.D. Aust. Microsomal electron transport. The role of reduced nicotinamide adenine dinucleotide phosphate-cytochrome c reductase in liver microsomal lipid peroxidation. J. Biol. Chem. 248: 7134-7141 (1973).

26. T. Omura and R. Sato. The carbon monoxide-binding pigment of liver microsomes. II. Evidence for its hemoprotein nature. J. Biol. Chem. 239: 2370-2378 (1964).

27. A. Horvat, J. Baxandall and O. Touster. The isolation of lysosomes from Ehrlich Ascites tumor cells following pretreatment of mice with Triton WR-1339. J. Cell Biol. 42: 469-479 (1969).

28. J. W. Halliday. Immunoassay of ferritin in plasma. In: Methods in Enzymology, Volume 84 (J. Langone and J. Van Vunakis, eds.) pp. 148-171, Academic Press, New York (1982).

29. T.S.L. Fan and F.S. Chu. An indirect enzyme-linked immunosorbent assay for detection of aflatoxin B_1 in corn and peanut butter. J. Food Protec. 47: 263-266 (1984).

30. T.C. Pederson and S.D. Aust. Aminopyrine demethylase. Kinetic evidence for multiple microsomal activities. Biochem. Pharmacol. 19: 2221-2230 (1970).

31. M.W. Kennedy, N.R. Carpentier, P.P. Dymerski, S.M. Adams and L.S. Kaminsky. Metabolism of monochlorobiphenyls by hepatic microsomal cytochrome P450. Biochem. Pharmacol. 29: 727-736 (1980).

32. J.A. Buege and S.D. Aust. Microsomal lipid peroxidation. In: Methods in Enzymology, Volume 52 (S. Fleischer and L. Packer, eds.) pp. 302-310, Academic Press, New York (1978).

33. P.E. Brumby and V. Massey. Determination of non-heme iron, total iron, and copper. In: Methods in Enzymology, Volume 10 (R.W. Estabrook and M.D. Pullman, eds.) pp. 463-474, Academic Press, New York (1967).

34. J.R. Gillette, B.B. Brodie and B.M. LaDu. Oxidation of drugs by liver microsomes: on the role of TPNH and oxygen. J. Pharmacol. Exp. Ther. 119: 532-540 (1957).

35. L.A. Morehouse, M. Tien, J.R. Bucher and S.D. Aust. Effect of hydrogen peroxide on the initiation of microsomal lipid peroxidation. Biochem. Pharmacol. 32: 123-127 (1983).

36. C. Beauchamp and I. Fridovich. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal. Biochem. 44: 276-287 (1971).

37. M. Tien, L.A. Morehouse, J.R. Bucher and S.D. Aust. The multiple effects of ethylenediaminetetracetate in several model lipid peroxidation systems. Arch. Biochem. Biophys. 218: 450-458 (1982).

38. A.L. Hunter and R.A. Neal. Inhibition of hepatic mixed-function oxidase activity *in vitro* and *in vivo* by various thiono-sulfur-containing compounds. Biochem. Pharmacol. 24: 2199-2205 (1975).

39. G.M. Bartoli, A. Muller, E. Cadenas and H. Sies. Antioxidant effect of diethyldithiocarbamate on lipid peroxidation assessed by low-level chemiluminescence and alkane production. FEBS Lett. 164: 371-374 (1983).

APPENDIX

Publications

Thomas, C.E., and S.D. Aust (1986), "Reductive release of iron from ferritin by paraquat and related bipyridyls". J. Biol. Chem., In Press.

Thomas, C.E., and S.D. Aust (1986), "Release of iron from ferritin by cardiotoxic anthracycline antibiotics". Arch. Biochem. Biophys., In Press.

Thomas, C.E., and S.D. Aust (1986), "Free Radicals and Environmental Toxins", Annals of Emerg. Med., In Press.

Thomas, C.E., and S.D. Aust (1986), "Role of metals in oxygen radical reactions and oxidative stress", In: Handbook of Biomedicine of Free Radicals and Antioxidants, (Miquel, J., ed.), CRC Press, Boca Raton, FL., In Press.

Thomas, C.E., L.A. Morehouse, and S.D. Aust (1985), "Ferritin and superoxide-dependent lipid peroxidation", J. Biol. Chem. 260: 3275-3280.

Thomas, C.E., and S.D. Aust (1985), "Rat liver microsomal NADPH-dependent release of iron from ferritin and lipid peroxidation", J. Free Rad. Biol. Med. 4: 293-300.

Saito, M., C.E. Thomas, and S.D. Aust (1985), "Paraquat and ferritin-dependent lipid peroxidation", J. Free Rad. Biol. Med. 1: 179-185.

Aust, S.D., L.A. Morehouse, and C.E. Thomas (1985), "Role of metals in oxygen radical reactions", J. Free Rad. Biol. Med. 1: 3-25.

Aust, S.D., C.E. Thomas, L.A. Morehouse, M. Saito, and J.R. Bucher (1985), "Active Oxygen and Toxicity", In: Biological Reactive Intermediates III, Pergamon Press, Inc., New York, NY., In Press.

Aust, S.D., C.E. Thomas, L.A. Morehouse, and M. Saito (1985), "Membrane Oxygen Radical Formation and Lipid Peroxidation", In: Cell Membranes and Cancer, (Galeotti, T., A. Cittadini, G. Neri, S. Papa, and L.A. Smets, eds.), Elsevier, Amsterdam, pp. 235-244.

Morehouse, L.A., C.E. Thomas, and S.D. Aust (1984), "Superoxide generation by NADPH-cytochrome P450 reductase: The effect of iron chelators and the role of superoxide in microsomal lipid peroxidation", Arch. Biochem. Biophys. 232: 366-377.

Abstracts

Thomas, C.E., and S.D. Aust (1986), "Radical release of iron from ferritin", The Toxicologist 6: 152.

Thomas, C.E., and S.D. Aust (1985), "Release of ferritin iron by redox active compounds", Conference on Radical Induced DNA Damage, East Lansing, MI.

Thomas, C.E., M. Saito, and S.D. Aust (1985), "Superoxide mediated, ferritin-dependent lipid peroxidation", Gordon Research Conference, Ventura, CA.

Thomas, C.E., L.A. Morehouse, and S.D. Aust (1984), "Ferritin-dependent microsomal and superoxide catalyzed lipid peroxidation", Fed. Proc. 43: 2029.

Thomas, C.E., L.A. Morehouse, and S.D. Aust (1984), "Ferritin and superoxide-dependent lipid peroxidation", Great Lakes Workshop on Oxygen Radicals in Medicine and Biology, East Lansing, MI.

Thomas, C.E., L.A. Morehouse, and S.D. Aust (1984), "Superoxide-dependent, ferritin catalyzed lipid peroxidation", Michgan Regional Chapter of the Society of Toxicology, East Lansing, MI.

Saito, M., C.E. Thomas, L.A. Morehouse, and S.D. Aust (1984), "Paraquat and ferritin-dependent lipid peroxidation", Great Lakes Workshop on Oxygen Radicals in Medicine and Biology, East Lansing, MI.

Morehouse, L.A., C.E. Thomas, and S.D. Aust (1983), "The effect of EDTA-Fe^{3+} on NADPH cytochrome P450 reductase-dependent superoxide generation and lipid peroxidation", Fed. Proc. 42: 2062.

Morehouse, L.A., J.A. Bumpus, R. Voorman, C.E. Thomas, and S.D. Aust (1984), "Superoxide production by reconstituted cytochrome P450-dependent mixed function oxidases", Fed. Proc. 43: 1477.