Microsomal Lipid Peroxidation As A
Mechanism of Cellular Damage

by

Douglas J. Kornbrust
Department of Radiation Biology and Biophysics

Submitted in Partial Fulfillment of the
Requirements for the Degree

DOCTOR OF PHILOSOPHY

MASTER

Supervised by Richard D. Mavis
Department of Radiation Biology and Biophysics

The University of Rochester
Rochester, New York

1979

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ABSTRACT

I. The NADPH/iron-dependent peroxidation of lipids in rat liver microsomes was found to depend on the presence of free ferrous iron. High concentrations of Fe^{2+} initiated microsomal lipid peroxidation at an initial rate which was independent of NADPH. With Fe^{3+} , lower Fe^{2+} concentrations, or longer incubation times, NADPH stimulated microsomal lipid peroxidation and this stimulation was blocked by cytochrome c which is capable of accepting electrons from the microsomal NADPH-cytochrome P-450 reductase. NADPH did not induce peroxidation in the absence of iron, nor did ferric iron in the absence of NADPH. Thus the role of NADPH appears to be maintenance of iron in the reduced Fe^{2+} state. The presence of superoxide dismutase, catalase, or the hydroxyl radical scavengers ethanol or thiourea had little or no effect on the iron-initiated peroxidation, ruling out dependence on reduced states of oxygen. Complete chelation of iron by EDTA completely inhibited peroxidation and no stimulation by either EDTA or ADP was observed over the peroxidation produced by free iron alone. Preincubation of microsomes in the absence of iron did not enhance the rate of peroxidation upon subsequent addition of iron, suggesting that iron acts by initiating peroxidative decomposition of membrane lipids rather than by catalyzing the breakdown of pre-formed hydroperoxides.

Liposomes of extracted microsomal lipid also underwent peroxidation in the presence of ferrous iron at a rate comparable to intact microsomes. Ascorbate stimulated iron-induced liposomal peroxidation but had no effect in the absence of iron. Hydrogen peroxide did not enhance iron-induced liposomal peroxidation and inhibited at high concentrations, ruling out a role for hydroxyl radicals produced by reduction of H_2O_2 by Fe^{2+} .

II. Carbon tetrachloride initiated lipid peroxidation in isolated rat liver microsomes in the absence of free metal ions. In contrast to the non-enzymatic process stimulated by ferrous iron, CCl_4 -induced peroxidation showed an absolute requirement for NADPH and appeared dependent on the integrity of cytochrome P-450 as reflected by measurement of aminopyrine demethylase and aniline hydroxylase activities. The peroxidation subsided after only 10 percent of the total peroxidizable polyunsaturated fatty acids had reacted, at which point the cytochrome P-450 activities were 85-95 percent inhibited, whereas NADPH-cytochrome P-450 reductase activity was not significantly affected. Ferrous iron, in contrast, caused peroxidation of 100 percent of the available polyunsaturated fatty acids, and produced decreases in cytochrome P-450 activities as well as loss of susceptibility to CCl_4 -induced peroxidation that approximately paralleled the extent of peroxidation. Thus, CCl_4 -induced peroxidation appeared to be ten times more potent in inhibiting cytochrome P-450 activities than

the peroxidation caused by iron. Boiled liver microsomes or liposomes prepared from extracted liver lipid underwent extensive peroxidation in the presence of untreated microsomes when ferrous iron was the initiating species. In contrast, the CCl_4 -induced peroxidative response was not affected by addition of these exogenous forms of lipid substrate and thus the initiating species appeared to be confined to the active microsomes. No detectable peroxidation was induced by CCl_4 in microsomes from brain, kidney, or lung, and microsomal aminopyrine demethylase and aniline hydroxylase activities were more than ten-fold lower in these tissues compared to liver. These results are consistent with activation of CCl_4 by cytochrome P-450 to a reactive short-lived radical which initiates peroxidation in the immediate vicinity of the cytochrome, and thereby inhibits enzyme activity either by destruction of essential lipids or by direct attack on the enzyme by reactive intermediates of the peroxidative process. Loss of cytochrome P-450 activity then results in cessation of the CCl_4 -induced peroxidative response prior to more extensive reaction of membrane polyunsaturated lipids.

III. Rates of in vitro lipid peroxidation of microsomes and homogenates were found to vary widely between different tissues and species. In rats and rabbits, lung microsomes peroxidized at a 25 to 50-fold lower rate than liver, kidney, testes, and brain microsomes. Heart microsomes peroxidized at a rate slightly

greater than, but most similar to, lung microsomes. Comparison of tissue homogenates also revealed the unique resistance of lung and heart to lipid peroxidation. Vitamin E levels in lung and heart microsomes were several-fold higher than in microsomes from the other tissues studied, which accounted for the relative resistance of lung and heart to lipid peroxidation. Liposomes of extracted rat lung microsomal lipid were also resistant to peroxidation and the amount of vitamin E contained in the lung lipid extract was sufficient to confer the same degree of resistance when incorporated into an equivalent amount of rat liver lipid. Higher rates of peroxidation in mouse lung microsomes relative to rabbit, rat, and human lung microsomes was similarly correlated with a lower concentration of vitamin E in mouse lung microsomes. These data provide strong support for the role of vitamin E as the major cellular antioxidant, with respect to lipid peroxidation, and demonstrate the utility of the microsomal system in characterizing tissue differences in susceptibility to peroxidative membrane decomposition.

IV. Rat lung and liver microsomes did not undergo lipid peroxidation in the absence of iron when incubated with NADPH and widely varying concentrations of paraquat. Paraquat also did not stimulate rat liver and lung microsomal peroxidation induced by added iron and NADPH, and was inhibitory at concentrations above 10 μ M. Similarly, no stimulation of peroxidation was produced by

paraquat in rabbit or human lung microsomes; however, under similar conditions, paraquat enhanced NADPH/iron-dependent peroxidation in mouse lung and liver microsomes, apparently by facilitating reduction of iron by the mouse microsomes. In lung microsomes obtained from rats sacrificed at 12, 18, and 24 hours following a lethal dose of paraquat (50 mg/kg, i.p.), there was no loss of vitamin E or increase in peroxidizability which would be expected if lipid peroxidation had occurred in vivo, although extensive lung damage developed during this time period. In addition, no decrease in peroxidizable polyunsaturated fatty acids was observed in lung homogenate or microsomes obtained from the paraquat-treated rats, although there was a significant reduction in the palmitate content of these fractions at 24 hours post injection. These results indicate that paraquat does not cause pulmonary toxicity by initiating peroxidation of lung lipids, and the decrease of palmitate in paraquat-damaged lungs is consistent with inhibition of fatty acid synthesis as an early event in the pathogenesis of paraquat toxicity.

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ABBREVIATIONS

APDM	-	aminopyrine demethylase
AH	-	aniline hydroxylase
BSA	-	bovine serum albumin
BHT	-	butylated hydroxytoluene
DPPC	-	dipalmitoyl phosphatidylcholine
MDA	-	malondialdehyde
NBT	-	nitroblue tetrazolium
pHMB	-	p-hydroxymercuribenzoic acid
PPUFA	-	peroxidizable polyunsaturated fatty acids
reductase	-	NADPH-cytochrome P-450 (or cytochrome <u>c</u>) reductase
SOD	-	superoxide dismutase
TBA	-	thiobarbituric acid

INTRODUCTION

Background on Lipid Peroxidation

A number of pathological conditions stemming primarily from exposure to toxic chemicals are believed to be manifested through a process of lipid peroxidation. This phenomenon has been most extensively studied in the liver, where peroxidative decomposition of cellular lipids has been associated with functional disturbances in subcellular structures, the classical example being the effect of carbon tetrachloride on the endoplasmic reticulum (100). Lipid peroxidation is also hypothesized to play a role in aging (93) as well as tissue damage resulting from vitamin E deficiency (89) or exposure to ionizing radiation (11).

Peroxidation of tissue lipids has long been equated with rancidity in the food industry, although it was not until relatively recently that its importance as a mechanism of cellular injury was recognized. This awareness is attributable primarily to the development of methods for quantitating peroxidative changes, stemming from the early observations of Kohn and Liversedge (56). In their report, they described the colorimetric reaction of thiobarbituric acid (TBA) with an unknown substance formed during the aerobic incubation of tissue homogenates, which was later identified by Patton and Kurtz (85) as malondialdehyde (MDA). Subsequently, it was shown that MDA was produced in greater amounts upon incubation of purified membrane fractions as well as isolated

lipids (46,70,87,94,133), and that its formation was associated with oxygen-dependent decomposition of polyunsaturated fatty acids (i.e.: those containing multiple double bonds) present as constituents of membrane phospholipids.

The reaction mechanism for the peroxidation of polyunsaturated fatty acids has been widely studied but is still not well understood. Dahle et al. (27) have proposed a theoretical sequence to account for the evolution of malondialdehyde and other products known to be generated during lipid peroxidation. As illustrated in the diagram below, the process is believed to be initiated by abstraction of a hydrogen atom from a carbon atom located between two double-bonded carbons, resulting in the formation of a lipid free-radical. This highly reactive species will then react with molecular oxygen and undergo a series of chain reactions during which various oxygen-containing free-radicals are generated, eventually resulting in multiple cleavage of the fatty acyl chain and degradation to numerous small molecular weight compounds, including malondialdehyde. Although MDA is considered to be a minor breakdown product of polyunsaturated fatty acids which contain three or more double bonds (27), its formation has been shown to be proportional to the amount of polyunsaturated fatty acids that have reacted (133).

The consequences of lipid peroxidation occurring in vivo are highly speculative but may involve loss of organelle function due to the structural defects incurred by the resulting loss of membrane

INITIATION

PROPAGATION

TERMINATION

lipids. Generation of highly reactive free radicals may be directly damaging to enzymes and cellular structures and there is some evidence that more stable toxic products may be formed which can migrate and elicit damage at sites remote from their origin (105,131). By these mechanisms, it has been postulated that initiation of lipid peroxidation in subcellular structures can lead to pathological states.

The induction of lipid peroxidation in isolated liver microsomes has been shown to require NADPH and molecular oxygen, and to be markedly stimulated by iron salts (46,70,133). The involvement of NADPH in the peroxidative process is mediated by the microsomal NADPH-cytochrome P-450 reductase, the purified form of which has also been shown to promote NADPH- and iron-dependent peroxidation of isolated lipid (liposomes) (87,94). The nature of the interaction between iron and NADPH which leads to peroxidation of lipids is unclear, however. Early evidence had suggested that nonheme iron would be active if present as a phosphate complex (46), although the requirement for phosphate was later disproven (133). Other observations of the effectiveness of inorganic iron led to speculation that its involvement might be simply to stimulate decomposition of lipid hydroperoxides (86,95), since it is well known that many transition metals accelerate homolysis of peroxidic materials (31,38,52). Due to lack of direct evidence, however, it remains uncertain whether this process plays an important role in peroxidation of microsomal lipids.

Current views on the action of iron have focused on its possible role in initiating the lipoperoxidative sequence. Some authors have postulated a direct electron transfer from NADPH to Fe^{3+} , catalyzed by the reductase, which yields Fe^{2+} as the initiator and propagator of microsomal peroxidation (15,81). Other authors, however, have proposed a more complicated involvement of the reductase in the production of superoxide anions ($\text{O}_2^{\bullet-}$), which dismutate to hydrogen peroxide (H_2O_2), and react with Fe^{2+} to yield hydroxyl radicals ($\text{OH}^{\bullet}$) as the ultimate initiator of peroxidation (59,60).

Studies reported in Results I were performed in order to characterize requirements for, and distinguish between the various possible mechanisms of, iron- and NADPH-dependent lipid peroxidation. It was felt that clarification of the molecular events involved in lipid peroxidation was a necessary prerequisite to meaningful investigation of lipid peroxidation a possible mechanism of toxicity.

Lipid Peroxidation As a Mechanism of Carbon Tetrachloride Toxicity

Carbon tetrachloride is a potent liver toxicant, producing fatty accumulation, necrosis, and other degenerative changes which are believed to stem from initial damage to hepatic endoplasmic reticulum. Functional and structural defects in this organelle system have been observed to occur relatively early following exposure to CCl_4 (100). Carbon tetrachloride has also been

reported to induce microsomal lipid peroxidation both in vitro (39,97,112) and in vivo (55,96,98), and this phenomenon has been the focus of numerous investigative attempts to relate the subcellular lesions observed to the large-scale organ pathology associated with CCl_4 poisoning (19,22,111,128).

Early studies yielded recognition of some of the conditions necessary for CCl_4 to promote lipid peroxidation in liver. Although a small amount of malondialdehyde was produced when liver homogenates were incubated with CCl_4 (25), it was subsequently shown that liver mitochondria were capable of metabolizing MDA (99), and thus isolated microsomal fractions were henceforth employed. Glende and Recknagel (39) characterized the requirement for NADPH, and Recknagel et al., (97,100) as well as Slater and Sawyer (112) presented evidence for the involvement of the microsomal NADPH-cytochrome P-450 reductase in the peroxidation process. In these early experiments, however, "endogenous" MDA formation (i.e. in the presence of NADPH but without CCl_4) was relatively high and the stimulatory effect of CCl_4 was not very dramatic. More recently, Masuda and Murano (67) reported conditions which yielded a very low background rate of MDA formation, against which a 3-10 fold stimulation of lipid peroxidation by CCl_4 was observed.

Certain experiments described in Results section II of this treatise were conducted in order to further characterize microsomal lipid peroxidation as promoted by CCl_4 , with regard to enzyme,

cofactor, and substrate requirements. These investigations have revealed major qualitative and quantitative differences between CCl_4 -and ferrous iron-induced peroxidation, thus facilitating determination of the independent effects of Fe^{2+} and CCl_4 on microsomal enzymes. Although CCl_4 has been previously observed to inhibit the activity of microsomal cytochrome P-450 both in vitro (9,40,67) and in vivo (18,115), due to the difficulty in resolving the effects of CCl_4 from ferrous iron a quantitative relationship between the extent of CCl_4 -induced peroxidation and the degree of impairment of this enzyme has not been established. Recognition of the discrepancies between the peroxidative activity of CCl_4 and Fe^{2+} , however, has allowed for this kind of determination, as described herein. It is hoped that generating information about the conditions under which CCl_4 is active in vitro will contribute to an understanding of how this compound or other chemical toxicants exert their deleterious effects in vivo.

Lipid Peroxidation in Extrahepatic Tissues

Liver microsomes have been most frequently employed in investigations of the mechanism of peroxidation of the fatty acyl moieties of membrane phospholipids. Little information is available, however, regarding the relative susceptibilities of membranes from extrahepatic tissues to undergo lipid peroxidation. Roback (102) observed that over 50 times more thiobarbituric acid-reactive material was produced during incubation of liver

homogenate than during incubation of lung or spleen homogenates. Willis and Recknagel (132) recently reported that in the presence of iron and ascorbate, lung microsomes were only 4 percent as active as liver microsomes in producing malondialdehyde, although the basis for the low activity of lung microsomes was not identified in that study.

Since lipid peroxidation has been implicated in the mechanisms of cellular damage caused by a number of different toxicants in a variety of different tissues, a comparison of the propensity of different tissues to undergo this process in vitro would be directly applicable to evaluation of these proposed mechanisms. Apparently different tissues vary widely in their susceptibility to lipid peroxidation, and the studies reported herein (Results III) have attempted to correlate these differences with tissue concentrations of the major cellular antioxidant, vitamin E.

Mechanisms of Paraquat Toxicity

Paraquat (methyl viologen), 1,1-dimethyl-4-4'-bipyridylium dichloride is a broad spectrum herbicide which has received widespread application. It is also highly toxic to mammalian species and results in the formation of delayed pulmonary lesions characterized by progressive fibrosis which is associated with a high degree of lethality in both animals and man (28). Initial symptoms of poisoning include burning of the mouth and throat, abdominal disturbances, vomiting frequently followed by renal and

hepatic failure. Respiratory impairment occurs within 2-5 days (62). Animals given substantial doses of paraquat become anorexic within a few hours and their breathing is shallow, punctuated by sudden gasping movements (37).

Histologically, the lungs show regions of alveolar collapse with widely dilated small airways (119), typical of Respiratory Distress Syndrome observed in premature infants. In general, the alveolar epithelial cells are most affected. At 8-16 hours following an i.v. dose of paraquat, there is extensive damage to both Type I and Type II cells, including sloughing of necrotic cells into the alveolar lumen, and loss of microvilli and a reduction in the number of lamellated inclusion bodies in the remaining Type II cells. Histopathological investigations have revealed no abnormalities in endothelial cells that would account for the edema which develops shortly after paraquat exposure (119). Subsequent pathogenesis is characterized by a progressive fibrosis, which appears to be predominantly intra-alveolar, and may develop in response to the degeneration of lung parenchyma (26).

The selective pulmonary toxicity of paraquat has been attributed to the unique ability of lung to accumulate paraquat in concentrations several-fold higher than in plasma or other tissues, an effect which has been demonstrated both in vivo (107) and in vitro (104). The mechanism of uptake into lung is not well understood but appears to be dependent on cellular energy production (104), and is not attributable to protein binding or metabolism (49).

Attempts to identify the mechanism by which paraquat exerts toxicity have focused on intracellular reactions of paraquat. Bus et al. (16) showed that incubation of paraquat with lung microsomes in the presence of NADPH under anerobic conditions resulted in a tremendous enhancement in the rate of NADPH oxidation and concomitant reduction of paraquat. The role of the microsomal NADPH-cytochrome P-450 reductase in mediating paraquat reduction was demonstrated using antibody prepared against the reductase (16). Previous studies on the herbicidal action of paraquat had revealed a requirement for oxygen and evidence of lipid peroxidation, as indicated by evolution of malondialdehyde following incubation of plant leaves with paraquat (30). Thus, the following mechanism was proposed to explain the toxic effects of paraquat on mammalian lung (16):

Reduction of paraquat by the microsomal reductase and subsequent reoxidation by molecular oxygen results in net transfer of electrons from NADPH to oxygen and formation of superoxide anion ($O_2^{\cdot-}$). This species of "activated oxygen" then spontaneously dismutates to singlet oxygen which reacts with unsaturated fatty acids (present as constituents of membrane phospholipids) and initiates a self-perpetuating and eventually destructive process of lipid peroxidation (16).

In support of this hypothesis, Bus et al. (16) showed that paraquat caused a concentration-dependent enhancement of malondialdehyde formation in an in vitro system consisting of a purified NADPH-cytochrome P-450 reductase and extracted lipid from rat liver microsomes. In contrast, Ilett et al. (49) found that paraquat inhibited peroxidation of whole lung microsomes from rats. In agreement with these latter investigations, Steffen and Netter (116) have shown that paraquat inhibits rat liver microsomal peroxidation. More recently, however, Talcott et al. (108,120) reported that paraquat stimulated the peroxidation of mouse lung and liver microsomes. This effect required the presence of free iron and was inhibited by superoxide dismutase, indicating that the stimulation by paraquat was not mediated by superoxide anions (120), as Bus et al. (16) had suggested. Diquat, a related herbicide, was found to be much more potent than paraquat in enhancing mouse microsomal peroxidation, although diquat is not lung-toxic. In addition, these same authors did not observe any

stimulation of lipid peroxidation by paraquat in rat lung microsomes under conditions identical to those which produced enhancement of peroxidation in mouse microsomes (108), yet rats and mice exhibit comparable sensitivity to paraquat-induced lung damage. Thus, in vitro studies on the ability of paraquat to induce lipid peroxidation have provided conflicting evidence for the lipid peroxidation hypothesis of paraquat toxicity.

In vivo investigations into this proposed mechanism have yielded inconsistent results. Bus et al. (17) showed that when animals were depleted of major cellular antioxidants, either by feeding a vitamin E-deficient diet or by administering diethyl maleate which depletes glutathione levels, sensitivity to paraquat apparently increased, as evidenced by an increased rate of mortality. In contrast, Shu et al. (108) reported that administration of the antioxidant, N,N'-diphenyl-p-phenylene diamine, to mice completely prevented paraquat-induced lipid peroxidation in subsequently isolated microsomes, but did not provide any protection against paraquat-induced lethality. In addition, conjugated dienes, which are normally formed when membrane lipids are peroxidized, were not elevated in the lungs of paraquat-treated rats. These observations, as well as results reported herein, do not favor the concept of lipid peroxidation as the mechanism of paraquat toxicity.

An alternative hypothesis previously advanced to account for the pulmonary toxicity of paraquat is that the herbicide may be

inhibiting production of pulmonary surfactant. Surfactant phospholipids are believed to be biosynthesized in alveolar Type II cells of the lung and these pneumocytes are severely damaged during paraquat poisoning. In fact, the symptomatic and morphologic changes induced by paraquat in lung have been described as being markedly similar to Infant Respiratory Distress Syndrome (66), a condition caused by lack of metabolic capacity to produce sufficient amounts of surfactant phospholipids. In addition to respiratory distress and atelectasis, an impairment of surfactant production could theoretically lead to edema formation (82), which is a prominent manifestation of paraquat poisoning.

Initial support for the hypothesis that paraquat causes a diminution in surfactant was derived from observations of a reduction in surface-active material (SAM) in the airways of paraquat-treated animals. For example, Robertson et al. (103) observed that the lavage fluid obtained from rat lungs two hours after paraquat administration did not promote the elasticity of a pulsating bubble as well as did the lung lavage fluid from control rats. Similarly, Fisher et al. (33), using a Wilhelmy surface balance, demonstrated that the amount of surface-active material obtained by lung lavage was significantly decreased three days after paraquat administration. Several physiological parameters of lung compliance were also altered in paraquat-treated animals, consistent with loss of surfactant (33).

In the studies of Robertson et al. (103), as well as Fisher et al. (33), paraquat administration did not produce any significant decreases in any phospholipid species, although in both cases the lack of effect observed was attributed to the high degree of variability in their lipid analyses. However, Fletcher and Wyatt (35) measured the incorporation of labeled palmitic acid into lung dipalmitoyl phosphatidylcholine (DPPC), the primary component of pulmonary surfactant, and found that paraquat had no effect on this synthesis pathway. In addition, paraquat apparently did not alter total lung lipids, total lipid phosphorus, lecithin phosphorus, or dipalmitoyl lecithin phosphorus content of whole lung homogenate. However, as noted by Fisher et al. (33), the alveolar compartment contains 1/8th of the total lung DPPC such that a depletion of surfactant from this compartment might not be detected when phospholipids are quantitated on a whole lung basis.

In support of this, Malmqvist et al. (65) showed that 24 hours following paraquat administration the phosphatidylcholine content of lung lavage fluid was only 25 percent of controls. Further experiments revealed a small but highly statistically significant reduction in total lung phospholipids, which was due primarily to a decrease in lung phosphatidylcholine. In more recent investigations, Fisher et al. (34) showed that incubation of rat lung slices with paraquat produced a 70 percent reduction in incorporation of [^{14}C]-acetate into lung lipids and that the appearance of labeled palmitate in DPPC of lung lavage fluid was

decreased by 37 percent 24 hours after paraquat administration. These findings suggest that paraquat may inhibit the synthesis of phospholipids in lung and thereby cause a depletion of the critical components of pulmonary surfactant. It remains to be demonstrated, however, whether these effects are responsible for, or merely a consequence of, the pulmonary lesions induced by paraquat.

The studies documented in Results III have revealed that, relative to other tissues, the lung is highly resistant to lipid peroxidation as induced in vitro by a variety of different stimulating agents. This protection against peroxidation is attributable to an unusually high concentration of vitamin E. These findings have served as the impetus for a re-evaluation of the lipid peroxidation hypothesis of paraquat lung toxicity, results of which are presented in section IV. The information derived from these experiments indicates that paraquat does not act by causing lipid peroxidation; however, changes in the fatty acid content of lung fractions following paraquat administration were noted that are consistent with an effect of paraquat on fatty acid synthesis and therefore supportive of the hypothesis that paraquat impairs surfactant production.

EXPERIMENTAL PROCEDURES

Source of Chemicals

acetaldehyde	E
acetylacetone	A
ADP	S
aminopyrine	A
ammonium acetate	A
ascorbic acid	S
bathophenanthroline	S
bovine serum albumin	S
butylated hydroxytoluene	S
catalase	S
cytochrome <u>c</u> (Type VI, horse heart)	S
EDTA	S
epinephrine	S
fatty acid methyl esters	N
Fe (NO ₃) ₃	S
FeSO ₄	S
glucose-6-phosphate	S
glucose-6-phosphate dehydrogenase	S
p-hydroxymercuribenzoic acid	S
metyrapone	S
NADPH	S

EXPERIMENTAL PROCEDURES (Continued)

paraquat (methyl viologen)	S
superoxide dismutase (Type I, bovine blood)	S
taurocholic acid	S
tetraethoxypropane	T
thiobarbituric acid	E
thiourea	E
α -tocopherol	S
trichloroacetic acid	S
xanthine oxidase (grade I, buttermilk)	S

- A - Aldrich Chemical, Rochester, New York
E - Eastman Chemicals, Rochester, New York
N - Nu Chek Prep, Elysian, Minnesota
S - Sigma Chemical Company, St. Louis, Missouri
T - Tridom Chemical, Hauppauge, New York

Animals

Rats were male Long Evans (200-300 g), mice were female BALB/c (20-30 g), and rabbits were male New Zealand albino (2-3 Kg). A sample of normal human lung tissue was obtained from tissue removed during a lobectomy.

Preparation of Microsomes

Animals were sacrificed by intraperitoneal injection of sodium pentobarbital¹, the chest was opened and a cannula was inserted into the trachea. Heart, lungs, and liver were perfused by injecting ice-cold 0.15 M NaCl into the right ventricle of the heart while simultaneously ventilating the lungs through the tracheal cannula. Lungs were excised, and the parenchyma was separated from the visible bronchi and blood vessels and minced thoroughly with scissors. Hearts, livers, kidneys, brains, and testes were removed and similarly minced. The minced tissues were washed several times in ice-cold buffered potassium chloride (0.15 M KCl, 5 mM Tris-maleate, pH 7.4) containing 1 mM EDTA and homogenized in the same, using a teflon-glass homogenizer. The homogenate was centrifuged successively at 300, 1600, 8000, and 30,000 x g for 10 minutes at each speed and the pellets discarded. The "cell-free homogenate" refers to the supernatant after centrifugation at 300 x g. Microsomes were obtained from the 30,000 x g supernatant by centrifugation at 100,000 x g for 1 hour. The microsomal pellet was washed by suspension in and resedimentation from buffered potassium chloride with no EDTA,

¹ The use of pentobarbital to sacrifice animals was found to have no effect on iron- or CCl₄-induced lipid peroxidation, or on NADPH-cytochrome c reductase, APDM, and AH activities. Pentobarbital sacrifice was used because of its greater convenience and reproducibility.

resuspended in the same and stored in liquid nitrogen. This washing procedure was performed with volumes that were adequate to assure that less than 10^{-2} μ M EDTA was present in the microsomal incubations. Protein concentrations were determined by the Lowry method (64).

Preparation of Liposomes

Lipids were extracted from microsomes by the method of Bligh and Dyer (10) and the chloroform phase was completely evaporated under nitrogen gas. The resulting lipid film was hydrated for 20 minutes followed by ultrasonic dispersion under N_2 for one minute or until a clear uniform suspension was obtained.

Incubation Conditions

Microsomes or liposomes were incubated for the specified lengths of time at 37°C . The total volume was usually 0.5 ml (or occasionally 1.0 ml) and contained 40 mM Tris-maleate buffer (pH 7.4) unless otherwise noted. The NADPH generating system consisted of 200 μ M NADPH, 1.5 mM glucose-6-phosphate, and 0.05 units of glucose-6-phosphate dehydrogenase. Iron was added either as $\text{Fe}(\text{NO}_3)_3$ or as FeSO_4 . Due to the instability of iron in solution, $\text{Fe}(\text{NO}_3)_3$ was prepared fresh and used immediately; FeSO_4 stock solutions were stabilized by acidifying with 0.1 mole of HCl per mole of iron, although when higher concentrations were used, a non-acid solution was freshly prepared so as not to perturb the pH of the buffered incubation mixture.

The xanthine oxidase system used to induce lipid peroxidation consisted of 0.8 mg (0.53 units) of xanthine oxidase and 5 mM acetaldehyde. Superoxide production by this system as well as by liver microsomes was measured by monitoring adrenochrome formation from epinephrine (4 mM) at 480 nm as described by Aust et al. (5). An extinction coefficient of $4.02 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ was used.

In experiments in which rates of peroxidation of microsomes from different tissues and species were compared (i.e. Results, III), the incubations contained, in addition to microsomes and Tris-maleate buffer, either 3.0 μM FeSO_4 and 250 μM NADPH or 1.0 μM FeSO_4 and 500 μM ascorbic acid, unless otherwise noted. Conditions for the incubation of homogenates are given in the figure legend.

Other reagents were added as described in the figure legends.

Measurement of Lipid Peroxidation

Lipid peroxidation was quantitated by colorimetric determination of malondialdehyde formation after complexation with thiobarbituric acid (15). Peroxidation was stopped by rapid addition of 50 μl of 0.2 percent butylated hydroxytoluene in ethanol followed by 0.15 ml of 20 percent trichloroacetic acid and 0.3 ml of 0.05 M TBA (or twice these volumes when the incubation volume was 1.0 ml). The final pH was 1.60 which was found to be optimal for formation of the MDA-TBA chromogen. Bovine serum albumin (0.5 mg) was added to facilitate precipitation of protein

during a 10 minute centrifugation, following which the resulting clear supernatant was removed, delivered to clean screw cap culture tubes which were then tightly capped and boiled for 8 minutes. The amount of colored product was measured by determining absorbance spectrophotometrically at 532 nm and converting to nmoles of MDA using a molar extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.

This value was verified using a tetraethoxypropane standard. None of the reagents present in the incubations affected the tetraethoxypropane-derived color. The absorbance spectrum for color produced during microsomal (or liposomal) incubations showed a maximum at 532 nm and thus represented true MDA-TBA color. Control (not incubated) blanks yielded a low background absorbance (0.02 - 0.04 OD units per 100 μg of protein) which was subtracted from sample absorbance. Limits of detection for MDA were assumed to be 0.005 OD units which is equivalent to 0.03 nmoles of MDA.

Lipid peroxidation was expressed in terms of nmoles of malondialdehyde per mg of protein or as "percent peroxidation" which is simply the percent of maximum MDA which would be produced by complete peroxidation of the peroxidizable polyunsaturated fatty acids (PPUFA)² present in the tissue fractions. Maximum MDA

² Peroxidizable polyunsaturated fatty acids (PPUFA), includes all of the detectable polyunsaturated fatty acids except linoleic acid (18:2) which we have demonstrated, in agreement with others (47,70) to be relatively resistant to peroxidation, and is not believed to evolve malondialdehyde (27).

formation was routinely determined for each liver microsomal preparation by incubating microsomes with a sufficient amount of FeSO_4 and NADPH to completely peroxidize microsomal lipids and give the maximum MDA production possible from each preparation, which varied between 65 and 120 nmoles MDA/mg microsomal protein. The amount of MDA produced during the experimental liver microsomal incubations was then expressed in terms of the percentage of this maximum MDA value, which has been shown to be equivalent to the percent depletion of peroxidizable lipid substrate (see Results I) and is referred to as percent peroxidation. Peroxidation in brain, kidney, testes, heart and lung microsomes was similarly quantitated in terms of the percentage of the maximum possible MDA formation, which was calculated by multiplying the value for the yield of MDA per mole of PPUFA in liver microsomes by the PPUFA content of the other fractions, as determined by phospholipid (20) and GLC fatty acid (69) analyses. In separate experiments, the yield of MDA per mole of PPUFA was found to be similar in all tissues studied, thus justifying the application of values obtained in liver microsomes to the determination of maximum MDA in other tissues.

Fatty Acid Analysis of Peroxidized Microsomes

In some experiments, fatty acids were quantitated following incubation of microsomes under the specified lipid peroxidizing conditions. Aliquots of the incubation mixture were removed for

MDA determinations just prior to stopping peroxidation by addition of 6 ml of 2:1 methanol-chloroform. Lipid was extracted by the Bligh and Dyer method (10), and fatty acid methyl esters were prepared as described by Mavis and Vagelos (69). Relative weight percent of the fatty acids present in the final extract were determined by gas liquid chromatography (Hewlett Packard, model 5830A) and identified by comparison to a series of methyl ester standards.

Enzyme Assays

NADPH-cytochrome c reductase was measured spectrophotometrically by recording the optical density change at 550 nm due to reduction of cytochrome c (130).

Aminopyrine demethylase activity was measured by a procedure similar to that described by Orrenius (83) and involved incubation of 200-400 μ g of microsomal protein with 40 mM Tris-maleate, pH 7.4, 300 μ M NADPH, 5 mM $MgCl_2$ and 8 mM aminopyrine (total volume 1.0 ml) for 30 minutes at 37°C. Formaldehyde was determined by the method of Nash (79). The procedure for measuring aniline hydroxylase activity was derived from Imai et al. (51), and included incubation of 300-500 μ g of microsomal protein with 80 mM Tris-maleate, pH 7.4, 300 μ M NADPH, 5 mM $MgCl_2$ and 8 mM aniline (total volume 1.0 ml) for 20 minutes at 37°C, following which p-aminophenol formation was determined.

Termination of NADPH/ Fe^{2+} - or CCl_4 -induced peroxidation prior to measurement of APDM or AH activities was accomplished simply by adding either aminopyrine or aniline, respectively, which have been shown to be powerful antioxidants (see Table 7). In experiments where CCl_4 -induced peroxidation was determined following peroxidation by NADPH/ Fe^{2+} , addition of EDTA, as specified in the figure legends, prevented subsequent iron-induced peroxidation without affecting MDA formation by CCl_4 .

Specific activities were expressed in nmoles per minute per mg microsomal protein. All enzyme assays were linearly proportional to time and added protein over the ranges used.

Vitamin E Determination

Vitamin E was measured using the spectrofluorometric technique of Taylor et al. (125). By this method, tissue samples are saponified and extracted yielding a hexane phase which contains partially purified vitamin E.

Incorporation of Vitamin E into Liposomes

Where indicated, vitamin E extracted from microsomes by the procedure of Taylor et al. (125) or authentic α -tocopherol subjected to the same procedure was incorporated into liver liposomes by mixing the hexane extract with the chloroform solution of liver microsomal lipids prior to drying and dispersing.

In Vivo Studies With Paraquat

Rats were injected intraperitoneally with 50 mg/kg of paraquat dissolved in distilled water, or an equal volume of distilled water only (controls), and sacrificed at 12, 18 and 24 hours post-injection. The lungs from some animals were prepared for microscopic examination using standard hematoxylin and eosin stains.

RESULTS I. The Role of Iron and NADPH

Incubation pH and buffer optima: Considerable differences in iron-dependent lipid peroxidation were observed with different buffer systems. When Tris-maleate was used, high rates of lipid peroxidation were produced by relatively low iron concentrations, as shown in Figure 1. A similar dependence on iron was observed with Tris-HCl (data not shown). In contrast, phosphate buffer greatly suppressed iron-induced peroxidation such that it was necessary to lower the phosphate concentration to less than 8 mM in order to observe stimulation of peroxidation by $1\ \mu\text{M}\ \text{FeSO}_4$ (data not shown). At higher phosphate concentrations, greater amounts of iron were required to stimulate peroxidation. This effect may be due to formation of a weak association between iron and phosphate which may impair the reactivity of iron towards microsomal lipids.

Optimal rates of MDA formation were obtained with 40-80 mM Tris-maleate, pH 7.4, although varying the pH between 6.4-8.0 had little effect on the rate of lipoperoxidative activity. At lower concentrations of (or in the absence of) Tris-maleate less peroxidation was observed. However, in the presence of an amount of KCl (0.4 M) sufficient to maintain ionic strength (and a small amount of phosphate buffer to stabilize pH), addition of up to 80 mM Tris buffer had no effect on peroxidation. Thus, stimulation of peroxidation by higher Tris concentrations in the absence of KCl can be attributed to an ionic strength effect. It has been

previously demonstrated that the lipid peroxidation reaction catalyzed by the purified reductase requires high ionic strength (87).

Characterization of iron and NADPH dependences: Microsomes prepared in the absence of EDTA peroxidized at a rapid rate upon addition of NADPH. This activity was completely inhibited by EDTA indicating dependence on free metal ions. Stimulation of peroxidation by ionic iron has been previously demonstrated (46,70,133). Consequently, microsomes were prepared in the presence of EDTA followed by washing to remove EDTA in order to eliminate metal ions and thus allow control over the iron concentration in our experiments. Figure 1 shows that peroxidation of these microsomes in the presence of NADPH was absolutely dependent upon added iron. Concentrations of ferrous iron as low as 0.5 μM were highly stimulatory and saturation was observed at 20 μM . Similar concentrations of ferric iron were nearly four-fold less effective and a gradual increase in peroxidation with increasing the Fe^{3+} concentration was observed which continued beyond the point of Fe^{2+} saturation.

Figure 2 illustrates the dependence of microsomal lipid peroxidation on NADPH at two different concentrations of ferrous iron. With 10 μM FeSO_4 , increasing NADPH concentration enhanced lipid peroxidation up to 150 μM NADPH, where the amount of MDA produced was about that expected for complete reaction of

membranous polyunsaturated fatty acids (see Table 4, below). At $1 \mu\text{M FeSO}_4$, a much lower concentration of NADPH ($40 \mu\text{M}$) was apparently saturating, and the magnitude of MDA formation associated with NADPH saturation was 4 times less than with $10 \mu\text{M}$ ferrous iron. Even a ten-fold increase in NADPH above $40 \mu\text{M}$ at the lower iron concentration did not produce any increase in peroxidation which suggests that Fe^{2+} concentration is the limiting factor in the extent of peroxidation. In fact, with $10 \mu\text{M FeSO}_4$, a significant amount of peroxidation occurred in the absence of NADPH.

Figure 3 shows the time course of this NADPH-independent microsomal lipid peroxidation as stimulated by free iron. Ferrous iron at $20 \mu\text{M}$ induced a fast initial rate of peroxidation which subsided after around 5-10 minutes. In contrast, the same concentration of ferric iron resulted in over a ten-fold lower initial rate, and the extent of peroxidation after 60 minutes was only around 25 percent of that produced by FeSO_4 . The ability of Fe^{2+} to promote a high initial rate of peroxidation in the absence of NADPH suggests that ferrous iron is capable of directly initiating the peroxidative process. However, this effect is apparently not catalytic since the reaction subsides prior to extensive depletion of membrane lipid substrate (compare Fig. 2 with Fig. 3). Therefore, enhancement of peroxidation by NADPH (Figure 2) may be simply due to its ability to stimulate reduction of Fe^{3+} to Fe^{2+} , as catalyzed by the microsomal reductase, thereby regenerating the active reduced state of iron.

Fig. 1 Effect of iron concentration on MDA formation in liver microsomes. Microsomal protein ranging from 45 to 160 μ g was incubated with 200 μ M NADPH and either FeSO_4 (●) or $\text{Fe}(\text{NO}_3)_3$ (○) for 15 minutes. Other conditions were as described in the Methods section. Each point represents the mean value from four (FeSO_4) or two ($\text{Fe}(\text{NO}_3)_3$) determinations performed in duplicate on microsomes derived from two microsomal preparations from 2 rats each.

Fig. 2 Effect of NADPH concentration on lipid peroxidation in liver microsomes as stimulated by 10 μM FeSO_4 (●) or 1 μM FeSO_4 (○). Microsomes (99-133 μg of protein) were incubated for 15 minutes with the indicated amounts of NADPH. Each point represents the mean value from two experiments performed in duplicate; the two experiments employed different microsomal samples derived from 2 or 3 rats.

Fig. 3 Time course of iron-stimulated microsomal lipid peroxidation in the absence of NADPH. Microsomes (45-144 μg of protein) were incubated with either 20 μM FeSO_4 (●) or 20 μM $\text{Fe}(\text{NO}_3)_3$ (○) but with no NADPH for the indicated lengths of time. Other conditions and procedures were as described in the Methods section. Each point represents the mean value from four (FeSO_4) or two ($\text{Fe}(\text{NO}_3)_3$) experiments performed in duplicate on microsomes derived from two (FeSO_4) or one ($\text{Fe}(\text{NO}_3)_3$) microsomal preparations from 2 or 3 rats.

In support of this mechanism it was found that 200 μM Fe^{3+} produced a 25 percent inhibition of microsome-catalyzed reduction of cytochrome c, presumably by competing with cytochrome c as an acceptor of electrons from the reductase. This effect was not attributable to direct oxidation of cytochrome c by Fe^{3+} since Fe^{3+} did not diminish the spontaneous reduction of cytochrome c (in the absence of microsomes, data not shown). Addition of 1.0 mM EDTA had no effect on reductase activity in the absence of iron, but appeared to enhance the inhibitory properties of 200 μM $\text{Fe}(\text{NO}_3)_3$, possibly by stabilizing the ferric iron in a form which is more readily reduced. More rapid reduction of EDTA-Fe^{3+} compared to free Fe^{3+} by the microsomal reductase has been previously reported (81,87), and is attributable to a shift in the half-cell potential of $\text{Fe}^{3+}/\text{Fe}^{2+}$ by EDTA (13). Ferrous iron was observed to be capable of directly reducing cytochrome c whether microsomes were present or not (data not shown), consistent with recent observations on the interaction of cytochrome c with Fe^{2+} (120).

Figure 4 shows the effectiveness of high concentrations of cytochrome c in preventing or stopping NADPH-dependent lipid peroxidation as stimulated by a low iron concentration (1 μM). Addition of cytochrome c at zero time or after 15 minutes of peroxidation drastically curtailed further peroxidation. This effect could be explained in three ways: 1) cytochrome c was competing with Fe^{3+} for electrons from the reductase, 2)

cytochrome c was directly oxidizing Fe^{2+} , or 3) cytochrome c was accepting electrons from the reductase and thereby preventing formation of some other peroxidation-inducing species, such as superoxide anion. In a related series of experiments we have shown that oxidized cytochrome c partially inhibits Fe^{2+} -stimulated peroxidation in the absence of NADPH (see Results, section II), which is consistent with its ability to directly oxidize ferrous iron to the inactive ferric form (120). However, peroxidation in the presence of NADPH was inhibited to a much greater extent by cytochrome c (Fig. 4), and this additional inhibition probably represents a competition between reduction of cytochrome c and either reduction of iron or of molecular oxygen (to form $\text{O}_2^{\cdot-}$) by the microsomal reductase.

We believe that the data in Figure 5 clearly show that NADPH is required only for maintenance of iron in the ferrous state, thus ruling out the involvement of superoxide in peroxidation. Using short incubation times and increasing concentrations of iron in the presence and absence of NADPH it was found that at 40-50 μM Fe^{2+} , the initial rate of lipid peroxidation is independent of NADPH. Thus ferrous iron alone is capable of promoting lipid peroxidation at a rate comparable to that occurring in the presence of NADPH if conditions are chosen such that regeneration of Fe^{2+} from Fe^{3+} is not required. The lack of stimulation of NADPH under these conditions seems to contradict previous suggestions that reductase-generated superoxide anions (4) or hydroxyl

Fig. 4 Effect of addition of cytochrome c at time zero or after 15 minutes of incubation on the rate of lipid peroxidation in rat liver microsomes as stimulated by 1.0 μ M FeSO_4 . Microsomes (45-114 μ g of protein) were incubated with an NADPH generating system and other reagents as described in the Methods section. Cytochrome c was added at a 200 μ M final concentration. Each point represents the mean value from 3 ($\bullet$, $\square$) or 4 ($\circ$) experiments in duplicate using microsomes derived from two microsomal preparations from 1 or 2 rats.

$\bullet$, control, no cytochrome c added; $\circ$, cytochrome c added at time zero; $\square$, cytochrome c added at 15 minutes.

Fig. 5 Effect of increasing concentrations of Fe^{2+} and Fe^{3+} with and without NADPH on initial rates of lipid peroxidation in rat liver microsomes. Microsomes (95-160 μg of protein) were incubated with either FeSO_4 with no NADPH ($\bullet$), FeSO_4 with 200 μM NADPH ($\circ$), $\text{Fe}(\text{NO}_3)_3$ with no NADPH ($\blacktriangle$), or $\text{Fe}(\text{NO}_3)_3$ with 200 μM NADPH ($\triangle$). Time of incubation was 100 seconds. Other conditions are described in the Methods section. Each point represents the mean value from five (FeSO_4) or 2 ($\text{Fe}(\text{NO}_3)_3$) experiments performed in duplicate using microsomes derived from two different microsomal preparations from 2 rats each. Points at 50 and 60 μM FeSO_4 are not statistically different at $p = 0.80$ whereas points ≤ 40 μM FeSO_4 are statistically different at $p = 0.20$ or less (as calculated using a two-tailed t test).

radicals (36,59,60) directly stimulate lipid peroxidation. Since sufficient amounts of reduced iron obviates the requirement for any input from the reductase, it appears that if activated oxygen is in fact produced through NADPH oxidation it does not initiate lipid peroxidation beyond that induced by iron alone.

Further evidence for the lack of involvement of activated oxygen in the initiation of microsomal lipid peroxidation was obtained by incubating microsomes in the presence of activated oxygen scavengers. According to the mechanism suggested by Lai and Piette (60), reductase-mediated oxidation of NADPH gives rise to $O_2^{\cdot -}$ which spontaneously dismutates to H_2O_2 . The hydrogen peroxide thus generated undergoes a Fenton reaction with reduced metal ions (i.e. Fe^{2+}) to yield $OH^{\cdot}$ as the ultimate initiator of lipid peroxidation. Contrary to this hypothesis, Table 1 shows that addition of superoxide dismutase (50-90 units) which would be expected to promote peroxidation by increasing the rate of $O_2^{\cdot -}$ dismutation to H_2O_2 , had no effect on microsomal peroxidation even though the iron concentration was low and the system was highly NADPH-dependent. The highest amount of SOD tested was capable of completely inhibiting adrenochrome formation from epinephrine as stimulated by 0.53 units of xanthine oxidase (data not shown, see Methods) and was thus highly active. These results confirm previous reports on the lack of effect of SOD on lipid peroxidation (54,88). Similarly, addition of catalase (550 units), alone or in combination with SOD had no effect on NADPH/ Fe^{2+} -

stimulated MDA formation (Table 1), which is inconsistent with a H_2O_2 intermediate, as postulated by Lai and Piette (60).

Moreover, amounts of ethanol (1-2 percent) and thiourea (4-8 mM) which have been reported to effectively scavenge hydroxyl radicals (8) did not inhibit NADPH-dependent peroxidation, although thiourea was observed to cause a slight reduction in NADPH-independent peroxidation (data not shown) and may, therefore, have weak antioxidant properties.

Mitochondria contain an active superoxide dismutase (72) and are variably present in different microsomal preparations. Thus it was considered that the lack of effect of activated oxygen scavengers on microsomal lipid peroxidation might be due to mitochondrial SOD contamination acting to obscure the effect of SOD or hydroxyl radical scavengers added to our microsomes. However, no activity of the mitochondrial marker, succinate-cytochrome c reductase, was detectable in our microsomes although the assay is sensitive enough to detect less than a 1 percent mitochondrial contamination under the conditions employed. Also, using the inhibition of xanthine oxidase-induced conversion of epinephrine to adrenochrome to quantitate the presence of SOD, we determined that the SOD content of our microsomes was less than 10^{-2} units per μg of protein. This amount of SOD is apparently insufficient to block superoxide-dependent phenomena since nearly 50 units of added SOD was required to completely inhibit oxidase-induced microsomal lipid peroxidation (see Table 2, below). Furthermore, in the presence of NADPH, these same microsomes stimulated epinephrine oxidation at a higher rate than that reported by other investigators (5,77), and

Table 1. The effect of activated oxygen scavengers and ADP on NADPH/Fe²⁺-induced lipid peroxidation. Microsomes (98-147 µg protein) were incubated for 15 minutes in the presence of 200 µM NADPH and 1.0 µM FeSO₄ plus the indicated amounts of scavengers or chelators. Each number represents the mean value ($\pm$ standard deviation) from 3-5 experiments performed in duplicate using microsomes derived from 2 or 3 different microsomal preparations from 1-3 rats each.

<u>Condition</u>	<u>Lipid Peroxidation (% of control MDA formation*)</u>
SOD,	
50 units	101.8 $\pm$ 1.7
90 units	104.7 $\pm$ 3.5
150 units	108.4 $\pm$ 2.6
catalase,	
550 units	99.7 $\pm$ 1.9
SOD, 150 units plus catalase, 500 units	102.0 $\pm$ 1.8
ethanol,	
1.0%	98.7 $\pm$ 0.9
2.0%	96.5 $\pm$ 1.8
thiourea,	
4 mM	98.0 $\pm$ 1.3
8 mM	94.3 $\pm$ 2.1
ADP,	
10 µM	101.0 $\pm$ 3.3
200 µM	101.8 $\pm$ 3.0
2 mM	100.0 $\pm$ 3.9

* control values averaged 22.6 $\pm$ 3 nmoles MDA/mg protein

this response was over 50 percent inhibited by 7.5 units of added SOD, thus demonstrating the viability of superoxide in our microsomal incubations in the absence of added SOD.

The possibility that Tris, with its ethanolic residues, was acting as a hydroxyl radical scavenger and thereby preventing hydroxyl radical-mediated peroxidation was also tested. Microsomes were prepared in the absence of Tris buffer and incubated with 1 μM FeSO_4 , 300 μM NADPH, 3 mM potassium phosphate (pH 7.4), 0.4 M KCl, and various concentrations of Tris-maleate (pH 7.4). Under these conditions, Tris had no effect on lipid peroxidation over the concentration range of 0.2 to 80 mM. Moreover, addition of ethanol (1 percent) or thiourea (8 mM) to Tris-free microsomes did not inhibit peroxidation. These results provide further support for the lack of involvement of $\text{OH}\cdot$ in NADPH-dependent microsomal peroxidation and justify the use of Tris buffers in the present studies as well as in those of other investigators (36,81,87,88,94).

Table 2 shows the ability of xanthine oxidase-generated $\text{O}_2\cdot^-$ to induce microsomal lipid peroxidation. In the absence of iron, addition of the xanthine oxidase system was associated with a relatively low level of lipid peroxidation which was not inhibited by SOD. Likewise, iron alone (1 μM) did not induce extensive peroxidation, but when xanthine oxidase and iron were both present a greater than additive amount of peroxidation was obtained. These results appear to support the contention that superoxide radicals are converted to peroxidation-initiating species

of activated oxygen in the presence of reduced iron. However, addition of SOD, which theoretically should have increased the conversion of $O_2^{\cdot-}$ to the postulated H_2O_2 intermediate (60), had an inhibitory effect on the xanthine oxidase/ Fe^{2+} -induced peroxidation. In addition, thiourea did not prevent this peroxidation, which seems to rule out the involvement of hydroxyl radicals. A more likely explanation, then, for the potentiating effects of xanthine oxidase and $FeSO_4$ on microsomal peroxidation is that xanthine oxidase-generated $O_2^{\cdot-}$ directly reduces iron and thereby maintains higher levels of the active ferrous form. Reduction of Fe^{3+} by superoxide radicals has been reported by a number of investigators (43,74,127). Since stimulation of peroxidation by xanthine oxidase-generated $O_2^{\cdot-}$ appears to involve enhancement of the effects of iron rather than conversion to hydroxyl radicals, it seems even more unlikely that NADPH-dependent microsomal peroxidation is initiated by $OH^{\cdot}$. In addition, we observed that H_2O_2 , tested over a hundred-fold concentration range, did not increase microsomal lipid peroxidation, even when iron was present (data not shown).

The concentration of thiourea which we tested (8 mM) was comparable to that which Lai and Piette (60) reported would inhibit the peroxidation of arachidonic acid in a "Fenton system" containing H_2O_2 and $FeSO_4$. It was assumed that MDA formation in that system was initiated by hydroxyl radicals produced in the Fenton reaction and that scavenging of $OH^{\cdot}$ by thiourea prevented the

Table 2. Effect of SOD and thiourea on xanthine oxidase/Fe²⁺-induced lipid peroxidation. Microsomes (196 μ g protein) were incubated for 30 minutes with the indicated reagents. The xanthine oxidase system used was shown to produce 8.1 nmoles of adrenochrome per minute in the presence of 4 mM epinephrine at 37°C (see Methods). Each number represents the mean ($\pm$ standard deviation) value from 3-5 experiments performed in duplicate using microsomes derived from 2-3 microsomal preparations from 1-3 rats.

<u>Condition</u>	<u>Lipid Peroxidation (nmoles MDA/mg microsomal protein)</u>
xanthine oxidase (0.53 units)	3.5 $\pm$ 1.7
xanthine oxidase plus SOD (45 units)	4.4 $\pm$ 1.8
FeSO ₄ (1 μ M)	2.8 $\pm$ 0.5
xanthine oxidase plus FeSO ₄	13.6 $\pm$ 1.6
xanthine oxidase plus FeSO ₄ plus SOD	4.2 $\pm$ 1.2
xanthine oxidase plus FeSO ₄ plus thiourea (8 mM)	14.1 $\pm$ 1.7

peroxidation. We have conducted similar experiments using lipid extracted from liver microsomes (liposomes). Table 3 shows that, as with whole microsomes, liposomal lipid underwent extensive peroxidation when exposed to ferrous iron alone. Addition of H_2O_2 , however, in concentrations similar to those employed by Lai and Piette (90-360 μM), did not enhance peroxidation and even inhibited at higher concentrations. Identical results were obtained whether the reaction was initiated by addition of liposomes or iron. Thus, production of MDA in a "Fenton system" appears to be due to the direct effect of Fe^{2+} rather than $OH\cdot$. Peroxidation by Fe^{2+} was also decreased by chelation of iron with an equivalent amount of EDTA, an effect which will be more fully presented below. Addition of 8 mM thiourea caused a 40 percent decrease in Fe^{2+} -induced liposomal peroxidation in the absence of H_2O_2 which we regard as a general antioxidant effect. Also, neither ethanol nor SOD inhibited MDA formation in this system. These findings, therefore, demonstrate that $O_2^{\cdot -}$ and $OH\cdot$ are neither initiators nor critical intermediates in liposomal or NADPH-dependent microsomal peroxidation.

Stimulation of liposomal peroxidation by 20 μM ferric iron was only 10 percent of that produced by the same concentration of ferrous iron. Also, in Figure 5, the initial rate of microsomal peroxidation produced by ferric iron alone (no NADPH) was more than 20 times lower than with ferrous iron, and a four-fold stimulation by NADPH was obtained even when the concentration of Fe^{3+} was

Table 3. Effects of various agents on lipid peroxidation in liposomes derived from rat liver microsomes. Liposomes were prepared and incubated (as described in the Methods section) for 10 minutes with the indicated reagents. Each number represents the mean value ($\pm$ standard deviation) from 3-5 experiments performed in duplicate using microsomes derived from 3 microsomal preparations of 1-3 rats.

	Iron	Additions	nmoles MDA/mg equivalent lipid*
control	----	-----	zero
FeSO ₄	10 μ M	-----	13.0 $\pm$ 5.1
	20 μ M	-----	17.7 $\pm$ 8.0
	10 μ M	H ₂ O ₂ , 90 μ M	12.7 $\pm$ 0.47
	10 μ M	H ₂ O ₂ , 180 μ M	11.4 $\pm$ 0.37
	10 μ M	H ₂ O ₂ , 270 μ M	9.5 $\pm$ 0.58
	10 μ M	H ₂ O ₂ , 360 μ M	8.0 $\pm$ 0.48
	10 μ M	H ₂ O ₂ , 180 μ M plus 10 μ M EDTA	5.9 $\pm$ 1.1
	10 μ M	thiourea, 8 mM	7.4 $\pm$ 0.71
	20 μ M	-----	1.7 $\pm$ 0.80
	3 μ M	-----	4.3 $\pm$ 0.44
FeSO ₄	-----	Ascorbate, 570 μ M	zero
	3 μ M	Ascorbate, 570 μ M	31.0 $\pm$ 5.5
	20 μ M	ADP, 10 μ M	17.9 $\pm$ 9.1
	20 μ M	ADP, 200 μ M	17.8 $\pm$ 9.3
	20 μ M	ADP, 2 mM	18.3 $\pm$ 9.8

* represents the lipid extracted from 1 mg of liver microsomal protein; recovery from extraction was 85-90 percent. The amount of liposomes added was 0.1 mg equivalent lipid in a 0.1 ml volume

40 μM . The slight NADPH-independent activity of ferric iron is probably due to a small amount of Fe^{2+} which may be present in solutions of Fe^{3+} , whereas addition of NADPH to the microsomal suspensions results in the production of more substantial amounts of the active Fe^{2+} species. Thus promotion of lipid peroxidation by ferric iron like that observed in Figures 1, 3 and 5 and in other laboratories (36,70,81,87,94,133), would appear to be due to the presence of ferrous iron, either added as a contaminant of ferric or produced by reduction of ferric during the incubation.

Table 3 also shows that peroxidation of liposomes by Fe^{2+} was enhanced by addition of ascorbate, whereas ascorbate in the absence of iron had no effect. Similar results were obtained with whole microsomes (data not shown). These findings suggest that stimulation of peroxidation by ascorbate, like that induced by NADPH and O_2^- (produced by xanthine oxidase), is attributable to its ability to reduce iron (84).

Effects of metal complexing agents: It has been previously reported that ADP-chelated iron will promote lipid peroxidation in whole microsomes (46,87,94). Similarly, for a system consisting of a purified reductase and isolated liver lipid, an EDTA-iron complex was reported to be required (87,94). We did not observe any enhancement over free ferrous iron-stimulated peroxidation by either of these complexing agents. Tested over the range of 0.01-2.0 mM, ADP had no effect on microsomal lipid peroxidation, as

stimulated by 1.0 to 20 μM ferrous iron in the presence of NADPH (Table 1), nor on the peroxidation of liposomes induced by ferrous iron alone (Table 3).

Figure 6 illustrates the effects of addition of increasing amounts of EDTA on ferrous iron-stimulated peroxidation in both liver microsomes and in liposomes composed of extracted liver lipid. In either system, none of the EDTA concentrations tested caused any increase in MDA formation over that produced by free iron³. In fact, excess EDTA produced a profound inhibition of peroxidative activity, revealed as a sharp downward deflection of the EDTA concentration curve, apparently reflecting chelation of iron. In whole microsomes, however, this response did not occur precisely at the equivalence point, which suggests that an amount of EDTA stoichiometrically equivalent to the amount of iron added to microsomes may not be sufficient to completely chelate all of the iron present. The reasons for this displacement from one-to-one EDTA-iron complexation are not clear but are probably related to other substances present in the incubations rather than iron contamination of the microsomes since the microsomes were washed with an EDTA-containing buffer to remove endogenous free iron.

³ It is unlikely that the lack of stimulation by added EDTA is due to the small amount (less than 10^{-2} μM) of EDTA present in our microsomal incubations, since the enhancement by other investigators (81,87,94) was observed at 10^4 -fold higher concentrations. In addition, we have prepared microsomes in the absence of EDTA and observed a similar lack of ability of added EDTA to promote free ferrous iron-induced peroxidation.

Fig. 6 Effect of increasing EDTA concentration on lipid peroxidation in rat liver microsomes and in liposomes derived from extracted liver lipid. Liposomes (lipid extracted from 100 μ g microsomal protein) were incubated with 20 μ M FeSO_4 and EDTA (Δ) as indicated under conditions described in the Methods section. Microsomes (58-103 μ g protein) were incubated with 20 μ M FeSO_4 with ($\bullet$) and without ($\circ$) 200 μ M NADPH, and with EDTA as indicated under conditions described in the Methods section. Time of incubation was 10 minutes. The equivalence point (see text) is indicated by an arrow. Each point represents the mean from 3 (liposomes) or 4 (microsomes) experiments performed in duplicate using microsomes (or liposomes) derived from 2 microsomal preparations from 1 or 2 rats each.

In particular, NADPH may bind iron in form which does not render the iron ineffectual as a peroxidizing species but rather serves to decrease the extent of iron binding to EDTA such that EDTA must be present in excess in order for complete inhibition of peroxidation. The ability of "auxiliary complexing agents" to interfere with the chelation of metal ions by EDTA is a well documented phenomenon (90). In support of this hypothesis, Figure 6 shows that for microsomal or liposomal peroxidation induced by iron alone (no NADPH) the EDTA inhibition curve was shifted towards the equivalence point. Conversely, addition of NADPH to the liposomal peroxidation system displaced the inhibition curve towards concentrations above the equivalence point (data not shown). Thus, the stimulation of peroxidation by relatively high concentrations of "EDTA-iron" observed by other investigators (81,87,94) may be attributable to a small but effective amount of free iron present in their incubations. Agents which reduce the extent of iron chelation by EDTA, for example, those substances containing phosphate groups, would be expected to stimulate peroxidation.

This may explain why ADP apparently promotes peroxidation in systems containing EDTA (81,87,94) whereas it has no effect in systems that do not contain EDTA, including our own and those of other investigators (70,133). In fact, the only evidence that ADP could stimulate peroxidation in the absence of EDTA (46) was discounted as being due to an artifact of iron contamination.

In the studies employing EDTA-iron (81,87,94) it was clearly shown that removal of EDTA from the system suppressed the peroxidative response, consistent with a requirement for EDTA. However, the concentration of iron used in these experiments was high (100 μM) relative to that which we have shown to be optimal for NADPH-dependent peroxidation (Fig. 1). We have observed that concentrations above 60 μM of ferrous iron promote less lipid peroxidation than do lower concentrations (data not shown)⁴. Consequently, addition of an equimolar amount of EDTA to a system containing high iron (and NADPH) may reduce the free iron concentration to a more effective level. For example, when we incubated microsomes with 100 μM FeSO_4 , MDA formation was considerably less than optimal. Upon addition of 100 μM EDTA, peroxidative activity was increased to a level corresponding to stimulation by about 20 μM free iron, thus appearing to promote peroxidation. As the concentration of EDTA was raised to a 40 μM excess, total inhibition of peroxidation occurred (data not shown).

Alternatively, in systems utilizing ferric iron, addition of an amount of EDTA insufficient to completely chelate the iron present might promote more rapid reduction of the free Fe^{3+} remaining and thereby augment peroxidation. EDTA-Fe^{3+} has been shown to be reduced at a much faster rate than free Fe^{3+} (81,87) and the EDTA-

⁴ It is not clear why inhibition occurs at high iron concentrations, although an increase in the destruction of free radicals by chain termination reactions, as described by Ingold (52), is suspected.

Fe^{2+} thus formed might exchange its Fe^{2+} ligand for free Fe^{3+} since the formation constant for the complexation of Fe^{3+} with EDTA is over ten orders of magnitude greater than for Fe^{2+} (90). Thus, the net effect would be increased production of free ferrous iron, the active initiator of microsomal lipid peroxidation. In this manner an apparent "requirement" for EDTA-chelated iron may be observed.

Further characterization of the role of iron: We have attempted to determine if there is any additional involvement of iron beyond initiation of the lipoperoxidative process. It has been previously suggested that the primary role of iron may be to catalyze decomposition of lipid hydroperoxides or other MDA precursors (86,95). According to this hypothesis, incubation of microsomes in the absence of iron should result in the formation and accumulation of peroxide intermediates, which would yield a faster rate of peroxidation upon addition of iron. However, Figure 7 shows when microsomes were preincubated with NADPH but without iron, the rate of peroxidation which occurred upon subsequent addition of iron was identical to the rate obtained when no preincubation was performed. In addition, Table 4 (below) shows that during incubation with EDTA no change in the fatty acid content of microsomal lipids occurred. Thus, it appears that the peroxidative sequence requires iron for its initiation. Moreover, stimulation of MDA formation by CCl_4 , as described in Results section II,

Fig. 7 Effect of preincubation without iron on the rate of microsomal lipid peroxidation. Incubations of 93 μg of microsomal protein were performed in the presence of 200 μM NADPH and 5 μM EDTA. FeSO_4 (20 μM) was added either at zero time (●) or at 15 minutes (▲). In some experiments (○), 1 mM EDTA was added after 5 minutes. Each point represents the mean value from two experiments performed in duplicate on microsomes derived from 2 rats.

occurs in the absence of iron, which provides strong evidence that iron is not required for the breakdown of MDA precursors or for any steps beyond initiation of peroxidation.

Figure 7 also shows that when excess EDTA was added to a rapidly peroxidizing system there was little or no delay in the cessation of MDA formation. This effect of EDTA must be due to iron chelation rather than any free radical scavenging property since this same amount of EDTA had no effect on CCl_4 -induced MDA formation (see Results II). Therefore, it appears that the peroxidative sequence leading to MDA formation occurs rapidly, relative to initiation, such that there is virtually no buildup of intermediates whose breakdown to MDA is detectable after "removal" of Fe^{2+} by EDTA.

As an additional note, when iron was excluded from the incubations but added prior to TBA, no colored product was observed. Incubating with iron followed by addition of a large excess of EDTA prior to the TBA procedure did not affect chromogen formation (data not shown). Therefore, iron is not involved in the reaction of MDA with TBA in our experiments.

Characterization of lipid substrate requirements: The time course of microsomal lipid peroxidation revealed an upper limit to MDA formation of 75-100 nmoles MDA/mg protein. This cessation of MDA production was not attributable to depletion of NADPH, iron, or to inhibition of NADPH-cytochrome c reductase and was therefore

postulated to represent total consumption of membrane polyunsaturated fatty acids. Table 4 shows the relationship between the percentage of maximum MDA formation and the percent decrease in the individual polyunsaturated fatty acids of liver microsomes.

Peroxidative membrane decomposition was associated primarily with loss of arachidonic acid (20:4) and decosohexaenoic acid (22:6), consistent with previous reports (70,134) and total depletion of these two fatty acids corresponded to maximal MDA formation. In fact, the percentage of decrease in 20:4 plus 22:6 very closely paralleled the percentage of maximal MDA formation over a wide range of peroxidizing conditions, thus confirming the reliability of MDA measurement as an indicator of the extent of lipid peroxidation.

Table 4. Association between loss of polyunsaturated fatty acids and MDA formation in liver microsomes. Experimental procedures are given in the Methods section, including contents of the NADPH-generating system. Microsomes (400-600 μ g protein) were incubated for 30 minutes (except for "not incubated"). MDA determinations were performed in duplicate and an average taken; fatty acid data are from single determinations. Maximum MDA formation varied from 70-105 nmoles of MDA per mg of microsomal protein. ND refers to none detected which is equivalent to less than 0.05 nmoles of MDA.

Condition	18:2	% total fatty acids				% maximum MDA formation	% decrease in 20:4 plus 22:6
		18:3	20:2	20:4	22:6		
not incubated	17.0	0.8	1.1	25.6	4.4	ND	zero
5 μ M EDTA 100 μ M NADPH	17.6	0.7	1.1	26.7	4.6	ND	zero
0.4 μ M FeSO ₄ 100 μ M NADPH	17.8	0.5	1.0	24.9	3.6	6.7	5.2
10 μ M FeSO ₄ 50 μ M NADPH	18.0	1.0	1.3	18.8	3.3	27.5	26.5
2 μ M FeSO ₄ NADPH gener- ating system	17.9	1.8	1.1	18.3	2.1	31.3	31.8
20 μ M FeSO ₄ 300 μ M NADPH	14.7	0.7	0.9	5.0	1.0	71.2	83.1
50 μ M FeSO ₄ 600 μ M NADPH	10.7	ND	ND	ND	ND	96.7	100

RESULTS II. Stimulation by Carbon Tetrachloride

Table 5 shows the effect of CCl_4 on microsomal lipid peroxidation occurring in the presence of increasing amounts of FeSO_4 . Peroxidation in the absence of CCl_4 is also shown such that stimulation by CCl_4 can be resolved from the background FeSO_4 -induced peroxidation. At iron concentrations below $2\ \mu\text{M}$ the component of lipid peroxidation due to CCl_4 appeared to be independent of the exact amount of iron which was present, whereas at higher FeSO_4 concentrations, the CCl_4 component was reduced. Loss of the CCl_4 component at higher iron concentrations appears to be due to a depletion of membrane polyunsaturated lipid substrate resulting from extensive iron-induced peroxidation in addition to a direct inhibitory influence described below. Stimulation of peroxidation by CCl_4 also occurred when no iron was present, and was identical in magnitude to the CCl_4 component of peroxidation obtained in the presence of low iron. These results indicate that CCl_4 -induced lipid peroxidation does not require reduced iron, or any free metal ion, since the same amount of stimulation by CCl_4 was observed in the presence of EDTA tested over the range of $5\ \mu\text{M}$ - $2.0\ \text{mM}$. Similar results were obtained with mouse liver microsomes (data not shown).

The time course of CCl_4 -induced peroxidation in the absence of free iron at different concentrations of NADPH is shown in Figure 8. Whereas Fe^{2+} was shown to be able to directly initiate

Table 5. The effect of CCl_4 and FeSO_4 on lipid peroxidation in rat liver microsomes. Microsomal protein (53-98 μg) was incubated in the presence of 200 μM NADPH and FeSO_4 as indicated with or without CCl_4 (10 μl of a 1 percent solution in ethanol) for 30 minutes in a total volume of 0.5 ml. "Percent peroxidation" refers to the percentage of maximum MDA formation obtainable (see Methods). Each number represents the mean value ($\pm$ standard deviation) from 3 experiments performed in duplicate using microsomes from 5 rats.

FeSO_4 , μM Added CCl_4	PERCENT PEROXIDATION		
	plus CCl_4	no CCl_4	increase with
none	10.0 $\pm$ 1.5	0.8 $\pm$ 0.6	9.2
none, 5 μM EDTA	9.4 $\pm$ 1.7	zero	9.4
100 μM EDTA	9.4 $\pm$ 2.1	zero	9.4
2 mM EDTA	9.7 $\pm$ 0.6	zero	9.7
0.3	21.5 $\pm$ 3.8	12.5 $\pm$ 2.5	9.0
0.5	27.8 $\pm$ 3.9	18.4 $\pm$ 1.8	9.2
1.0	37.0 $\pm$ 4.4	27.8 $\pm$ 7.2	9.2
2.0	59.0 $\pm$ 6.0	52.0 $\pm$ 11.0	7.0
3.0	73.9 $\pm$ 7.5	70.8 $\pm$ 7.6	3.1
4.0	85.5 $\pm$ 7.1	84.0 $\pm$ 8.3	1.5
5.0	88.5 $\pm$ 2.4	89.9 $\pm$ 5.3	---
10.0	96.4 $\pm$ 1.5	97.9 $\pm$ 0.4	---
20.0	96.7 $\pm$ 1.9	98.9 $\pm$ 1.4	---

peroxidation in the absence of NADPH (Results I), CCl_4 activity is absolutely NADPH-dependent, which attests to a requirement for microsomal metabolism of CCl_4 to peroxidation-initiating free radicals, as has been previously postulated (97,100,112). Neither ascorbate nor NADH would substitute for NADPH (data not shown), which is consistent with suggestions that the mixed function oxidase system is the site of CCl_4 metabolic activation (76,97,100,106,107,112).

Peroxidation by CCl_4 exhibited a maximal rate at 200 μM NADPH, or with a NADPH-generating system, but was observed to subside after 30 minutes even when NADPH was saturating. MDA formation at this point indicated that only 9 percent of the membrane polyunsaturated fatty acids had been peroxidized, and this was confirmed by GLC fatty acid analysis (data not shown). Therefore, unlike iron-stimulated peroxidation which continues until all of the available PUFA's have reacted, peroxidation by CCl_4 appears to be highly limited. At saturating NADPH concentrations, maximal CCl_4 -induced peroxidation was proportional to the amount of microsomal protein over the range of 0.05 to 2.5 mg (data not shown).

Attempts to increase the extent of CCl_4 -induced peroxidation by varying the manner in which the haloalkane was added to the incubation system were not successful. The techniques employed included delivery of CCl_4 to the aqueous microsomal suspensions either directly, as an ethanol solution, or as a sonicated

Fig. 8 The time course of CCl_4 -induced peroxidation in the absence of iron at different NADPH concentrations. Microsomal protein (147-182 μg) was incubated at 37°C for the indicated times with 50 μM EDTA and 10 μl of a 1 percent solution of CCl_4 in ethanol in the presence of the following concentrations of NADPH: none, ($\blacklozenge$); 12.5 μM , ($\odot$); 25 μM , ($\blacktriangle$); 50 μM , ($\blacksquare$); 100 μM , ($\circ$); 200 μM , ($\triangle$); 400 μM , ($\square$); NADPH-generating system (see Methods), ($\blacklozenge$ ---). The total volume was 0.5 ml. Each point represents the mean value from 5 experiments performed in duplicate using microsomes from two different microsomal preparations from 2 rats each.

suspension in sodium taurocholate, as well as via gaseous diffusion, accomplished by mixing CCl_4 with mineral oil and placing a drop on the inside of the teflon-coated caps of the culture tubes. In general, these methods were equally effective in terms of the extent of peroxidation produced and the particular procedure chosen was largely a matter of convenience.

Figure 9 shows the effect on lipid peroxidation obtained by varying the amount of CCl_4 in the microsomal incubations. Similar curves were obtained either by varying the concentration of CCl_4 in a fixed volume of ethanol or by adding increasing volumes of a sodium taurocholate solution containing 0.5 percent CCl_4 . Although stimulation by CCl_4 was maximal at $0.5 \mu\text{l CCl}_4/\text{mg}$ protein with ethanol as solvent and $2.0 \mu\text{l CCl}_4/\text{mg}$ protein when taurocholate was used, the maximum level of peroxidation in both cases did not exceed 11 percent peroxidation, which was comparable to that obtained in Figure 8.

Further attempts to augment peroxidation by CCl_4 involved additions of various combinations of ADP- or EDTA-chelated iron. Studies by Pederson, Buege, and Aust (87) have suggested that these complexes might function as initiators or intermediary electron transport components in NADPH/iron-dependent microsomal lipid peroxidation, although we did not observe any enhancement of CCl_4 -induced peroxidation by these agents (data not shown). The influence of pH on CCl_4 -induced peroxidation was also investigated. As was the case for ferrous iron-stimulated

Fig. 9 Dependence of microsomal lipid peroxidation on the concentration of CCl_4 . Microsomes (196 μg) were incubated for 20 minutes with 50 μM EDTA, 200 μM NADPH and CCl_4 , added either as increasing concentrations in 10 μl of ethanol ($\bullet$) or as increasing volumes of a 0.5 percent aqueous solution of CCl_4 in 4.65 mM sodium taurocholate ($\circ$). The total volume was 0.5 ml. Each point represents the average of two experiments performed in duplicate using microsomes obtained from 2 rats.

peroxidation, CCl_4 activity did not show any remarkable sensitivity to pH when varied one unit in either direction from the maximal at pH 7.4 (data not shown). Thus, lack of extensive peroxidation by CCl_4 does not appear to be attributable to less than optimal CCl_4 concentration, absence of ADP- or EDTA-iron complexes, or suboptimal pH.

Alternatively, the termination of CCl_4 -induced peroxidation prior to exhaustion of membrane PUFA may be due to loss of the capacity to metabolically activate CCl_4 . A number of investigators have demonstrated that liver microsomal cytochrome P-450 activity is markedly inhibited following in vitro incubation with (9,40,67), or in vivo administration (18,115) of CCl_4 . Figure 10 compares the time course of CCl_4 -induced peroxidation with the aminopyrine demethylase, aniline hydroxylase, and NADPH-cytochrome P-450 reductase (i.e., NADPH-cytochrome c reductase) activities of liver microsomes. Control values were obtained by incubating microsomes with CCl_4 in the absence of NADPH which produced no lipid peroxidation, as in Figure 8, and resulted in only a 13 and 14 percent loss of APDM and AH activities, respectively, over the time course of the experiment (data not shown)⁵.

⁵ With higher amounts of CCl_4 the direct effect of CCl_4 (i.e.: in the absence of NADPH) on cytochrome P-450 activities was more pronounced; no direct effect of CCl_4 on NADPH-cytochrome P-450 reductase activity was observed.

Fig. 10 Correlation between rate of CCl_4 -induced lipid peroxidation ($\blacktriangle$) and NADPH-cytochrome c reductase ($\blacksquare$), aminopyrine demethylase ($\bullet$), and aniline hydroxylase ($\circ$) activities. For APDM and AH experiments, microsomes (300-500 μg protein) were first incubated in the presence of 100 μM EDTA, an NADPH-generating system (see Methods), and 0.5-0.8 μl CCl_4 /mg protein (in ethanol) for the indicated lengths of time, following which an aliquot was removed for MDA determination and a mixture of the enzyme assay reagents (see Methods) was immediately added to the remaining suspension. Reductase experiments were conducted similarly except that only 10-20 μg of protein was used. The presence of aniline or aminopyrine was sufficient to terminate further peroxidation in the cytochrome P-450 assays, whereas 0.01 percent butylated hydroxytoluene was added prior to measurement of reductase activity. (This concentration of BHT partially inhibited the reductase activity, as shown in Table 3. However, the same lack of effect of lipid peroxidation on reductase activity was observed when no BHT was added. In fact, the reductase was found to be only 30 percent inhibited following incubation for 24 hours under strong peroxidizing conditions). APDM and AH activities in the absence of CCl_4 were 10.9 ± 0.7 (mean $\pm$ standard deviation) and 0.99 ± 0.09 nmoles product per min. per mg protein, respectively, whereas APDM and AH activities following incubation with CCl_4 in the absence of NADPH (i.e.: control activities) were 9.5 ± 0.6 and 0.85 ± 0.06 nmoles product per min. per mg, respectively, after 40 minutes. Control activity of the reductase was 262 ± 4 nmoles cytochrome c reduced per min. per mg protein. Each point represents the mean value from 2 (reductase) or 4 (APDM and AH) experiments performed in duplicate using microsomes derived from two microsomal preparations from 1-2 rats each.

In the presence of NADPH, however, increasing peroxidation by CCl_4 with time was accompanied by a parallel loss of both APDM and AH activities, and the peroxidative activity of CCl_4 subsided at the point where cytochrome P-450 was almost totally inhibited. In contrast, CCl_4 -stimulated peroxidation was associated with only a slight effect on NADPH-cytochrome P-450 reductase activity. Thus, NADPH-dependent activation of CCl_4 to peroxidation-initiating free radicals appears to depend on the integrity of cytochrome P-450 and loss of the metabolic activity of this enzyme during CCl_4 -induced peroxidation may account for the cessation of the peroxidative response after reaction of only 10 percent of the membrane PPUFA.

Inhibition of cytochrome P-450 during CCl_4 -induced peroxidation may occur through: 1) direct attack of CCl_4 free radical metabolites on the cytochrome; 2) direct effect of toxic products generated during peroxidative decomposition of membrane lipids, or 3) indirect inactivation stemming from peroxidation of lipids required for enzyme activity (118). Consistent with the second and third hypotheses, cytochrome P-450 has been shown to be inhibited by peroxidation elicited by a number of different agents, including NADPH/iron, ascorbate, or ionizing radiation (47,135). Figure 11 shows that increasing peroxidation by NADPH/iron produced losses in APDM and AH activities which closely approximated the extent of peroxidation such that at 100 percent peroxidation, these activities were nearly totally inhibited. NADPH/iron-stimulated

Fig. 11 Effect of NADPH/Fe²⁺-induced peroxidation on NADPH-cytochrome c reductase (■), aminopyrine demethylase (●) and aniline hydroxylase (○) activities and on the peroxidative response to CCl₄ (▲----). For APDM and AH experiments, microsomes (300-500 μg) were incubated for 30 minutes with 0-20 μM FeSO₄ and an NADPH-generating system (see Methods), following which aliquots were removed for MDA determination and enzyme assay reagents were added as in Fig. 10. For CCl₄ experiments, an analogous procedure was employed where NADPH/Fe²⁺-induced peroxidation was terminated by addition of 100 μM EDTA. The remaining microsomal suspension was then incubated for 30 minutes with 2.1 μl CCl₄/mg protein (added as an aqueous solution in sodium taurocholate). Reductase experiments were similar to APDM and AH determinations except that 10-20 μg of protein was used. Control incubations were performed in the absence of iron (i.e.: with 100 μM EDTA, which had no direct effects on the enzymes) for 30 minutes and yielded activities of 9.3 ± 1.1 (mean ± standard deviation), 0.93 ± 0.3 and 231 ± 33 nmoles product per min. per mg protein for APDM, AH, and reductase, respectively. Control CCl₄-induced peroxidation obtained in this manner was 7.61 ± 0.7 percent peroxidation. Each point represents the mean value from 3-4 experiments performed in duplicate using microsomes derived from 2 microsomal preparations from 1-2 rats each.

peroxidation was also accompanied by an inhibition of the peroxidation-inducing activity of CCl_4 which roughly paralleled the loss of cytochrome P-450 activities such that after 80 percent peroxidation by NADPH/iron the peroxidative response to CCl_4 was completely abolished⁶. In contrast, microsomal reductase activity was less than 10 percent inhibited even after 100 percent peroxidation by NADPH/iron. Results similar to Figure 11 were also obtained when microsomal peroxidation was induced by ascorbate/iron (data not shown).

The results to this point strongly support the involvement of cytochrome P-450 in the initiation of CCl_4 -induced lipid peroxidation, since susceptibility to CCl_4 -induced peroxidation is strongly correlated with the activity of cytochrome P-450, whereas there is no association with NADPH/cytochrome P-450 reductase activity. These data also demonstrate the lability of cytochrome P-450 to lipid peroxidation initiated by three different systems, including CCl_4 , which supports the hypothesis that lack

⁶ Since 20 percent of the peroxidizable PUFA remained in the Fe^{2+} -peroxidized membranes that had been rendered insensitive to CCl_4 , the lack of a CCl_4 effect in these preparations cannot be attributed to a complete lack of peroxidizable lipid. Inhibition of cytochrome P-450 activity towards CCl_4 by the partial peroxidation of membrane lipids seems a more likely explanation. The relatively low APDM and AH activities remaining in the membranes which were apparently rendered incapable of CCl_4 activation is consistent with a small portion of these activities residing in a form(s) of cytochrome P-450 which cannot metabolize CCl_4 . Multiple forms of cytochrome P-450 with differing substrate specificities have been reported (42).

of extensive peroxidation by CCl_4 is due to a peroxidation-induced loss of the requisite metabolic activity of cytochrome P-450. However, comparison of Figures 10 and 11 reveals that maximal inhibition of APDM and AH activities occurs at a ten-fold lower level of lipid peroxidation when CCl_4 is the initiating agent rather than FeSO_4 . This difference may be accounted for by the relative reactivities of the initiating species. Whereas Fe^{2+} is a relatively stable ion which may freely diffuse throughout the microsomal suspensions, free radical metabolites of CCl_4 may be considerably less stable and would probably react only in the immediate vicinity of their formation. Peroxidation of membrane lipids which are closely associated with the cytochrome P-450 could lead to relatively rapid inactivation of the enzyme and a termination of peroxidation prior to extensive loss of microsomal PUFA. Support for this hypothesis was obtained by investigating the effects of addition of exogenous lipid on CCl_4 -induced microsomal peroxidation, results of which are shown in Table 6. Peroxidation of liposomes derived from extracted liver-lipid was elicited by addition of ferrous iron alone (as was observed in Results I) whereas CCl_4 alone was incapable of directly initiating peroxidation in liposomes. When liposomes were added to liver microsomal suspensions containing NADPH and FeSO_4 peroxidation of the liposomal lipid occurred, as reflected by MDA formation in excess of that obtained with microsomes alone. Liposomes in the presence of microsomes were peroxidized to a five-

Table 6. Peroxidation of exogenous lipid by liver microsomes plus iron or CCl₄. Liver microsomes (117 µg) were incubated for 20 minutes in the presence of 200 µM NADPH and either 1.0 µM FeSO₄ or CCl₄ (2.5 µl/mg protein) in sodium taurocholate, with and without liposomes derived from 100 µg of liver microsomes or with and without boiled liver microsomes (98 µg) as indicated in the table. The direct effect of Fe²⁺ or CCl₄ on liposomes was also tested. Peroxidation of the liposomal lipid was quantitated by subtracting the amount of MDA formed in the absence of liposomes and dividing by the number of milligrams of microsomal protein from which the liposomal lipid was prepared (assuming 85 percent of the lipid in the original microsomes was recovered as liposomal lipid). Percent peroxidation values are then obtained in the same manner as with microsomal peroxidation. Each number represents the mean value from 2-4 experiments performed in duplicate using microsomes derived from 2 microsomal preparations from 1-2 rats each. ND indicates no detectable MDA which is equivalent to less than 0.05 nmoles of MDA (see Methods).

CONDITION	MDA (nmoles/20 min.)	PERCENT PEROXIDATION microsomal lipid	"exogenous" lipid
Liver Liposomes			
plus FeSO ₄	.33	---	4.6
plus CCl ₄	ND	---	ND
Liver Microsomes			
plus FeSO ₄	1.69	31.4	---
plus FeSO ₄ plus liposomes	3.53	31.4	26.5
plus CCl ₄	.77	8.6	---
plus CCl ₄ plus liposomes	.75	8.4	ND
plus CCl ₄ plus boiled liver microsomes	.74	8.3	ND

fold greater extent than in the presence of Fe^{2+} alone which is consistent with the role of the microsomal reductase in catalyzing the reduction of iron, thereby generating higher levels of the active ferrous form. Apparently this process, which promotes high rates of peroxidation in microsomes, will also enhance peroxidation of exogenously added lipid.

In contrast, addition of liposomes to a CCl_4 -stimulated liver microsomal system (no iron) does not result in any additional MDA formation, which indicates that the peroxidation induced by CCl_4 is confined to the microsomes. Similarly, addition of boiled liver microsomes to otherwise untreated microsomes did not result in any enhancement of peroxidation, which also demonstrates that the extent of CCl_4 -induced peroxidation is independent of the amount of peroxidizable lipid substrate added to the incubations. Thus, it appears that CCl_4 may induce relatively focal peroxidative damage, most likely involving those lipids closely associated with the cytochrome P-450 site of CCl_4 activation, which would account for the greater potency of CCl_4 in inhibiting the enzyme.

The effects of various inhibitors on iron- and CCl_4 -induced microsomal peroxidation and on microsomal reductase and cytochrome P-450 activities are presented in Table 7. As previously shown (Results I), cytochrome c inhibits NADPH/Fe^{2+} -induced peroxidation, possibly by competing with iron for electrons transferred from the reductase enzyme but also apparently by directly oxidizing Fe^{2+} , as indicated by the inhibitory effect of

Table 7. Effect of various potential inhibitors on NADPH/Fe²⁺-, Fe²⁺- and CCl₄-induced lipid peroxidation and on NADPH-cytochrome c reductase, APDM and AH activities. Microsomes were incubated with the indicated concentrations of inhibitors and either 20 μ M FeSO₄ (Fe²⁺ only), 1 μ M FeSO₄ plus 200 μ M NADPH (NADPH/Fe²⁺), or 100 μ M EDTA, 0.4 μ l CCl₄/mg protein plus an NADPH generating system (CCl₄) for 10, 15, and 15 minutes, respectively. Control (in the absence of inhibitor) levels of peroxidation were 28.8 ± 3.7 (mean $\pm$ standard deviation), 23.4 ± 3.1 , and 7.7 ± 0.8 percent peroxidation for Fe²⁺-, NADPH/Fe²⁺- and CCl₄-induced peroxidation, respectively. The effect of inhibitors on enzyme activities was obtained simply by addition of the inhibitors to the normal assay (see Methods). Control (in the absence of inhibitor) enzyme activities were 9.8 ± 2.5 , 0.92 ± 0.1 , 206 ± 6 nmoles product per min. per mg protein for APDM, AH and reductase activities, respectively. Each number represents the mean value from 2-6 experiments performed in duplicate using microsomes derived from 1-3 microsomal preparations from 1-3 rats each.

EFFECT ON LIPID PEROXIDATION

(% control MDA formation)

EFFECT ON ENZYME ACTIVITIES

(% control activity)

INHIBITOR	by NADPH/Fe ²⁺	by Fe ²⁺ only	by CCl ₄	reductase	APDM	AH
cytochrome c 2 mg/ml (200 μ M)	26.9	58.8	10.1	----	20.2	8.3
BSA, 2 mg/ml (30 μ M)	99.1	99.6	97.1	----	99.7	96.3
α -tocopherol, 46 μ M	23.8	34.2	18.3	87.3	97.9	97.0
BHT, 182 μ M	8.9	12.5	10.6	77.8	55.3	81.3
SOD, 90 units	104.7	107.9	99.1	104.5	101.6	100.0
catalase, 550 units	101.6	101.9	100.1	104.1	99.4	98.9
SOD, 90 units plus catalase, 500 units	99.7	100.4	102.0	102.7	103.3	102.0
aniline, 8 mM	7.8	2.0	10.3	102.1	---	---
aminopyrine, 8 mM	15.0	8.4	17.2	----	---	---
metyrapone, 40 mM	39.5	97.9	6.9	104.4	6.2	28.1
pHMB, 200 nmoles/mg protein	83.6	104.1	8.5	104.9	8.2	11.0

cytochrome c on the peroxidation stimulated by iron alone. This same amount of cytochrome c also caused a 90 percent reduction in peroxidation initiated by CCl_4 which is again probably due to the ability of cytochrome c to compete for electrons from the reductase, in this case, with the cytochrome P-450, as reflected by the substantial depression in cytochrome P-450 activities in the presence of cytochrome c.

Reduction of CCl_4 -stimulated peroxidation was also produced by addition of the free-radical scavengers butylated hydroxytoluene and α -tocopherol at concentrations which effectively inhibited $\text{Fe}^{2+}/\text{NADPH}$ - and Fe^{2+} -induced peroxidation (Table 7). The loss of cytochrome P-450 activities associated with CCl_4 -induced peroxidation was partially prevented by α -tocopherol (data not shown). BHT appeared to have a direct inhibitory effect on APDM and AH activities (Table 7) and thus did not appear to afford as much protection against CCl_4 -induced loss of cytochrome P-450 as did α -tocopherol (data not shown).

Previous investigations in which both CCl_4 -associated peroxidation and enzyme damage were prevented by incubation under anerobic conditions (9,40) have led to speculation that these biochemical alterations may be mediated by a form of activated oxygen produced during microsomal metabolism of CCl_4 (21). Table 7, however, shows that neither superoxide dismutase nor catalase protected against CCl_4 -induced peroxidation and were likewise ineffective against $\text{NADPH}/\text{Fe}^{2+}$ - or Fe^{2+} -induced peroxidation,

as was observed in Results I. CCl_4 -induced loss of APDM and AH activities was also not prevented by SOD or catalase (data not shown)⁷. Therefore, peroxidative or enzymic changes in microsomes produced by CCl_4 which we have observed do not appear to be mediated by $\text{O}_2^{\cdot -}$ or H_2O_2 .

Table 7 also shows that the cytochrome P-450 substrates, aminopyrine and aniline, greatly suppressed CCl_4 -induced lipid peroxidation. Similar effects were previously observed and were interpreted to represent competitive inhibition of cytochrome P-450-mediated metabolism of CCl_4 (67). However, our results show that these substrates will also drastically inhibit peroxidation initiated by NADPH/Fe^{2+} or even Fe^{2+} alone, and thus their effects on CCl_4 -induced peroxidation may also be attributed to their antioxidant properties.

In contrast, the competitive inhibitor of cytochrome P-450, metyrapone, had no effect on Fe^{2+} -stimulated peroxidation, although peroxidation induced by NADPH/Fe^{2+} or CCl_4 was 60 and 93 percent inhibited, respectively. The suppression of CCl_4 -induced peroxidation is probably due to a reduction in the

⁷ Experiments in which a xanthine oxidase system was employed to generate superoxide, as quantitated by conversion of epinephrine to adrenochrome, have verified the integrity of superoxide dismutase activity in the presence of CCl_4 , EDTA, and other conditions employed to obtain CCl_4 -induced peroxidation (data not shown). Thus, lack of effect of SOD in Table 7 cannot be attributed to its inactivation under these conditions.

extent of metabolic activation of CCl_4 , since metabolism of aniline and aminopyrine were highly inhibited by metyrapone. Inhibition of NADPH-dependent peroxidation may be due to an increase in microsomal electron flow from the reductase to cytochrome P-450, away from reduction of iron, although metyrapone did not inhibit microsomal-catalyzed reduction of cytochrome c (i.e., NADPH-cytochrome c reductase activity)⁸.

Addition of pHMB produced a 90 percent loss of cytochrome P-450 activities and caused a similar reduction in the extent of peroxidation induced by CCl_4 , although it had little or no effect on NADPH/ Fe^{2+} - or Fe^{2+} -stimulated peroxidation or on the microsomal reductase. These results strongly support the contention that cytochrome P-450 is the site of conversion of CCl_4 to peroxidation-initiating free radicals.

Further experiments were conducted to test whether CCl_4 could elicit peroxidation in microsomes from other organs, particularly those which appear to be damaged by CCl_4 . As shown in Table 8, both kidney and brain microsomes will undergo iron-stimulated lipid peroxidation resulting in substantial depletion of membrane PUFA's, whereas lung microsomes appear to be only slightly peroxidizable by FeSO_4 .

⁸ This discrepancy may be accounted for by the high concentration of cytochrome c present (85 μM) relative to iron (1 μM).

Table 8. Lipid peroxidation in rat brain, kidney, lung, and liver microsomes induced by FeSO₄ or CCl₄. For the peroxidation experiments, microsomes (up to 500 µg protein) were incubated with 300 µM NADPH and either 5 µM FeSO₄ or 2.6 µl CCl₄/mg protein for 30 min. Percent peroxidation was quantitated as described in the Methods sections. APDM and AH activities were obtained using up to 812, 964, and 1134 µg of protein for brain, lung, and kidney microsomes, respectively. Each number represents the average value from two experiments performed in duplicate using microsomes derived from two microsomal preparations from 1-2 rats each. ND indicates not detectable which is equivalent to less than 0.05 nmoles of MDA or less than 0.6 and 0.3 nmoles product for APDM and AH activities, respectively.

Source of Microsomes	Method of Peroxidation	Percent Peroxidation	Cytochrome P-450 activities (nmoles product/min-mg.)	
			APDM	AH
Brain	FeSO ₄	73.2	ND	ND
	CCl ₄	ND		
Kidney	FeSO ₄	94.4	0.55	0.02
	CCl ₄	ND		
Lung	FeSO ₄	8.2	0.40	0.09
	CCl ₄	ND		
Liver	FeSO ₄	88.5	10.9	0.99
	CCl ₄	9.3		

However, no peroxidation was produced by CCl_4 in any of the microsomal fractions other than liver, although histopathological changes induced by CCl_4 have been observed in kidney (117) and in lung (41,126). Similarly, Villarruel, *et al.* (128) did not observe any increase in the amounts of conjugated dienes present in the microsomal lipids from either of these organs following in vivo administration of CCl_4 . Instead, their study revealed a qualitative correlation between indications of ultrastructural lesions, cytochrome P-450 content, and irreversible binding of ^{14}C from $^{14}\text{CCl}_4$ to microsomal lipids, which suggested that cytochrome P-450-mediated metabolism of CCl_4 and subsequent binding of the metabolites to cellular components might be responsible for the CCl_4 -induced renal, pulmonary, and even hepatic injury.

However, as Table 8 also shows, APDM and AH activities, which were well correlated with the peroxidation-inducing capacity of CCl_4 in liver microsomes (Fig. 11), were found to be very low in kidney and lung microsomes, which suggested that these tissues have very little capacity to metabolically activate CCl_4 . It therefore appears that the histopathological alterations in lung or kidney occurring during CCl_4 poisoning are not a consequence of metabolism of CCl_4 within those tissues, but may be due to some other disturbances such as restricted blood flow (75); or they may be secondary to hepatic dysfunction. This interpretation obviously does not account for the binding of $^{14}\text{CCl}_4$ to the microsomal

lipids of these tissues observed by Villarruel, et al. (128), since this phenomenon presumably reflects metabolic activation of CCl_4 to reactive free radicals (19). However, we find it even more difficult to explain how, if such activation were occurring in kidney microsomes, no peroxidation would be induced, since kidney microsomes are readily peroxidizable by iron (Table 8) and therefore not resistant to lipid peroxidation. Thus, the mechanism by which CCl_4 binds to kidney microsomal lipids remains obscure.

RESULTS III. Tissue and Species Differences in Microsomal Lipid Peroxidation and Vitamin E Content

Figure 12 shows the time course of lipid peroxidation in rat liver, brain, kidney, testes, heart, and lung microsomes, as induced by NADPH and ferrous iron. The rate of peroxidation of lung microsomes was extremely low, relative to liver, brain, kidney, and testes microsomes. Heart microsomes exhibited a rate of peroxidation slightly higher than lung microsomes but still twenty-fold lower than liver microsomes at the early time points. These differences were not due to differences in lipid composition of the microsomes, since their polyunsaturated fatty acid content was comparable and the data are expressed in terms of the percentage of the peroxidizable lipids that had reacted.

Although the NADPH-dependent peroxidation is known to be mediated by the microsomal NADPH-cytochrome P-450 reductase (87), differences in peroxidizability could only be partially accounted for by tissue differences in the activity of this enzyme. Using cytochrome c as an electron acceptor, specific activities were found to be 205, 46, 37, 25, 30, and 91 nmoles cytochrome c reduced per min per mg protein for liver, brain, kidney, testes, heart and lung microsomes, respectively. These values obviously do not correlate with the pattern of microsomal peroxidation observed in Figure 12.

Fig. 12 Rate of NADPH/Fe²⁺-induced lipid peroxidation in microsomes from various tissues of rat. (●) liver, (■) brain, (▲) kidney, (□) testes, (△) heart, (○) lung microsomes. Each point represents the mean value from 4 determinations performed in duplicate using microsomes derived from two microsomal preparations from 5 and 6 rats each. One hundred percent peroxidation is equivalent to 108 ± 11 , 67 ± 15 , 54 ± 9 , 36 ± 4 , 62 ± 14 , and 92 ± 8 nmoles of MDA per mg of protein for liver, brain, kidney, testes, heart, and lung microsomes, respectively. The PPUFA contents of these same microsomes were 803 ± 92 , 495 ± 145 , 403 ± 62 , 271 ± 31 , 458 ± 82 , and 677 ± 62 nmoles of PPUFA per mg of protein, respectively.

PERCENT PEROXIDATION

In order to more accurately assess the relative peroxidizability of the microsomes, rates of peroxidation were determined using ascorbate instead of NADPH. The ascorbate/iron system involves a direct, rather than enzyme-catalyzed reduction of iron (84 and Results I) and is therefore not dependent on the microsomal NADPH-cytochrome P-450 reductase activity. Results of these experiments are shown in Figure 13. Rates of ascorbate/iron-induced peroxidation were similar to those obtained with NADPH/iron (Fig. 12), except that liver microsomes peroxidized less rapidly in the ascorbate-stimulated system. This difference is probably due to the much higher reductase activity of liver microsomes which may enhance the rate of NADPH-dependent peroxidation in this fraction relative to the microsomes from other tissues.

In order to determine whether the rates of peroxidation in microsomes were representative of the whole tissue or organ, we performed similar measurements using tissue homogenates. As shown in Figure 14, heart and lung homogenates were peroxidized to much lesser extent than homogenates of the other tissues studied, a result which demonstrates the uniqueness of these tissues as well as the validity of using microsomes as an indicator of whole tissue peroxidizability.

Attempts to elucidate the nature of these apparent tissue differences in susceptibility to peroxidation focused on the differences between lung and liver microsomes. Fatty acid analysis of lung and liver microsomes following incubation revealed that the

Fig. 13 Rate of ascorbate/ Fe^{2+} -induced lipid peroxidation in microsomes from various tissues of rat. Symbols are the same as in Figure 12. Each point represents the mean values from 4 determinations performed in duplicate using microsomes derived from two microsomal preparations from 5 and 6 rats each. One hundred percent peroxidation and PPUFA content are the same as in Figure 12.

Fig. 14 Rate of Fe^{2+} -induced lipid peroxidation in cell-free homogenates of various rat tissues. Homogenates were prepared as described in Methods and incubated with 40 mM Tris-maleate (pH 7.4) and 40 μM FeSO_4 (Note: The homogenates contained EDTA which was present as a 30 μM concentration such that the concentration of free iron, which is effective in inducing peroxidation, was around 10 μM). Symbols are the same as in Figure 12. Points are the mean values from 2 determinations performed in duplicate using homogenate obtained from two different preparations from 5 and 6 rats each.

polyunsaturated fatty acids of the lung microsomes were only slightly decreased under conditions which resulted in total reaction of the peroxidizable lipids of the liver microsomes (data not shown). Increasing concentrations of NADPH or ascorbate did not increase the rate of lung microsomal peroxidation. As shown in Figure 15, peroxidation of lung microsomes changed only slightly as a function of increasing iron concentration, whereas liver microsomal peroxidation increased dramatically. In addition, varying the incubation pH did not augment lung microsomal peroxidation relative to liver microsomal peroxidation (data not shown). Thus, the lack of peroxidation of lung microsomes does not appear to be attributable to limiting concentrations of iron, NADPH or ascorbate, or to suboptimal pH.

One possible explanation for the different rates of peroxidation in the different tissues is that the microsomes contain different amounts of the biological antioxidant, vitamin E. In order to test this hypothesis, we measured levels of vitamin E in the different microsomes, results of which are shown in Table 9. Vitamin E was quantitated on a per mg of protein basis as well as per mg of peroxidizable polyunsaturated fatty acids, the latter of which probably more accurately represents the antioxidant capacity of the tissue. Calculated on this basis, lung microsomes contained 6 to 8 times more vitamin E than liver, kidney, testes, and brain microsomes, which is consistent with the much slower rate of peroxidation in lung microsomes (Fig. 13). Heart microsomes,

Fig. 15 Lipid peroxidation in rat liver and lung microsomes as a function of ferrous iron concentration. Liver (●) and lung (○) microsomes were incubated for 15 minutes with 250 μ M NADPH and FeSO_4 as indicated. Points are the mean values from 4 determinations performed in duplicate using microsomes derived from two microsomal preparations from 1 and 2 rats (liver) and 4 and 6 rats (lung). One hundred percent peroxidation is equivalent to 85 ± 8 and 70 ± 2 nmoles of MDA per mg of protein for liver and lung microsomes, respectively. The PPUFA contents of these same microsomes were 633 ± 41 and 520 ± 59 nmoles of PPUFA per mg of protein, respectively.

which peroxidized faster than lung but slower than liver, kidney, testes, or brain microsomes contained an intermediate amount of vitamin E.

We also studied rates of peroxidation and vitamin E content in microsomes from different species. As shown in Figure 16, peroxidizability of rabbit microsomes from various tissues was very similar to rat microsomes (Fig. 13) and again there was an inverse correlation with vitamin E content, shown in Fig. 17. Some variation between species in these parameters was observed, however. Figure 18 summarizes the data on lung microsomes from rats, rabbits, mice, and humans. Rabbit lung microsomes contained the highest amount of vitamin E, and practically no lipid peroxidation was detectable after a 30 minute incubation. In contrast, peroxidation was by far the highest in mouse lung microsomes, and vitamin E content was nearly five times less than in rabbit lung microsomes. Peroxidizability and vitamin E content of human lung microsomes was most similar to rat lung microsomes. These correlations suggest that microsomal susceptibility to lipid peroxidation is determined primarily by the concentration of vitamin E present in the microsomal membranes. The relative resistance of lung microsomes to peroxidation, then, appears to be due to the high levels of vitamin E.

In order to further substantiate this hypothesis we measured the rate of ascorbate/iron-induced peroxidation in liposomes prepared from extracted microsomal lipid. Figure 19a shows that

Table 9. Vitamin E content of microsomes from various tissues of rat. Values are the means $\pm$ standard deviations from 4 experiments performed in duplicate using microsomes derived from two microsomal preparations from 5 and 6 rats each.

Source of Microsomes	μg Vitamin E	μg Vitamin E
	<u>mg protein</u>	<u>mg PPUFA</u>
Lung	0.77 ± 0.01	4.3 ± 0.4
Heart	0.26 ± 0.05	1.9 ± 0.6
Liver	0.16 ± 0.03	0.73 ± 0.01
Kidney	0.09 ± 0.02	0.69 ± 0.14
Testes	0.10 ± 0.04	0.67 ± 0.07
Brain	0.09 ± 0.04	0.50 ± 0.15

Fig. 16 Rate of ascorbate/ Fe^{2+} -induced lipid peroxidation in microsomes from various tissues of rabbit. Symbols as in Figure 13. Points are the mean values from 4 determinations performed in duplicate using microsomes derived from two microsomal preparations from 1 rabbit each. One hundred percent peroxidation is equivalent to 81 ± 11 , 109 ± 14 , 62 ± 22 , 57 ± 5 , 37 ± 5 , and 82 ± 9 nmoles of MDA per mg of protein for liver, brain, kidney, testes, heart, and lung microsomes, respectively. The PPUFA contents of the same microsomes were 501 ± 25 , 752 ± 106 , 423 ± 88 , 394 ± 12 , 251 ± 49 , and 562 ± 37 nmoles of PPUFA per mg of protein, respectively.

PERCENT PEROXIDATION

Fig. 17 Vitamin E content of microsomes from various tissues of rabbit. PPUFA refers to peroxidizable polyunsaturated fatty acids (18:3, 20:3, 20:4, 20:5, 22:4, 22:5, and 22:6, see Methods). Values shown are means and standard deviations from 3 determinations performed in duplicate using microsomes derived from two microsomal preparations from 1 rabbit each. PPUFA contents are the same as in Figure 16.

Fig. 18 Lipid peroxidation and vitamin E content of lung microsomes from different species. Lipid peroxidation was determined following a 30 minute incubation of microsomes with the ascorbate/ Fe^{2+} system. Other procedures are described in the Methods section. PPUFA is defined in Figure 17. Values shown are means and standard deviations from 4 (rat), 3 (rabbit) or 2 (human and mouse) determinations performed in duplicate using microsomes derived from 2 (rat, rabbit, mouse) or 1 (human) microsomal preparation from 5 and 6 (rat), 1 (rabbit and human) and 10 and 16 (mouse) animals each. One hundred percent peroxidation is equivalent to 82 ± 9 , 92 ± 8 , 82 , and 81 ± 13 nmoles of MDA per mg of protein for rabbit, rat, human, and mouse lung microsomes, respectively. The PPUFA contents of these same microsomes were 562 ± 37 , 677 ± 62 , 533 , and 536 ± 64 nmoles of PPUFA per mg of protein, respectively.

Fig. 19 Peroxidation of liver and lung liposomes by ascorbate/iron. Liposomes were prepared or vitamin E extracted from aliquots of lung or liver microsomes containing equal amounts of PPUFA. (a) liver liposomes (●), lung liposomes (○). (b) liver liposomes with incorporated vitamin E from lung microsomes (○), liver liposomes with incorporated authentic vitamin E equal to that in lung microsomes (□), liver liposomes with incorporated vitamin E from an equal additional sample of liver microsomes (△), liver liposomes plus vitamin E extract of a water blank (⊙). Each point is the mean value from 3 determinations performed in duplicate using microsomal lipid derived from two microsomal preparations from 5 and 6 (lung) and 1 and 2 (liver) rats.

liver liposomes were over 50 percent peroxidized in 30 minutes while lung liposomes under the same conditions were almost completely resistant to peroxidation. As shown in Figure 19b, incorporation of the vitamin E extracted from an equivalent amount of lung microsomes, or an amount of authentic vitamin E equal to that found in the lung microsomes, into liver lipid produced liver liposomes which were also resistant to peroxidation. In contrast, vitamin E extracted from an equivalent amount of liver microsomes and added to liver lipid (essentially doubling the vitamin E content of the liver liposomes) had only a modest effect on the rate of peroxidation relative to control liver liposomes. These findings demonstrate that the six-fold greater amount of vitamin E in lung compared to liver (Table 9) is sufficient to account for the observed resistance of lung liposomes and microsomes to peroxidation.

RESULTS IV. The Mechanism of Paraquat Toxicity

The ability of various agents to induce lipid peroxidation in rat liver and lung microsomes is summarized in Table 10. As shown in Results I, free ferrous iron ($1\text{ }\mu\text{M}$) produced a small amount of peroxidation in liver microsomes, whereas much greater amounts of peroxidation were produced by the same iron concentration in the presence of NADPH, ascorbate, or a superoxide-generating (xanthine oxidase) system. The stimulatory properties of the latter agents have been attributed to their ability to promote reduction of iron (see Results I). Stimulation of peroxidation by these agents was also observed in rat lung microsomes (Table 10), although the amount of peroxidation produced in lung was 20- to 30-fold less than in liver microsomes, reflecting the 6- to 8-fold higher vitamin E content of lung microsomes (see Results III).

Table 10 also shows that peroxidation of liver microsomes was induced by carbon tetrachloride in the presence of NADPH, but in the absence of free ferrous iron. No peroxidation of lung microsomes by CCl_4 was detected, however, most likely since the lung, in addition to its higher vitamin E content, contains relatively little cytochrome P-450, which is believed to be required for activation of CCl_4 to peroxidation-initiating free radicals (see Results II).

Paraquat did not stimulate peroxidation in either lung or liver microsomes, when tested over a 10^5 -fold concentration range under

Table 10. Stimulation of Lipid Peroxidation in Rat Liver and Lung Microsomes by Various Agents^a

<u>Stimulating Agents(s)</u>	<u>Percent Peroxidation^b</u>	
	<u>Liver Microsomes</u>	<u>Lung Microsomes</u>
FeSO ₄ , 1 μ M	3.2 $\pm$ 0.5	ND
FeSO ₄ , 1 μ M NADPH, 250 μ M	72.5 $\pm$ 12.7	2.3 $\pm$ 0.3
FeSO ₄ , 1 μ M Ascorbic Acid, 500 μ M	76.3 $\pm$ 9.3	3.4 $\pm$ 1.9
FeSO ₄ , 1 μ M Xanthine Oxidase, 0.53 units Acetaldehyde, 2.5 mM	15.7 $\pm$ 2.1	0.64 $\pm$ 0.2
CCl ₄ , 0.5-0.8 μ l/mg protein NADPH, 300 μ M EDTA, 100- μ M	9.3 $\pm$ 1.3	NDC
Paraquat, 10 ⁻⁷ -10 ⁻² M NADPH, 400 μ M EDTA, 100 μ M	ND	ND

^a Microsomes were incubated as described in Experimental Procedures for 30 minutes with the above indicated agents.

^b mean $\pm$ standard deviation from 4-8 determinations performed in duplicate. One hundred percent peroxidation is equivalent to 76-107 (liver) and 70-83 (lung) nmoles of MDA per mg of microsomal protein.

^c ND-none detected, the limits of detection being 0.05 nmoles of MDA which is equivalent to less than 0.5 percent peroxidation.

conditions identical to those which were found to be optimal for CCl_4 -induced liver microsomal peroxidation (i.e.: in the absence of iron). Figure 20 shows that, in agreement with the lack of malondialdehyde formation (Table 10), there was no loss of polyunsaturated fatty acids from rat lung microsomes following incubation with NADPH and widely varying concentrations of paraquat. Lack of stimulation of peroxidation by paraquat in the absence of iron was also observed in several different buffer systems and was not attributable to suboptimal incubation pH (data not shown).

Earlier reports have demonstrated reduction of paraquat by microsomes in the presence of NADPH, catalyzed by the NADPH-cytochrome P-450 reductase (16). Thus, we considered the possibility that reduced paraquat might donate electrons to free ferric iron and thereby stimulate peroxidation by maintaining iron in the ferrous state. We found that paraquat greatly increased the rate of NADPH-dependent microsomal reduction of Fe^{3+} to Fe^{2+} (data not shown) in the presence of bathophenanthroline as an indicator of ferrous iron (24). However, the iron in this experiment was present as a bathophenanthroline chelate, which is not effective in initiating lipid peroxidation, and is reduced at a different rate in this system than free iron⁹.

⁹ Noguichi and Nakano (81) have shown that the rate of microsomal-catalyzed reduction of chelated iron differs markedly from that of free iron reduction, as indicated by NADPH consumption.

Fig. 20 Polyunsaturated fatty acid content of rat lung microsomes incubated with various concentrations of paraquat. Rat lung microsomes (400-500 μ g of protein) were incubated with Tris-maleate buffer (pH 7.4), 500 μ M NADPH, 100 μ M EDTA and paraquat as indicated for 30 minutes. Each point represents the mean $\pm$ the standard deviation from three determinations in duplicate using microsomes isolated from 6 rats.

In order to test whether paraquat could facilitate reduction of free iron and thereby stimulate lipid peroxidation, we incubated microsomes with paraquat and NADPH in the presence of ferrous iron. As shown in Figure 21, paraquat did not stimulate peroxidation of rat lung or liver microsomes beyond that produced by iron and NADPH alone, and was even inhibitory at concentrations above 10 μ M. Thus, paraquat is apparently not capable of mediating reduction of free iron at a rate comparable to the direct reduction of iron by the NADPH-cytochrome P-450 reductase of rat microsomes, and may even compete with iron for electrons from the reductase, as suggested by the inhibition of peroxidation with higher paraquat concentrations.

The ability of paraquat to stimulate peroxidation in the presence of NADPH and ferrous iron in microsomes from different species is shown in Figure 22. As observed in Figure 21, 1 mM paraquat produced a 50 percent inhibition of peroxidation in rat liver and lung microsomes. In rabbit or human lung microsomes, paraquat had no effect on peroxidation. However, paraquat produced a 1.4 and 3.0-fold increase in peroxidation in mouse liver and lung microsomes, respectively, at the same concentration which produced inhibition of peroxidation in rat microsomes. Both control and paraquat-stimulated peroxidation of mouse microsomes was completely inhibited by EDTA, and superoxide dismutase had no effect on these activities (data not shown), indicating that the stimulation of peroxidation by paraquat was dependent on iron but was not mediated by superoxide anions.

Fig. 21 Effect of paraquat on the rate of lipid peroxidation in rat liver (●) and lung (○) microsomes. Microsomes were incubated with Tris-maleate buffer (pH 7.4), 300 μ M NADPH, 1.0 μ M FeSO_4 , and paraquat as indicated for 20 minutes (liver microsomes) and 60 minutes (lung microsomes). Each point represents the mean value from three determinations in duplicate using microsomes derived from two microsomal preparations from 4 and 6 rats (lung) and 2 rats (liver) each. One hundred percent peroxidation is equivalent to 91 ± 8 and 72 ± 8 nmoles of MDA per mg of protein for liver and lung microsomes, respectively. The PPUFA contents of these same microsomes were 680 ± 51 and 550 ± 39 nmoles of PPUFA per mg of protein, respectively.

Fig. 22 Effect of paraquat on lipid peroxidation in microsomes from different species. Microsomes were incubated for 30 minutes with 300 μ M NADPH, 1.0 μ M FeSO_4 , with and without 1.0 mM paraquat. Values are the mean $\pm$ the standard deviation from three determinations in duplicate using microsomes derived from 2 (rat, rabbit, mouse) or 1 (human) microsomal preparations from 1-2 (rat liver), 5-6 (rat lung), 1 (human, rabbit), 10-12 (mouse lung) and 3-4 (mouse liver) animals in each preparation. One hundred percent peroxidation is equivalent to 89 ± 13 , 92 ± 8 , 82 , 82 ± 9 , 81 ± 13 , and 102 ± 21 nmoles of MDA per mg of protein for rat liver, rat lung, human lung, rabbit lung, mouse lung, and mouse liver microsomes, respectively. The PPUFA contents of these same microsomes were 644 ± 47 , 677 ± 62 , 533 , 562 ± 37 , 536 ± 64 and 672 ± 48 nmoles of PPUFA per mg of protein, respectively.

The reason why paraquat produced stimulation of peroxidation in mouse, but not in rat, rabbit or human microsomes is not completely clear. Mouse lung microsomes were found to contain 3 to 5-fold lower concentrations of vitamin E than rat, rabbit or human lung microsomes (see Results III). However, lack of stimulation of peroxidation by paraquat in these latter fractions cannot be entirely accounted for by their high vitamin E content since paraquat also did not stimulate peroxidation in rat liver microsomes (Figure 22), or in lung microsomes from vitamin E-deficient rats (data not shown), both of which contain relatively low amounts of vitamin E. A more likely explanation for these findings is that in the mouse microsomes, under the conditions employed, paraquat can mediate reduction of free iron at a rate which exceeds that of the direct reduction of iron. This might be expected if the NADPH-cytochrome P-450 reductase of mouse microsomes was relatively ineffective in catalyzing direct reduction of free iron. In support of this hypothesis, Table 11 shows that in mouse lung and liver microsomes, the amount of peroxidation induced by ascorbate and iron, which does not depend on the reductase activity, was 6.3 and 4.7 times greater, respectively, than the amount induced by NADPH and the same concentration of iron, whereas the ratio was 1.1 to 1.5 for microsomes from other species. These results suggest that mouse microsomes contain a relatively low free-iron reductase activity which may account for the ability of paraquat to stimulate peroxidation in mouse microsomes.

Table 11. Comparison of NADPH/Iron- and Ascorbate/Iron-Induced Lipid Peroxidation in Microsomes from Different Species

<u>Source of Microsomes</u>	<u>Percent Peroxidation</u>		<u>Ratio Ascorbate/Iron NADPH/Iron</u>
	<u>by NADPH/Iron^b</u>	<u>by Ascorbate/Iron^c</u>	
Rat Liver	72.5 $\pm$ 12.7	76.3 $\pm$ 9.3	1.1
Rat Lung	2.3 $\pm$ 0.3	3.4 $\pm$ 1.9	1.5
Rabbit Lung	0.3 $\pm$ 0.3	0.4 $\pm$ 0.2	1.3
Human Lung	3.9 $\pm$ 0.8	5.2 $\pm$ 1.0	1.4
Mouse Liver	19.9 $\pm$ 1.4	92.7 $\pm$ 1.6	4.7
Mouse Lung	7.6 $\pm$ 0.7	48.0 $\pm$ 5.5	6.3

- a mean $\pm$ standard deviation from 2-4 experiments performed in duplicate.
- b Microsomes were incubated with Tris-maleate buffer (pH 7.4), 250 μ M NADPH, and 1.0 μ M FeSO₄ for 30 minutes
- c Conditions were the same as NADPH/Iron except that 500 μ M ascorbate was used in place of NADPH

Notwithstanding the apparently anomalous effect of paraquat in stimulating mouse microsomal peroxidation, it is evident that paraquat does not stimulate lipid peroxidation in microsomes from rats or humans, both of which are quite vulnerable to the toxic effects of paraquat. Thus, overall, the in vitro evidence indicates that paraquat does not exert toxicity by causing lipid peroxidation.

We also investigated the ability of toxic doses of paraquat to elicit peroxidative changes in vivo. Administration of a single 50 mg/kg dose of paraquat intraperitoneally to rats resulted in extensive histopathological damage similar to that described by other investigators (119), including widespread edema, disruption of normal cellular architecture, and focal hemorrhage, in the lungs of animals sacrificed at 24 hours post-injection. No evidence of cellular necrosis or repair was observed at this 24 hour time point, however.

Figure 23 shows that there was no difference in the rate of in vitro lipid peroxidation in lung microsomes isolated from paraquat-treated and control rats 24 hours after injection. These findings suggest that the vitamin E level of rat lung microsomes was not altered by paraquat exposure. Consistent with this, Table 12 shows that there were no differences in vitamin E content of lung cell-free homogenates or microsomes between paraquat-treated and control animals. A similar lack of change in lung vitamin E or microsomal peroxidizability was observed at 12 and 18 hours following

Fig. 23 Rate of ascorbate/iron-induced lipid peroxidation in lung microsomes from rats treated with paraquat (50 mg/kg, i.p.). Microsomes isolated from paraquat-treated (n=6) and control (n=7) rats were incubated with Tris-maleate buffer (pH 7.4), 500 μ M ascorbate, and 1.0 μ M FeSO_4 . Each point represents the mean $\pm$ the standard deviation from four determinations in duplicate. (○, control; ●, paraquat-treated). One hundred percent peroxidation is equivalent to 64 ± 7 and 72 ± 19 nmoles of MDA per mg of protein for control and paraquat-treated lung microsomes, respectively. The PPUFA contents of these same microsomes were 436 ± 69 and 496 ± 134 nmoles of PPUFA per mg of protein, respectively.

TABLE 12

Vitamin E Content of Cell-Free Homogenate and Microsomes
From Rats Exposed to Paraquat (50 mg/kg, i.p.)^a

<u>PPUFA</u> ^b	<u>μg Vitamin E/mg protein</u>	<u>μg Vitamin E/mg</u>
Control Homogenate	0.12 ± 0.02	-----
Paraquat Homogenate	0.12 ± 0.02	-----
Control Microsomes	0.67 ± 0.1	5.04 ± 0.6
Paraquat Microsomes	0.63 ± 0.2	4.94 ± 0.7

^a mean ± standard deviation from 4 determinations using tissue fractions obtained from 6-7 animals

^b peroxidizable polyunsaturated fatty acids (18:3, 20:3, 20:4, 20:5, 22:4, 22:5, and 22:6)

administration of paraquat. Thus, paraquat does not stimulate lipid peroxidation in vivo to an extent which results in depletion of lung vitamin E.

More direct evidence that paraquat did not induce peroxidation in the lungs of poisoned animals was obtained by analysis of the fatty acid composition of lung fractions following paraquat exposure. Figure 24 shows that exposure to paraquat did not produce any losses in any of the peroxidizable polyunsaturated fatty acids of rat lung homogenate or microsomes when measured 24 hours after dosing. In fact, there was an apparent elevation of the relative content of many of the polyunsaturated fatty acids with paraquat exposure, which was apparently due to a 17 and 27 percent reduction in the palmitate content of homogenate and microsomes from paraquat-treated animals. At 12 and 18 hours following injection with paraquat, no changes in the fatty acid composition of lung homogenate or microsomes were observed (data not shown).

Fig. 24 Fatty acid content of lung cell-free homogenate and microsomes from control and paraquat-treated rats. Fatty acids were determined as described in Experimental Procedure. Values are the mean $\pm$ the standard deviation from four determinations using microsomes derived from two microsomal preparations from 3 and 4 (control) and 3 (paraquat-treated) rats each.

DISCUSSION I. The Role of Iron and NADPH

A number of reports have appeared that described enhancement or stimulation of lipid peroxidation by iron or iron-containing solutions, although there has not been consistency as to which form of iron was found to be most effective. Early suggestions that iron-containing proteins such as hemoglobin, cytochromes, or hematin would catalyze peroxidation of unsaturated lipid (123) eventually gave way to more conclusive evidence that protein-bound iron was not effective and could in fact be inhibitory (133). In agreement, we have observed that oxidized cytochrome c can inhibit NADPH-dependent MDA formation (Fig. 4), and that hemoglobin will not support peroxidation (data not shown). The involvement of inorganic iron has been repeatedly demonstrated (46,70,87,94,133), but it has not often been clear whether the ferric or ferrous form was preferred or at which stage in the peroxidative process effects were exerted. Our data strongly indicate that Fe^{2+} is the required species (Fig. 1) and that it acts to initiate peroxidation of membrane lipids.

Most of our own as well as other investigator's observations of the requirements for lipid peroxidation can be explained in terms of effects on Fe^{2+} concentration. For example, suppression of MDA formation when a potassium phosphate buffer was employed, as was also reported by Masuda and Murano (67), is most likely attributable to formation of an iron-phosphate complex. Enhancement

of microsomal peroxidation by addition of liver supernatant (39) may be due to the presence of free metal ion in that fraction, and the apparent requirement of metal complexing agents like ADP or EDTA may represent an indirect effect of these agents on free ferrous iron concentration. Promotion of peroxidation by ascorbate, as well as the enzyme-catalyzed peroxidative reactions induced in microsomes by NADPH (Fig. 2) or xanthine oxidase (Table 2), appear to involve simply a potentiation of the effects of iron through maintaining its reduced state. These reactions bear considerable resemblance to the conventional autoxidation of isolated unsaturated fatty acids, studies of which have revealed enhancement by ferrous iron(31) as well as a preference for oxidation of polyenoic fatty acids over those of lesser unsaturation (27).

Although we have hypothesized that Fe^{2+} elicits peroxidation by reacting directly with membrane lipids and oxygen, other investigators have proposed that the role of iron is to catalyze formation of hydroxyl radicals as the ultimate peroxidation - initiating species. In support of this, Lai and Piette (60) observed that addition of ESR spin-trapping agents which react with hydroxyl radicals would inhibit microsomal peroxidation. However, concentrations of the spin-trap which produced maximal ESR signal intensity in their experiments only produced around 70 percent inhibition of MDA formation. Thus, the peroxidation they observed cannot be considered entirely dependent on $\text{OH}\cdot$ and the possibility

remains that the spin-trapping compounds partially inhibited lipid peroxidation by reacting with free radical intermediates other than $\text{OH}\cdot$. Similarly, *p*-nitrosodimethylaniline, an efficient scavenger of radiolytically-generated hydroxyl radicals, has been shown to react nonspecifically in several biochemical systems (12). It was concluded from these studies that under biochemical conditions, as opposed to radiolysis or photolysis, no freely diffusable $\text{OH}\cdot$ radicals are formed.

It might still be argued, however, that hydroxyl radical-dependent peroxidation might be somehow suppressed under the conditions we employed, although we have ruled out the possibility that Tris may exert this effect. On the contrary, the maximal amount of MDA produced in our experiments was over one hundred-fold greater than the maximal amount of MDA formation observed by Lai and Piette (60), and it is therefore highly unlikely that our experiments were conducted under suboptimal conditions for NADPH/Fe^{2+} -induced peroxidation. Thus, the apparently $\text{OH}\cdot$ -independent peroxidative activity we have observed probably represents a major fraction of, if not the total, NADPH-dependent activity.

The hydroxyl radical mechanism, as proposed by Lai and Piette (60), involves the assumption that $\text{O}_2^{\cdot-}$ is generated by the microsomal reductase, evidence for which was derived primarily from studies in which conversion of epinephrine to adrenochrome was induced by microsomes and NADPH, and was inhibited by SOD (5).

Although oxidation of epinephrine can be elicited by xanthine oxidase-generated $O_2^{\cdot-}$ (71), it has also been shown that epinephrine will undergo spontaneous autoxidation to adrenochrome by a mechanism involving $O_2^{\cdot-}$ as a critical intermediate rather than as an initiating species (78). Consistent with this latter finding, we observed that EDTA- Fe^{3+} induces adrenochrome formation in the absence of microsomes and that this reaction is inhibited by SOD (unpublished observations). Hence, epinephrine oxidation in the presence of microsomes does not unequivocally demonstrate that $O_2^{\cdot-}$ is generated by the microsomes. On the contrary, several lines of evidence indicate that microsomes do not evolve significant amounts of superoxide. Microsomal reduction of cytochrome c is not inhibited by SOD (77) and is therefore not superoxide-mediated, while xanthine oxidase-induced reduction of cytochrome c is completely blocked by addition of SOD (71). Similarly, reduction of nitroblue tetrazolium, which is taken to be an indicator of the presence of $O_2^{\cdot-}$ (73), was induced by microsomes but apparently by a largely superoxide-independent mechanism since SOD had only a slight inhibitory effect on the microsome-catalyzed reduction of NBT (77). Moreover, partial inhibition by SOD of reductase-mediated NBT reduction has been attributed to enhancement of a side reaction in which radical intermediates of NBT are reoxidized by molecular oxygen (3), and does not appear to support the involvement of superoxide. Thus microsomes evidently do not produce an amount of superoxide which

can compete to any significant extent with the direct reduction of cytochrome c or NBT by the microsomal reductase. In addition, superoxide generated by a xanthine oxidase system was shown to stimulate microsomal peroxidation in the presence of Fe^{2+} , and this peroxidation was inhibited by SOD (Table 2). In contrast NADPH-dependent peroxidation was not inhibited by SOD (Table 1) which indicates that no superoxide was produced under these conditions. Thus, hypothetical mechanisms of lipid peroxidation based on the assumption that superoxide is generated by the microsomal reductase appear tenuous.

An alternative hypothesis for the role of iron in microsomal lipid peroxidation is that it may catalyze decomposition of pre-existing membrane lipid hydroperoxides resulting in the production of chain - initiating free radicals (44,53). These radicals would then further react with membrane lipids yielding more extensive formation of lipid peroxides and their breakdown products, including malondialdehyde. Under certain conditions, Fe^{2+} has been observed to promote decomposition of purified hydroperoxides by what appears to be a free radical mechanism (31,38,52). Also, peroxidation was observed to be more rapid in "aged" lipid suspensions (53).

Conversely, it has been shown that fatty acid hydroperoxides present in microsomes or in purified form will undergo free radical-type decomposition in the absence of any free metal ions (i.e.: with EDTA present) (27,61). Since EDTA completely abolishes

MDA formation (Fig. 6), and also prevents peroxidative loss of membranous polyunsaturated fatty acids (Table 4), it appears that no peroxidation can occur under these conditions. Therefore, hydroperoxide decomposition seems unlikely to be an initiating event since it proceeds in the absence of iron but peroxidation does not. Furthermore, Tam and McCay (122) have shown that no peroxides are detectable in microsomes that have been incubated with EDTA, which makes the existence of pre-formed lipid hydroperoxides seem unlikely. Rather, it appears that the role of iron is precursory to hydroperoxide formation. Similarly, when we "aged" our EDTA-treated microsomes by preincubating in the absence of iron at 37°C (Fig. 7), the rate of peroxidation produced by subsequent addition of iron was no different than the rate observed in non-preincubated microsomes. Thus, conditions which might be expected to increase membrane hydroperoxides did not lead to subsequent enhancement of the iron-stimulated peroxidation, which suggests that iron reacts directly with intact membrane lipids and oxygen to initiate peroxidation. Convincing stoichiometric evidence has been recently reported suggesting formation of a free-radical complex between arachidonic acid, Fe^{2+} and oxygen which could result in peroxidation of the hydrocarbon chain (91). Breakdown of peroxidized lipid to a variety of carbonyl compounds, including malondialdehyde, occurs most likely without further metal ion catalysis, as evidenced by MDA evolution in a CCl_4 -initiated, iron-free system (see Results II).

Although the results reported here were obtained using rat liver microsomes, a similar pattern of Fe^{2+} -stimulated MDA formation correlated with polyunsaturated fatty acid loss was observed with microsomes from rat kidney or brain, as well as from mouse liver, (data not shown). Peroxidation of liver liposomes was also elicited by addition of FeSO_4 , and other investigators have demonstrated ferrous iron-stimulated lipid peroxidation in mitochondria (7) and homogenates (6,7) derived from various tissues. Therefore, this phenomenon appears to be quite ubiquitous, at least in vitro.

The extent to which this process occurs in intact biological systems, however, is questionable since appreciable decomposition of membrane lipids would impair cellular functions. Microsomes that are prepared in the absence of EDTA and incubated with NADPH exhibit a fairly rapid rate of MDA formation, which is inhibitable by EDTA, and this response has been shown to be greater in microsomes prepared from animals that have been fasted for 24 hours (63). Similarly, MDA formation was observed upon incubation of isolated hepatocytes, and was enhanced in cells obtained from 24 hour-starved animals (48). In vivo, a loss of cytochrome P-450 accompanies states of starvation and dietary iron overload, and this effect has been attributed to peroxidative membrane decomposition (29). Increased susceptibility to lipid peroxidation associated with starvation may be due to depletion of intracellular antioxidants which are normally present in amounts sufficient to

suppress iron-induced peroxidation. During a dietary iron overload, however, these antioxidant protective mechanisms may be overcome. Consistent with these proposals, Sirivech et al. (110) have shown that biological reductants such as dihydroflavins can mediate iron release from ferritin at rates which may be physiologically significant. This release is believed to be induced by the reduction of ferritin-bound Fe^{3+} to Fe^{2+} (80). In addition, it has been suggested (57) that a number of chemical toxicants may exert their injurious effects by forming relatively lipophilic complexes with endogenous metal ions which may penetrate hydrophobic membrane barriers and initiate "autoxidative" damage. Thus, the potential for iron-stimulated lipid peroxidation apparently exists in vivo but is probably suppressed through protein binding, compartmentalization, or antioxidant factors. Deterioration of these protective systems may occur in pathological states, however.

DISCUSSION II. Stimulation by Carbon Tetrachloride

Unlike NADPH, ascorbate or xanthine oxidase-generated superoxide anions which apparently promote lipid peroxidation by maintaining iron in the reduced state, carbon tetrachloride appears to initiate microsomal peroxidation by a mechanism which is independent of exogenous iron, an observation which has not been previously documented. In fact, Benedetti et al. (9) observed very little MDA formation by CCl_4 in the presence of EDTA, and Masuda and Murano (67) reported that EDTA partially inhibited CCl_4 -induced peroxidation, although the inhibition was not complete even at 10 mM EDTA. The reasons for these discrepancies are not fully understood but may be attributable to greater metal ion contamination of the microsomal preparations used in the previous studies, as indicated by the level of endogenous peroxidative activity (i.e., in the absence of CCl_4) occurring during their incubations. In contrast, peroxidation in our EDTA - washed microsomes is absolutely dependent on addition of iron or CCl_4 . Thus, in microsomes highly contaminated with iron, a certain portion of the iron may be trapped or protein-bound and therefore inactive during the control incubations (no CCl_4 or no NADPH) but may be released during CCl_4 -induced peroxidation and thereby generate an EDTA-sensitive component of the CCl_4 -initiated peroxidative response. However, we believe that the peroxidative influence of metal ions released during CCl_4 -induced peroxidation

is probably not of physiological significance since, in vivo, free metal ions would most likely be rapidly scavenged and inactivated by endogenous chelators like citrate or histidine, or proteins like ferritin, which are absent in a purified microsomal preparation. Furthermore, in the studies by Masuda and Murano (67), addition of a phosphate buffer greatly suppressed their endogenous peroxidative activity but had very little effect on CCl_4 -induced peroxidation, which suggests that stimulation of peroxidation by CCl_4 is essentially independent of metal ions.

An alternative explanation for the apparently EDTA-inhibitable component of CCl_4 -induced peroxidation observed by other investigators is that the presence of contaminating metal ions during the colorimetric determination of TBA can lead to additional chromogen formation. It has recently been shown that lipid hydroperoxides present in peroxidized microsomes can react with ferrous iron during the boiling step to yield TBA-reactive products (2). In the studies by both Benedetti et al. (9) and Masuda and Murano (67), acid insoluble factors were not removed prior to boiling, and would consequently be available to react with contaminating ferrous ions. EDTA may prevent this reaction and would thus appear to partially inhibit CCl_4 -induced peroxidation. Since acid-insoluble factors were removed by centrifugation in the present studies, this effect of EDTA would not be observed.

Although the inverse relationship between CCl_4 -stimulated peroxidation and cytochrome P-450 activity has been studied by other investigators (9,40,67), in general an adequate quantitative assessment of the extent of peroxidation induced by CCl_4 and its impact on cytochrome P-450 has been thwarted by the lack of control over iron concentration. There appears to be at least three ways in which a high background level of iron-induced peroxidation may obscure the effect of CCl_4 : 1) by establishing a competition for electrons arising from NADPH oxidation by the microsomal reductase such that CCl_4 activation by cytochrome P-450 is reduced, 2) by producing a rapid loss of polyunsaturated membrane lipids such that peroxidizable substrate is limiting, and 3) by contributing to deterioration of cytochrome P-450, thus halting conversion of CCl_4 to peroxidation-initiating free radicals (Fig. 11). We have avoided these difficulties by using EDTA-washed microsomes and incubating with EDTA.

The fact that cytochrome P-450 was labile to lipid peroxidation regardless of the initiating species employed suggests that loss of lipid required for activity (118) may be responsible for the inhibitions observed. However, these results do not rule out the possibility that the enzyme may be directly inhibited by CCl_4 metabolites or by toxic products formed during the peroxidative breakdown of membrane lipids. Evidence that CCl_4 metabolites do not directly affect microsomal enzymes was obtained by Glende et al. (40) who showed that incubation of microsomes under anerobic

conditions prevented both CCl_4 -induced lipid peroxidation and the CCl_4 -induced loss of cytochrome P-450 and glucose-6-phosphatase activities, while metabolism of CCl_4 was apparently not reduced under these conditions. These results indicated that peroxidation of membrane lipids, rather than direct attack of CCl_4 metabolites, was responsible for the enzyme inhibitions.

Studies by Wills (135) have indicated that loss of membrane lipids was responsible for the inhibition of cytochrome P-450 and glucose-6-phosphatase occurring during NADPH/iron- and ascorbate/iron-induced peroxidation. However, other investigators (63) have shown that microsomal lipid peroxidation is associated with breakdown of the cytochrome P-450 heme, which is suggestive of a direct attack on the enzyme by free-radical intermediates of the peroxidative process. Thus, the mechanism by which CCl_4 -induced peroxidation inhibits cytochrome P-450 activity has not been clearly identified.

In vivo, CCl_4 has been shown to produce a 20 percent decrease in the PUFA content of liver microsomes (50), which is approximately twice that which we observed in vitro and may reflect the presence of soluble protective mechanisms acting to diminish the extent of deterioration of cytochrome P-450. Nevertheless, these in vivo results are consistent with our interpretation that CCl_4 -induced peroxidation constitutes a relatively focal lesion, involving loss of only a small portion of total membrane lipid (3-6 percent) and selective disruption of membranous enzymes. Viewed as

such, these findings may not appear to support the hypothesis that the toxicological manifestations of CCl_4 are due to its ability to initiate lipid peroxidation. However, it is conceivable that even a small loss of microsomal membrane constituents in vivo could disrupt membrane fluidity or alter permeability (45) to an extent which impairs microsomal function. In addition, recent evidence indicates that lipid peroxidation results in the formation of certain products having hemolytic (131) or hormone-like (105) properties which could account for cellular damage in regions remote from the center of peroxidative activity.

DISCUSSION III. Tissue and Species Differences

The vitamin E content of various tissues is a somewhat ambiguous concept depending upon the choice of denominators. Previous studies of tissue vitamin E content expressed as μg per gm of tissue (1,32) did not reveal some of the striking differences among the tissues studied here. Since vitamin E is probably located in cell membranes, it seems most useful to quantitate its presence in tissues as a function of some parameter of the tissue membrane content. Thus, we have chosen to express vitamin E content on the basis of peroxidizable polyunsaturated fatty acids (PPUFA) of the microsomes as well as per mg of microsomal protein. The values obtained by this method correlate with microsomal susceptibility to lipid peroxidation and can account for the high resistance of lung and heart microsomes to peroxidation. High concentrations of vitamin E per mg of microsomal protein have been previously reported (125) for lung and heart relative to kidney and liver.

The presence of vitamin E in lung and heart microsomes at levels sufficient to almost totally prevent peroxidation under conditions which resulted in complete peroxidation of microsomes from other tissues raises serious questions regarding lipid peroxidation as a mechanism of pathological or toxicological damage in lung or heart. It would appear that peroxidation of lung or heart microsomes could only occur after depletion of their relatively high content of vitamin E.

Vitamin E has long been recognized as a membrane-soluble antioxidant (124). Its importance as a biological protective agent relative to soluble antioxidant factors such as glutathione or superoxide dismutase has not been established. Our results show that, in the absence of soluble factors, the susceptibility to peroxidation of microsomes from several tissues and species correlates with the microsomal vitamin E content. Since lung and heart microsomes are highly resistant to lipid peroxidation in the absence of soluble factors, the need for soluble antioxidants in these tissues is not apparent, with respect to protection of membrane lipids. Interestingly, the peroxidizability of whole tissue homogenates (Fig. 14) also reflected microsomal vitamin E content. Lung and heart homogenates underwent very little peroxidation under conditions which produced several-fold greater peroxidation in homogenates of brain, kidney, liver, and testes. Thus, it is clear that in the tissues containing lesser amounts of vitamin E, there are no soluble factors which are effective under these conditions in protecting against lipid peroxidation to the same extent that vitamin E protects lung and heart.

The protection of lung and heart against peroxidation by a high content of membranous vitamin E is teleologically sound considering the relatively high oxygen tension in the lung and the exposure of heart to freshly oxygenated blood from the lungs. The mechanism of vitamin E uptake and retention by tissues is unknown and thus the biochemical basis for high levels of membranous vitamin E in lung

and heart remain to be elucidated. The cellular distribution of the lung vitamin E also remains an intriguing area for future investigations, since the lung contains a number of different cell types (129).

DISCUSSION IV. The Mechanism of Paraquat Toxicity

The ability of various chemical toxicants, including carbon tetrachloride and related halogenated hydrocarbons, to stimulate microsomal lipid peroxidation in vitro has been repeatedly demonstrated (92,113). Studies on the ability of paraquat to promote microsomal peroxidation are relatively less numerous and have yielded contradictory results. Bus et al. (16) found that paraquat stimulated malondialdehyde formation in a system containing microsomal lipid and NADPH-cytochrome P-450 reductase purified from rat liver microsomes, which suggested that paraquat could react similarly in vivo to produce lung damage. However, we have shown that paraquat inhibits peroxidation in whole lung microsomes from rats, in agreement with a previous report (49) as well as in rat liver microsomes, and that it has no effect on peroxidation of rabbit or human lung microsomes (Fig. 22). These results suggest that the reconstituted liver microsomal system employed by Bus et al. responds differently than whole microsomes and may, therefore, not be an appropriate model for biochemical alterations in lung with respect to paraquat toxicity.

Recently, Talcott et al. (120) reported stimulation of lipid peroxidation in mouse lung and liver microsomes by paraquat. The authors did not feel that their results supported the lipid peroxidation hypothesis of lung toxicity, however, since diquat, which is not lung-toxic, was much more potent than paraquat in

stimulating microsomal peroxidation on a molar basis and was thus comparable to paraquat even after correcting for the greater ability of lung to accumulate paraquat. Therefore, the ability of bipyridylum herbicides to stimulate lipid peroxidation in vitro did not correlate with their in vivo capacity to elicit lung damage.

In agreement with Talcott et al., we have demonstrated enhancement of peroxidation in mouse lung and liver microsomes by paraquat, and have characterized the dependency of this reaction on iron and the apparent lack of involvement of superoxide anions. However, this response was not observed in lung microsomes from other species known to be susceptible to paraquat-induced lung damage (i.e.: rats and humans), indicating that stimulation of peroxidation by paraquat may be peculiar to mouse microsomes. Consistent with this, Shu et al., (108) found that paraquat had no effect on peroxidation in rat lung microsomes under conditions which had revealed stimulation in mouse lung microsomes. In attempting to account for the apparently anomalous effect of paraquat on mouse microsomal peroxidation, we have postulated that, in the mouse microsomes, paraquat can facilitate electron transfer from the NADPH-cytochrome P-450 reductase to free iron, whereas in microsomes from other species tested, the reductase catalyzes iron reduction directly at a much faster rate than that mediated by paraquat. Although the amount of peroxidation produced by NADPH and iron was the highest in mouse lung microsomes, this is most likely attributable to the relatively low vitamin E content of mouse

lung microsomes (Fig. 18) and is probably not a function of microsomal iron-reductase activity. Comparison of NADPH/iron- and ascorbate/iron-induced peroxidation, however, indicated that the mouse microsomes have a low capacity to undergo NADPH-dependent (reductase-catalyzed) peroxidation relative to their ability to be peroxidized by an ascorbate-containing (non-enzymatic) system (Table 11). Although we have not been able to measure rates of free iron reduction in microsomes due to the reactivity of free ferrous iron, we have found that microsomal-catalyzed reduction of cytochrome c varies considerably between different species and tissues (unpublished observations), which suggests that rates of iron reduction may be similarly variable, depending on the source of microsomes.

In contrast to our results, as well as those of Shu et al., Steffen and Netter (116) have recently reported that paraquat has an inhibitory effect on mouse liver microsomal peroxidation. These apparently contradictory findings may be attributable to the fact that different strains of mice were employed in the different studies.

In addition to the lack of correlation between the occurrence of pulmonary toxicity and the ability of paraquat to stimulate microsomal peroxidation in vitro, we did not observe any changes indicative of lipid peroxidation in the lungs of rats following in vivo exposure to toxic doses of paraquat. Lung vitamin E levels were unaltered by paraquat treatment, as indicated by the lack of

change in microsomal peroxidizability (Fig. 23) as well as by direct measurement (Table 12). Decreased lung vitamin E and increased malondialdehyde has been observed following ozone inhalation (23), however, which suggests that peroxidative changes occurring in vivo may be detectable by these methods. By the same argument, a loss of polyunsaturated fatty acids in liver microsomes was observed following administration of CCl_4 (50), whereas we showed that there was no loss of peroxidizable fatty acids in paraquat-damaged lungs that would indicate that lipid peroxidation had occurred (Fig. 24). In vivo evidence that paraquat does not cause lipid peroxidation was also obtained by Shu et al. (108), who showed that conjugated dienes were not elevated in the lungs of mice given large doses of paraquat. Moreover, administration of the antioxidant N, N'-diphenyl-p-phenylene diamine to mice completely blocked the stimulation of microsomal peroxidation by paraquat, although the animals were not protected against the lethal effects of paraquat. In view of these findings, lipid peroxidation does not appear to be an adequate explanation for paraquat-induced lung damage.

Our observation that paraquat caused a decrease in lung palmitate content (Fig. 24) is consistent with the hypothesis that paraquat exerts toxicity by producing a diminution in dipalmitoyl-phosphatidylcholine (DPPC), the primary component of pulmonary surfactant (65). Buechler and Rhoades (14) have shown that a large portion of the palmitate present in DPPC is derived from de novo

synthesis, and an inhibition of fatty acid synthesis in lung has been observed at early times following administration of paraquat (114). Decreased fatty acid synthesis may be a consequence of the reduced levels of NADPH, most likely resulting from the uncoupling of microsomal (136) as well as mitochondrial (58) electron transport. Alternatively, or concomitantly, depletion of NADPH and NADH may have an effect on metabolism of prostaglandins. The lung is a major site of synthesis of prostaglandins, and considerable evidence has accumulated which indicates that the ratio of reduced to oxidized pyridine nucleotides may control both the interconversion and the first step in the metabolism of prostaglandins (68). Alterations in prostaglandin levels would have profound effects on both respiratory and nonrespiratory lung function. Thus, the edema, bronchodilation, and hemorrhage associated with paraquat poisoning could conceivably be mediated by excessive levels of certain prostaglandins, arising from a paraquat-induced decrease in reduced pyridine nucleotides. In connection with this hypothesis, Reddy et al. (101) recently reported that administration of aspirin and indomethacin, inhibitors of prostaglandin synthesis, provided protection against the lethal effects of paraquat.

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