LIPID PEROXIDATION:
ENZYME-CATALYZED PEROXIDATION OF MEMBRANE LIPIDS AND THE ROLE OF CHELATED IRON

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ABSTRACT

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By

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NADPH-cytochrome c reductase from rat liver was solubilized from the microsomal membrane with either bromelain or a crude pancreatic lipase. Lipase-solubilization of the enzyme was shown to be catalyzed by a protease contaminant of the lipase preparation, since protease inhibitors prevented solubilization. When the crude lipase preparation was subjected to gel filtration, the solubilizing activity was found only in the fractions containing protease activity. The bromelain solubilized enzyme was purified to homogeneity. An antibody directed against the purified NADPH-cytochrome c reductase inhibited both the enzyme's cytochrome c reducing activity and NADPH-dependent liver microsomal lipid peroxidation.

NADPH-dependent lipid peroxidation in lung microsomes was shown to occur much slower than NADPH-dependent peroxidation in liver microsomes. Since NADPH-cytochrome c reductase catalyzes NADPH-dependent lipid peroxidation in liver microsomes, the corresponding lung enzyme was purified and compared to the liver enzyme. Both enzymes had

identical ionic strength optima and were equally inhibited in the reduction of cytochrome c and ferricyanide by antibody directed against the liver enzyme. Double diffusion immunoprecipitation on Ouchterlony plates of the detergent-solubilized liver and lung microsomes resulted in converging precipitin lines indicating similar antigenic sites. The molecular weights of the detergent-solubilized and bromelain-solubilized enzymes from both liver and lung microsomes were 79,000 and 71,000 daltons, respectively. Both enzymes were equally effective in catalyzing the peroxidation of liposomal membranes in the presence of NADPH and chelated Fe^{+++}.

Peroxidation in microsomes catalyzed by NADPH-cytochrome c reductase requires the presence of ADP chelated Fe^{+++}. However, ADP-Fe^{++} is much more effective than ADP-Fe^{+++} in catalyzing lipid peroxidation in the absence of enzyme, thus suggesting that the enzyme functions during lipid peroxidation to reduce ADP-Fe^{+++}. During the course of reductase catalyzed lipid peroxidation, lipid hydroperoxide levels build up early in the reaction, followed by a decrease in hydroperoxide levels, indicating that net breakdown of hydroperoxides is occurring.

Lipid peroxidation in liposomes catalyzed by NADPH-cytochrome c reductase and ADP-Fe^{+++} is greatly enhanced by the addition of Fe^{+++} chelated by EDTA. However, EDTA-Fe^{++}, unlike ADP-Fe^{++}, has no ability to initiate lipid peroxidation in the absence of enzyme. In the presence of NADPH-cytochrome c reductase, EDTA-Fe^{+++} catalyzed the rapid breakdown of cumene hydroperoxides, probably through a lipid radical generating process. It is proposed that the enzyme-catalyzed reduction of

ADP-Fe^{+++} causes the initiation of NADPH-dependent lipid peroxidation and that enzymatically reduced EDTA-Fe^{+++} greatly enhances lipid peroxidation via its interaction with lipid hydroperoxides to produce new lipid radicals.

Lactoperoxidase, in the presence of H_2O_2 and I^-, also catalyzes the peroxidation of microsomal and liposomal membranes. Fe^{+++} is not required for the initiation of lipid peroxidation in this system, but does function to assist in the breakdown of lipid hydroperoxides when the membranes are exposed to heat. The free radical trapper BHT abolishes the appearance of hydroperoxide breakdown products in liposomes and decreases their appearance in microsomes.

Lactoperoxidase-catalyzed lipid peroxidation is not initiated by the formation of singlet oxygen, superoxide, hydroxyl radicals or triiodide, since scavengers of these species do not inhibit enzyme-catalyzed lipid peroxidation. An oxidized product of I^- may be responsible for lactoperoxidase-catalyzed lipid peroxidation.

The antibacterial activity of the lactoperoxidase, H_2O_2, I^- system is not mediated by the peroxidation of bacterial membrane lipids since antioxidants do not prevent lactoperoxidase-catalyzed bacterial killing. In addition, other lipid peroxide forming enzyme systems do not cause bacterial killing.

Soybean lipoxygenase catalyzes lipid hydroperoxide formation in γ-linolenic acid micelles and in detergent-solubilized microsomes and liposomes. In liposomes, the breakdown of lipoxygenase formed lipid hydroperoxides occurs primarily when the membranes are heated in the presence of Fe^{+++}. BHT blocks the appearance of lipid hydroperoxide

breakdown products. In microsomes, a factor is present which facilitates the breakdown of lipid hydroperoxides without the requirement for heat and Fe^{+++}.

Lipid hydroperoxides formed by lipoxygenase in microsomes interact with cytochrome P-450 to catalyze the metabolism of the drug, aminopyrine. Recent evidence suggests that cytochrome P-450 is the microsomal factor responsible for the breakdown of lipid hydroperoxides during enzyme-catalyzed lipid peroxidation. The reduction of EDTA-Fe^{+++} by NADPH-cytochrome c reductase in liposomes may act to mimic the hydroperoxide breakdown function proposed for cytochrome P-450.

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To Barbara

And My Parents

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ABBREVIATIONS

ADP	adenosine-5'-diphosphate
BHA	2,6-di-tert-butylanisole
BHT	butylated hydroxytoluene
CHP	cumene hydroperoxide
DBB	o-dibenzoylbenzene
DEAE-	diethylaminoethyl-
DOC	sodium deoxycholate
DPIF	1,3-diphenylisobenzofuran
EDTA	ethylenediaminetetracetate
IgG	immunoglobulin-G
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NDHG	nordihydroguarietic acid
PMSF	phenylmethylsulfonylfluoride
SDS	sodium dodecylsulfate
STI	soybean trypsin inhibitor
TBA	thiobarbituric acid
TCA	trichloroacetic acid
TMPD	N,N,N',N'-tetramethyl-p-phenylenediamine
Tris	Tris(hydroxymethyl) aminomethane

INTRODUCTION

Lipid peroxidation is an example of a biological, free radical process that is deleterious to living organisms. Damage to biological components, particularly unsaturated fatty acids, through a peroxidative attack by molecular oxygen has been implicated in a wide variety of pathological states and may be an integral part of the overall degenerative processes involved in aging. Both enzymatic and nonenzymatic initiators of lipid peroxidation have been discovered.

My interest in the processes involved in lipid peroxidation began in an effort to continue the work begun by Dr. Thomas Pederson on the characterization of NADPH-dependent lipid peroxidation in microsomes. It had been suggested that NADPH-dependent peroxidation of microsomal membrane lipids was enzymatically catalyzed by the flavoprotein, NADPH-cytochrome c reductase.

Chapter I deals with the study of the reaction mechanism involved in microsomal lipid peroxidation catalzyed by NADPH-cytochrome c reductase. This study was greatly faciliated by the development of an assay that would permit the detection of membrane lipid hydroperoxides. Prior to the development of this assay, the standard methodology for monitoring membrane lipid peroxidation permitted only the detection of the lipid peroxidation breakdown product, malondialdehyde. It could be demonstrated that malondialdehyde production was not always a valid indicator of the peroxidative events occurring during

membrane lipid peroxidation. Extensive use was also made of a model peroxidation system composed of artificial membranes containing lipids extracted from microsomal membranes. The use of the model peroxidation system permitted the precise examination of the factors involved in enzyme-catalyzed lipid peroxidation without interference from components present in the microsomal membrane.

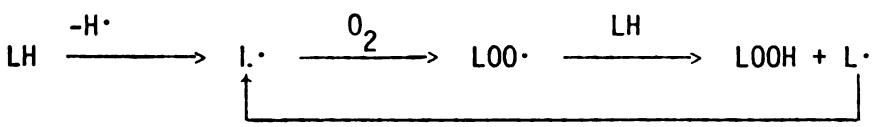
A major problem in characterizing the mechanism involved in the initiation of enzyme-catalyzed lipid peroxidation was the catalytic, radical propagating interference demonstrated by free and chelated forms of metal ions. The NADPH-dependent peroxidation system appeared to require chelated forms of ferric ion for the initiation of membrane lipid peroxidation. The ferric ions also contributed to the free radical, chain propagation processes involved in lipid peroxidation, thus making the study of the initiation phase of the peroxidation reaction difficult. Chapters II and III deal with the characterization and the subsequent use of two new lipid peroxidation catalyzing enzyme systems to study the initiation phase of enzyme-catalyzed lipid peroxidation. Neither enzyme system appeared to require chelated metals for the initiation of membrane lipid peroxidation. It was possible, through the use of free radical trappers and metal chelators, to separate the events occurring in the initiation phase of lipid peroxidation from events occurring in later phases of lipid peroxidation.

Recent evidence is examined concerning the possible involvement of a major hemoprotein constituent of microsomes, namely cytochrome P-450, in the overall control and propagation of microsomal lipid peroxidation. A comparative study of the enzyme-catalyzed peroxidation

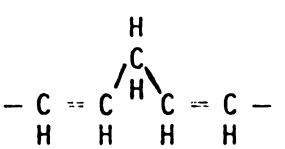
of microsomal membranes versus liposomal membranes permits the assignment of a new, participating role for cytochrome P-450 in microsomal membrane lipid peroxidation.

LITERATURE REVIEW

A characteristic property of unstaturated fatty acids is that in the presence of free radical initiators and molecular oxygen, they undergo oxidative deterioration (1-4). Membranes are particularly susceptible to peroxidative attack since they contain a large amount of unsaturated lipid and are bathed in an oxygen-rich, metal containing fluid (5, 6). Oxidation of unsaturated lipid involved the formation of semistable peroxides from the reaction between lipid radicals and molecular oxygen (7). The simplified scheme below illustrates the reactions leading to the formation of lipid peroxides, starting with the abstraction of a hydrogen from the lipid chain (8, 9).



Unsaturated fatty acids are usually considered the site of initiation of lipid radical formation since the divinyl methane group of the fatty acid chain is particularly susceptible to hydrogen abstraction (9-11).



The lipid radical resulting from hydrogen abstraction readily reacts with oxygen to yield a peroxy radical. Uri (7) points out that this

reaction proceeds so quickly that termination of the peroxy radical brought about by a bimolecular collision is unlikely, if oxygen is present in sufficient amount. The peroxy radical formed can abstract a hydrogen atom from a neighboring lipid to produce a lipid hydroperoxide plus another lipid radical, thus propagating the radical chain reaction (7). Lipid hydroperoxides can also form radicals either by homolytic scission (12-14);

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

or by bimolecular recombination (15-17).

$$2LOOH \longrightarrow LOO\cdot + LO\cdot + HOH$$

The latter two reactions are less likely to occur in biological tissues because of competition for the hydroperoxide by other oxidizable species (18). One group of oxidizable species commonly associated with lipid membranes is free or chelated metals. Metals undergo univalent redox coupling with hydroperoxides to produce radicals (12, 19-22).

Once initiated, lipid peroxidation in biological membranes could become autocatalytic and proceed until the lipid substrate was exhausted, except for the presence of tissue components which prematurely terminate the chain reaction (23-24). Water and lipid soluble radical scavengers, proteins, and nucleic acids all react with lipid radicals to interrupt the radical chain reaction. In addition, lipid radicals interact to produce non-radical recombination products (9, 25-28).

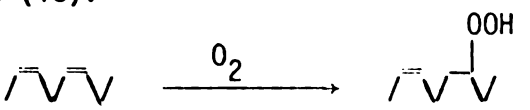
$$2L\cdot \longrightarrow L - L$$

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

$$2LOO\cdot \longrightarrow LOOL + O_2$$

Therefore, the extent of lipid peroxidation depends on the factors governing the initiation, propagation, and termination of the radical reactions involved.

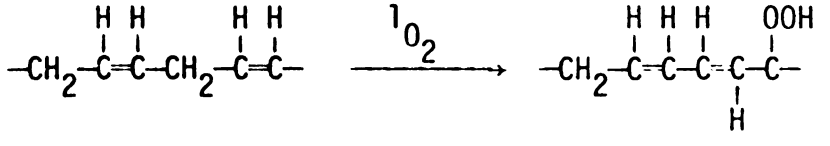
Initiation of lipid peroxidation may occur through an autoxidative attack on unsaturated fatty acids by molecular oxygen, or by formation of free radical initiators by enzymatic or non-enzymatic reactions (28-35). Compounds with allylic or benzylic hydrogens are readily oxidized by molecular oxygen to form hydroperoxides in fairly good yields (18).



This is not surprising since molecular oxygen has the electronic distribution of a diradical (36).

In addition to direct interaction of oxygen with unsaturated lipids, non-enzymatic mechanisms exist that result in initiation of lipid peroxidation via radical formation. Ionizing radation produces cation radicals and electrons as primary products, which decay to produce a variety of charged or neutral radicals (37-40). Irradiation can initiate radical reaction <u>in vivo</u>, which lead to peroxidation of unsaturated fatty acids (9, 41, 42). The primary initiation reaction <u>in vivo</u> appears to be one in which electrons, hydrogen atoms and hydroxy radicals produced by radiolysis of water react with cell constituents (43).

Photolysis of chemical bonds can also produce radicals (44). In animals, individuals with light pigmentation may be particularly sensitive to sunlight. The accelerated aging of human skin in areas exposed to sunlight is a result of direct photolysis and photoinitiated autoxidation of collagen (45). Alcohols and ethers are photooxidized fairly readily by ketonic sensitizers. These reactions are probably initiated through hydrogen abstraction by sensitized ketones, resulting in free radicals (48). The resulting radicals can then react with olefins containing allylic hydrogens, as in unsaturated lipids, to give lipid radicals (49). In addition to light stimulated radical production, lipid hydroperoxides can be formed by another photodynamic process. Fatty acids and cholesterol have been shown to give photooxidation products, including hydroperoxides, in which the double bond shifts to the allylic position during an attack on the lipid by photodynamically produced singlet oxygen (50, 51).



Lipid peroxidation catalyzed by either the photoproduction of radicals or singlet oxygen is probably the cause of damage to membranes commonly reported as a result of photooxidation (52-54).

Ozone, at levels present in normal air (0.01-0.02 ppm), initiates autoxidation of polyunsaturated fatty acids <u>in vivo</u> (30, 55). The peroxidation of linolenic acid exposed to 0.02 ppm ozone occurs substantially faster than control lipids exposed to pure air (43). The observed autoxidation was inhibited by radical scavengers, such a 2,6-di-tert-butylanisole (BHA) and α-tocopherol. Although ozone, unlike oxygen, does not display the characteristics of a diradical, it

does react with virtually every type of organic molecule to produce radicals (43, 56). The reaction mechanism by which ozone reacts with aldehydes, alkanes, and amines consistently includes the proposal of a radical intermediate. The reaction of ozone with polyunsaturated fatty acids yields an esr signal indicating the presence of a radical (57).

Nitrogen dioxide, like ozone, is present in normal air and is an important pollutant in smog (58, 59). It is a free radical sufficiently stable to exist in high concentrations but reactive enough to initiate free radical chain reactions. It can initiate autoxidation of olefins at levels as low as 0.1 ppm (60). Like ozone, it appears to exert its physiological effect, at least in part, by initiating radical reactions (61). Vitamin E protects rats against damage caused by exposure to nitrogen dioxide (62) and the esr signal observed in radical reactions is present when olefins react with nitrogen dioxide (63, 64).

Chlorinated hydrocarbons, such as chloroform and carbon tetrachloride, cause toxic reaction <u>in vivo</u> that are mediated by radical formation (6, 65, 66). Although these compounds are considered stable and not likely to undergo unimolecular homolysis at biological temperatures, they do appear to react with enzymes of the liver to produce radicals. Carbon tetrachloride, when added to microsomes in the presence of NADPH, results in an increase in lipid peroxidation. Recknagel suggests that the microsomal NADPH-dependent drug metabolism system forms $CCl_3\cdot$ radicals via the metabolism of carbon tetrachloride. These radicals subsequently attack lipid molecules and initate lipid peroxidation (67).

Ethanol metabolism may also result in free radical production. DiLuzio demonstrated that concomitant oral administration of ethanol and an antioxidant mixture containing BHT, BHA, and propyl gallate, protected rats against increased liver fat (68, 69). Since ethanol intoxication results in increased peroxide content in liver extracts, it was concluded that ethanol damage to liver occurs via lipid peroxidation (70, 71).

Metal ions (e.g. Cu^{+2}, Fe^{+2}), particularly in the presence of reducing agents like ascorbate and cysteine, catalyze an increase in lipid peroxidation (73-75). This non-enzymatic reaction may proceed via the production of perhydroxy radicals ($HO_2\cdot$) or its anion (O_2^-) via the interaction of reduced metal ions and molecular oxygen (6).

Enzymatic reactions are also known to produce radicals, which can diffuse from the enzyme's surface before they are oxidized or reduced to an even electron species (76). Sequential, one electron transfers resulting in a transient radical, may be an intermediate step in many two electron transfer reactions (77). The NAD· radical can be produced by one electron oxidation of NADH as well as the one electron reduction of NAD^+ (78, 79). Oxygen may react with NAD· by a simple one electron transfer to produce superoxide (78).

$$NAD\cdot + O_2 \longrightarrow NAD^+ + O_2^-$$

A one electron internal transfer has been postulated for the oxidation of reduced cytochrome c. The one electron oxidation results in the formation of a ferrocytochrome II cation radical with the odd electron located in the porphyrin ring (80). An electron transfer follows, resulting in ferricytochrome c.

$$Fe^{II}\ \text{Cyt c} \xrightarrow{-e^-} [Fe^{II}\ \text{Cyt c}]^{+} \longrightarrow Fe^{III}\ \text{Cyt c}$$

Cyclic oxidation and reduction of the flavin coenzymes of the electron transport chains in intracellular organelles may result in flavin semiquinone radicals (81-83).

$$FH \xrightarrow{H\cdot} FH_2\cdot \xrightarrow{H\cdot} FH_3$$

Evidence exists that indicates that chelated metals effect the equilibrium between quinones and hydroquinones, and semiquinone radicals. Chelated metals strongly favor the existence of the semiquinone radical (84, 85).

Enzymes that have been demonstrated to mediate lipid peroxidation <u>in vitro</u> included NADPH-cytochrome c reductase (86-39), NADH-cytochrome b_5 reductase (87), xanthine oxidase (88-92), lipoxygenase (93-95), superoxide dismutase (96) and lactoperoxidase (97). Although the mechanisms of action of these peroxide forming reactions are not clearly understood, it is likely that the transfer of electrons resulting in the formation of radicals in involved in all these enzyme systems.

Once initiation of lipid peroxidation has begun, autocatalytic radical chain reactions occur. The radical propagation involved in lipid peroxidation, in which the net number of radicals in conserved, can be divided into three reaction types (30, 55, 98).

atom transfers $\quad R\cdot + R'H \longrightarrow RH + R'\cdot$

additions $\quad R'\cdot + R_2-C=CH_2 \longrightarrow R_2-\dot{C}-CH_2-R'$

β scission $\quad R-\underset{R'}{\overset{R}{C}}-O\cdot \longrightarrow R-\overset{R}{C}=O + R'\cdot$

Atom transfer is the most common radical reaction and virtually all radical reaction systems demonstrate this reaction (43). In autoxidation of lipids, hydrogen abstraction is an important part of the chain propagation reaction (27, 43).

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

Under conditions where oxygen is abundant, the concentration of lipid peroxy radicals in high.

In the autoxidation of methyl linolenate, the rate of hydrogen abstraction by methyl linolenate peroxy radical is seven orders of magnitude greater than the rate for the bimolecular, self-annihilation reaction between two peroxy radicals (27).

Metals also contribute to radical chain propagation by reacting with lipid hydroperoxides and lipid radicals to produce more radicals. Metals accelerate autoxidation in a complex series of reactions leading to the transfer of electrons between lipid molecules. The ferrous ion catalyzed decomposition of hydrogen peroxide (Fenton's system) illustrates this point (18, 35, 98-100):

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

$$Fe^{+++} + H_2O_2 \longrightarrow Fe^{++} + HO_2\cdot + H^+$$

$$Fe^{++} + OH\cdot \longrightarrow Fe^{+++} + OH^-$$

$$Fe^{++} + HO_2\cdot \longrightarrow Fe^{+++} + HO_2^-$$

$$Fe^{+++} + HO_2^- \longrightarrow Fe^{++} + O_2^- + H^+$$

$$OH\cdot + H_2O_2 \longrightarrow HO_2\cdot + H_2O$$

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

Hematin and hemoproteins also accelerate lipid peroxidation, but are not thought to be involved in initiation of peroxidation (55, 101-103). Many transition metal complexes which occur in cells *in vivo* catalyze the decomposition of lipid hydroperoxides (104, 105). *In vitro* studies of the autoxidation of cell membranes also indicate that metal containing compounds act as prooxidants in lipid peroxidation (106, 107). Finally, many of the symptoms of iron toxicity are similar to those conditions produced by conditions leading to *in vivo* lipid peroxidation (108, 109).

Metals may also enhance lipid autoxidation in a manner not directly dependent upon radical propagation. Experiments with autoxidized blood plasma lipids indicate that chemiluminescence results upon addition of transition metals to the peroxidizing system (110). The resulting chemiluminescence is also observed in microsomes and mitochondria when NADPH plus ferric ion or ferrous ion are used to promote lipid peroxidation (111, 112). These findings have led to the belief that metals react with lipid hydroperoxides to produce electronically excited species. Singlet oxygen (88) and excited, triplet carbonyl functions (113) have been proposed as the light emitting species. Both of these excited species are capable of interacting with unsaturated lipids to produce more hydroperoxides (114).

In view of the damaging effect radicals can have on biological systems, it is not surprising that mechanisms exist for the termination of radical-catalyzed chain reactions. As in all radical reactions, termination of free radicals may occur through collision of two radical species (25, 26). In membrane lipid peroxidation, collision of two lipid radicals would result in cross-links between lipid chains. This

could be particularly destructive, since covalent bonds between lipid chains would drastically effect the structure and properties of the membrane (115-118).

A number of endogenous substances exist *in vivo* that, at physiological concentrations, act to terminate radical processes. Water-soluble radical scavengers include vitamin C, thio containing compounds, proteins, and purine bases (119, 120). Vitamin C is commonly used as a food preservative and acts to trap two radicals in a two electron sequential reduction to form dihydroascorbate (121, 122).

Thio compounds are well known scavengers of radicals (6). Thio compounds interrupt radical processes by acting as hydrogen donors, followed by dimerization.

$$2RSH + 2L\cdot \longrightarrow 2RS\cdot \longrightarrow RSSR + 2LH$$

Cysteinamine, glutathione, ergothioneine and related thios all impart radical scavenging activity (6). The sulfhydryl groups present in proteins may function in the same manner. It is noteworthy that glutathione also functions to inhibit lipid peroxidation in the presence of glutathione peroxidase. Glutathione peroxidase reduces lipid peroxides to alcohols by oxidizing reduced glutathione (23).

Purine bases may also play a role in controlling radical reactions. Uric acid, inosine and RNA have all been shown to inhibit lipid autoxidation of linolenic acid *in vitro* (124, 125). The high concentration of inosine found in the endoplasmic reticulum suggests that inosine functions to protect this membrane from peroxidative attack (126).

A number of antioxidants present in plants may act as radical scavengers when ingested by animals. Gallic acid, eugenol, and nordihydroguarietic acid (NDHG) all act as plant-derived radical scavengers (6). NDHG is commonly used as a food preservative in lard, oil, and baking mixes (6).

Animals also possess a number of lipid-soluble antioxidants. Vitamin E (α tocopherol) is perhaps the most studied lipid-soluble antioxidant (127-128). It has been shown to inhibit in vitro lipid peroxidation. Vitamin E deficiency in vivo increases lipid peroxidation in erythrocytes, adipose and adrenals exposed to hyperoxia (129).

Ubiquinone, from the mitochondrial respiratory chain, acts to terminate free radical reactions. Like many of the quinones, it acts as an antioxidant and, when administered to rats, protects against ethanol induced fatty livers (130). Ubiquinol-6 is as effective as α tocopherol at inhibiting free radical reactions (131).

Carotenes also protect against radical mediated reactions. Forbes and Taliaferro (132) demonstrated that rats maintained on diets rich in carotenes were protected against carbon tetrachloride-induced liver injury, which is mediated by lipid peroxidation. β Carotene has been used to inhibit in vitro, enzymatic lipid peroxidation in microsomes (88).

Exogenous antioxidants are commonly ingested as additives to food stuffs. BHT, BHA, propyl gallate, ethoxyquin, and NDHG are commonly used as food preservatives to prevent oxidative deterioration in lipid-rich foods (6). Some foods are particularly susceptible to changes in flavor, color and odor due to peroxidation, because of the high fat and chelated metal content (133).

Drug antioxidants like chloropromazine are very effective lipid radical scavengers, since they are lipid soluble and likely to penetrate lipid membranes (134, 135).

The inhibition of lipid peroxidation by the previously mentioned antioxidants is generally accomplished through the antioxidants' ability to form stable, non-reactive radicals or by hydrogen donation by one of the following pathways (6).

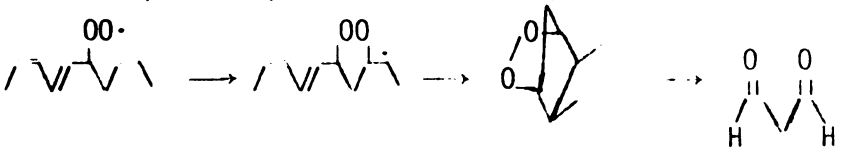
$$LOO\cdot + AH_2 \longrightarrow LOOH + AH\cdot$$

$$2AH\cdot \longrightarrow AH_2 + A$$

or

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

Lipid hydroperoxides are intermediate products of lipid peroxidation, and their breakdown leads to a variety of secondary products, such as volatiles, polymers, and oxygenated compounds (136). Hydroperoxide breakdown can proceed by either homolytic or heterolytic mechanisms. Metal-catalyzed homolytic cleavage of hydroperoxides is common in foods, where transition metals and metaloproteins are abundant (137). The ferrous ion induced decomposition of purified linoleic acid hydroperoxides results in the production of at least 20 detectible compounds, including alcohols, ketones, and ethers (12). When polyunsaturated fatty acids with three or more methylene interruped double bonds undergo autoxidation, a breakdown product is formed which reacts with thiobarbiture acid (TBA) to give a colored chromophore (10, 138, 139). This product has been identified as malondialdehyde and is derived from lipid endoperoxides (11, 140).



Detection of malondialdehyde via the reaction with thiobarbituric acid is a commonly used assay for lipid peroxidation. An intermediate in the formation of malondialdehyde from lipid endoperoxides is a prostaglandin-like compound. In purified lipid emulsions, this prostaglandin-like compound decomposes to yield malondialdehyde both thermally and under the mild acid conditions of the TBA assay (11).

In biologically active materials, hydroperoxides are also degraded enzymatically by mechanisms which are little understood (136). The liver cytosol and the mitrochondrial matrix in animals contain the previously mentioned hydroperoxide metabolizing enzyme, glutathione peroxidase (123, 141). Hydroperoxide isomerase in plants metabolizes hydroperoxides to α ketols (142, 143). Flour-water suspensions contain an enzyme that converts lipid hydroperoxides to epoxides (144, 145), while an enzyme found in potato tubers converts peroxides to vinyl ethers (146).

The major sites within a cell that are susceptible to lipid peroxidation are the biomembranes, especially those of the subcellular organelles. Membrane associated enzymes and proteins are particularly susceptible to oxidative damage during lipid peroxidation. Among the consequences of lipid peroxidation on membrane associated proteins are: loss of enzyme activity (147), loss of solubility due to aggregation or complex formation (148), chain scission (149), as well as loss of specific amino acids. Cysteine, lysine, histidine, and methionine are particularly susceptible (150, 151). The inactivation of sulfhydryl containing amino acids usually occurs through the formation of cross-links (152). Polymers of cross-linked protein are formed when human serum albumin is stored (153). This is not surprising since 25% of the

fatty acids bound to human serum albumin are unsaturated and susceptible to autoxidation. The cross-linked serum albumin also exhibits a fluorescence absent in freshly prepared samples. This fluorescence has been observed in lipid-protein systems undergoing lipid peroxidation (154). When freshly prepared serum albumin was exposed to either peroxidized linoleic acid or to carbonyl compounds similar to those produced during the breakdown of lipid peroxides, the fluorescent spectra resulted. It was subsequently demonstrated that amines and imines present in lysine and histidine react with lipid peroxidation breakdown products, including malondialdehyde, to produce fluorescent chromophores. Biologically important amines like RNA, DNA, and phospholipids form cross-links with malondialdehyde and other carbonyl compounds produced during lipid peroxidation to form fluorescent chromophores with Shiff base structures (155).

$$R - N = CH - CH = CH - NH - R$$

These fluorescent lipid-protein pigments accumulate in animals and are called lipofusin, ceroid, or aging pigments (156, 157). Lipofusin pigments are especially found in heart and brain and their formation is a function of age, oxidative stress, and antioxidant deficiency (8).

Reichel studied the correlation between the appearance of lipofusin pigments and age in rat testes (158). The increased appearance of lipofusin pigments in testes was found to be proportional to age. When rats were fed a diet supplimented with vitamin E, methionine, and BHT, a 44% reduction in the appearance of lipofusin pigments was observed.

In vitro, factors which promote lipid peroxidation can be shown to cause damage to lipid-protein membrane preparations. When ascorbate

and ferrous ions were incubated with erythrocytes a rapid breakdown of the membranes occurred (6). Ionizing radiation and the radical initiator, dialuric acid, also increased erythrocyte hemolysis (159, 160). Hemolysis paralleled the formation of malondialdyhyde. Antioxidants like α tocopherol and BHT prevented hemolysis. These findings have led others to suspect that the use of hyperoxia as a clinical treatment may increase peroxidative damage to erythrocytes, since lipid peroxidation increases in vitro with increased oxygen partial pressure (129).

Erythrocytes are also sensitive to light. When erythrocytes were incubated with small amounts of photosensitizers, like Rose Bengal, Eosin, or Neutral red, in the presence of light of a wavelength greater than 350 nm, hemolysis occurred within minutes (161).

Isolated mitochondria, like erythrocytes, are adversely effected by ferrous ions and reducing agents. Mitochondrial swelling, which parallels lipid peroxidation, produces irreversible damage under these conditions (162).

Waldschmidt et al. (163) demonstrated that freshly prepared mitochondria contained a large number of free radical centers, as indicated by esr signals. The number of free radical centers increased with increasing age of the animal and could be decreased in numbers by inhibitors of mitochondrial respiration. Such centers probably arise from the transfer of electrons from the respiratory chain to radical forming species. Pacer et al. (162) demonstrated that NADPH increased mitochondrial lipid peroxidation and that cyanide and azide inhibited lipid peroxidation.

Hyperbaric pressure also increases the appearance of malondialdehyde in mitochondria, either in organ cultures or in suspension (164).

The increase in lipid peroxidation was largely prevented by addition of EDTA to the medium.

The endoplasmic reticulum is particularly susceptible to lipid peroxidation owing to the high concentrations of $C_{20:4}$ and $C_{22:6}$ unsaturated fatty acids. Microsomes from liver, kidney, and brain all undergo lipid peroxidation at 37° C, especially in the presence of cell sap or ferrous ions (6). Microsomal peroxidation is inhibited by metal chelators, antioxidants, and inhibitors of cytochrome P-450 (75). NADPH greatly stimulates microsomal lipid peroxidation (165) and carbon tetrachloride further increases lipid peroxidation in the presence of NADPH (166).

Recent evidence indicates that paraquat (methyl viologen) induced pulmonary lesions in mammals result from paraquat mediated lipid peroxidation (167, 168). NADPH is required and both superoxide dismutase and singlet oxygen scavengers inhibit peroxidation. NADPH-cytochrome c reductase was shown to mediate the paraquat-induced peroxidation (169, 170).

When microsomal membranes are incubated with ascorbate and ferrous ions, glucose 6 phosphatase activity decreases as lipid peroxidation increases (171). The loss of activity of the enzyme results as a consequence of the loss of membrane structure essential for the enzyme's activity. Amino acid incorporation into rough endoplasmic reticulum in vitro is also inhibited concurrent with lipid peroxidation (172).

Hatefi and Hansten (173) have studied the effect of chaotrophic agents on mitochondria and microsomes. Their results indicate that the destructive processes that promote lipid peroxidation are built into the machinery of these two organelles. Under normal conditions,

molecular oxygen cannot find access to the membrane lipids to catalyze autoxidation. However, under conditions which lead to the destabilization of the membrane, such as addition of chaotrophic agents, lipid peroxidation occurs. α Tocopherol and metal chelators protect the destabilized membranes from peroxidative damage.

Desai has studied the effect of ultra violet radiation on lysosomal membranes. A correlation was observed between lipid peroxidation, free radical production, and release of lysosomal enzymes (174). A similar release of enzymes was observed with exposure of lysosomes to γ-radiation. Fong et al. (91) have demonstrated that incubation of lysosomes with enzyme systems known to promote lipid peroxidation results in release of acid phosphatase. The xanthine:xanthine oxidase peroxidation system and the NADPH-cytochrome c reductase system both cause peroxidation damage to lysosomes and the release of acid phosphatase.

Lysosomes contain large amounts of lipofusin pigments. It has been proposed that lysosomes engulf lipid-rich particles and that these particles slowly peroxidize. Lysosomes particularly rich in lipofusin pigments have low levels of acid phosphatase, suggesting that hydroperoxide breakdown products cross-link with lysosomal enzymes to produce lipofusin pigments. Lysosomal lipofusin pigments stain histochemically for acid phosphatase. Goldfischer et al. (176) have suggested that lipid peroxidation in lysosomes is accelerated when heme-rich mitrochondria or microsomes are engulfed, owing to the prooxidant properties of hemes and chelated metals.

METHODS AND MATERIALS

Material Sources:

Male Sprague-Dawley rats (225-250 g) were obtained from Spartan Research Animals, Inc., Haslett, Michigan. Rats were fasted for 24 hours before sacrifice.

Lactoperoxidase (milk), lipoxygenase (soybean), glucose oxidase (Sigma type II), and catalase (beef liver) were obtained from Sigma Chemical Company, St. Louis, Missouri. Bromelain was obtained as a gift from the Dole Company, Honolulu, Hawaii. Lipase (pancreatic) was obtained from Nutritional Biochemistry Company, Cleveland, Ohio.

A stock culture of Escherichia coli was obtained from Mr. William Litchfield, Department of Biochemistry, Michigan State University, East Lansing, Michigan.

ADP (Sigma fermentation grade), cytochrome c (Sigma type IV), ascorbate, butylated hydroxytoluene, o-dianisidine (dihydrochloride), EDTA, Coomassie blue, dithiothieitol, heparin (Grade I), γ-linolenic acid, D-mannitol, NADPH, sodium dodecyl sulfate, phenylmethylsulfonyl-fluoride, trypsin inhibitor (soybean), thiobarbituric acid, Tween 20, and Tris base were obtained from Sigma Chemical Company, St. Louis, Missouri. 1,3-Diphenylisobenzofuran was obtained from Aldrich Chemical Company, Milwaukee, Wisconsin. Freund's adjuvant (complete) and agar (special Noble grade) were obtained from Difco Laboratories, Detroit, Michigan. Trypticase soy broth and trypticase soy agar were obtained

from BBL, Division of Becton-Dickerson Company, Cockeysville, Maryland. Phenobarbitol was obtained from Merck and Company, Inc., Rahway, New Jersey. Aminopyrine and cumene hydroperoxide were obtained from K and K Laboratories, Plainview, New York. Casein (Hammersten) was obtained from Schwartz Bioresearch Inc., Orangeburg, New York. Phenol reagent was obtained from Harleco, Philadelphia, Pennsylvania.

Preparation of Rat Liver and Lung Microsomes:

Liver microsomes were isolated from perfused rat livers as previously described (177). Lung microsomes from the same animals were obtained by a similar procedure with slight modifications. Lung from male rats (225-250 g) were removed, blotted dry, and weighed. The weighed lung tissue was cut into fine pieces with scissors and homogenized in 4 volumes of 1.15% KCl, 0.2% nicotinamide, using 9 passes of a Potter-Elvehjem homogenizer with Teflon pestle. The homogenate was centrifuged at 9,000 x g for 20 minutes. The supernatant was carefully decanted and centrifuged at 105,000 x g for 90 minutes. The microsomal pellets from both liver and lung were washed by resuspending the pellets in 0.3 M sucrose, 0.1 M sodium pyrophosphate, pH 7.5 and recentrifuging at 105,000 x g for 90 minutes. Microsomes thus obtained were resuspended by homogenization and stored at -20° C under N_2 in 0.05 M Tris-HCl, pH 7.5, containing 50% glycerol. The average yield of microsomal protein per 250 g rat was 128 mg for liver tissue and 4 mg for lung tissue.

Lipase Solubilization of NADPH-Cytochrome c Reductase:

A crude pancreatic lipase preparation has previously been used to release NADPH-cytochrome c reductase from the microsomal membrane

surface (178). Crude pancreatic lipase was subjected to gel filtration and the eluting fractions tested for their ability to solubilize NADPH-cytochrome c reductase. Lipase was dissolved in 0.1 M sodium phosphate, pH 7.5 at a concentration of 20 mg/ml. One ml of the lipase was applied to a Sephadex G-100 column (20 x 250 mm) and 1.2 ml fractions were collected. Aliquots (0.05 mls) from each column fraction were mixed with 10 mg microsomal protein in 1.95 ml of 0.05 M Tris-HCl, then diluted with ice-cold 0.05 M Tris-HCl, pH 7.5 and centrifuged at 105,000 x g for 90 minutes. The supernatant was decanted and the pellet resuspended in Tris buffer. NADPH-cytochrome c reductase activity was assayed in both supernatant and microsomal fractions and the percent of the enzyme solubilized was calculated. NADPH-cytochrome c reductase was assayed as described under "Methods."

Assay of Protease Activity:

Protease activity was measured by following the increase in TCA-soluble peptides released from casein. Casein (3.0 mg) in 1.0 ml of 0.1 M sodium phosphate, pH 7.5, was mixed with enzyme (in 0.05 ml) and incubated for six minutes in a 37° C water bath. The reaction was terminated by the addition of an equal volume of 10% TCA. The mixture was mixed and centrifuged for 20 minutes at 1,000 x g. One ml of the supernatant was neutralized by the addition of 2.0 ml of 0.5 N NaOH followed by 0.6 ml of 1 N phenol reagent. The solution was mixed well and allowed to stand for 30 minutes at room temperature. The absorbance of the solution at 750 nm was determined against a blank containing all the reagents except enzyme. Protease activity was expressed as μmoles of TCA-soluble tyrosine equivalents released per mg casein per minute.

Assay of Lipase Activity:

Lipase activity was assayed by monitoring the enzyme-catalyzed hydrolysis and release of fatty acids from a solution of Tween 20. Five ml of a solution of 2.0% Tween 20 in 0.05 M sodium acetate, pH 7.7 at 25° C, was placed in the reaction vessel of a Radiometer, Type III titrigraph with mechanical stirrer. Enzyme (0.05 ml) was added to the Tween 20 and the release of fatty acids recorded by titrating the solution automatically with 0.02 N NaOH. Lipase activity was expressed as μequivalents of free fatty acids released per minutes.

NADPH-Dependent Peroxidation of Membrane Lipids:

NADPH-dependent lipid peroxidation of microsomal membranes was assayed as follows. Microsomes (1.0 mg protein/ml) were incubated in a Dubnoff shaker at 37° C with 0.1 mM ADP, 0.1 mM $FeCl_3$, and 0.2 mM NADPH in 0.05 M Tris-HCl, pH 7.4. The reaction was initiated by the addition of NADPH. The extent of lipid peroxidation was determined by removing 1.0 aliquots at various time intervals and measuring either the amount of hydroperoxides present of the amount of malondialdehyde formed.

NADPH-dependent lipid peroxidation in liposomes was determined as follows. Liposomes, prepared from extracted microsomal lipids, were prepared as previously described (87). The amount of lipid phosphate present was determined according to Bartlett (179). Liposomes (0.5 μmoles lipid phosphate/ml) were incubated in a Dubnoff shaker at 37° C with 0.1 mM ADP, 0.1 mM EDTA, 0.2 mM $FeCl_3$, purified NADPH-cytochrome c reductase, and 0.2 mM NADPH in 0.05 M Tris-HCl, pH 7.4. The reaction was initiated by the addition of NADPH.

Malondialdehyde formation in both microsomes and liposomes was determined as previously described (86). The malondialdehyde

concentration of the sample was calculated using an extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$. Lipid hydroperoxide concentrations were determined as described below.

Measurement of Lipid Hydroperoxides by the Iodometric Assay:

Reduction of hydroperoxides by iodide is a useful tool in determining the amount of lipid hydroperoxides present in a membrane sample. The procedure is based on the ability of I^- to reduce peroxides by the following reaction (23):

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

Under the conditions of the assay used here, only lipid hydroperoxides react with I^-, thus excluding from the reaction the endoperoxides that break down to form malondialdehyde. The amount of hydroperoxides present in a membrane sample was determined by extracting the membrane lipids from the aqueous reaction mixture followed by reacting the lipids with iodide. One ml of reaction mixture was added to 5.0 ml of chloroform: methanol (2:1) and vortexed for one minute. The mixture was centrifuged in a clinical centrifuge for one minute to accelerate the separation of the two layers. Most of the upper, aqueous layer was aspirated, and 3.0 ml of the lower, chloroform layer was removed using a syringe and placed in a small test tube. The chloroform was evaporated off under a stream of N_2. While still under a stream of N_2, 1.0 ml of acetic acid: chloroform (3:2) was added to the dried lipid residue, followed immediately by 0.05 ml of KI (1.2 g/ml H_2O). The solution was stoppered and quickly mixed and placed in the dark for exactly five minutes. At the end of this time, 3.0 ml of 0.5% cadmium

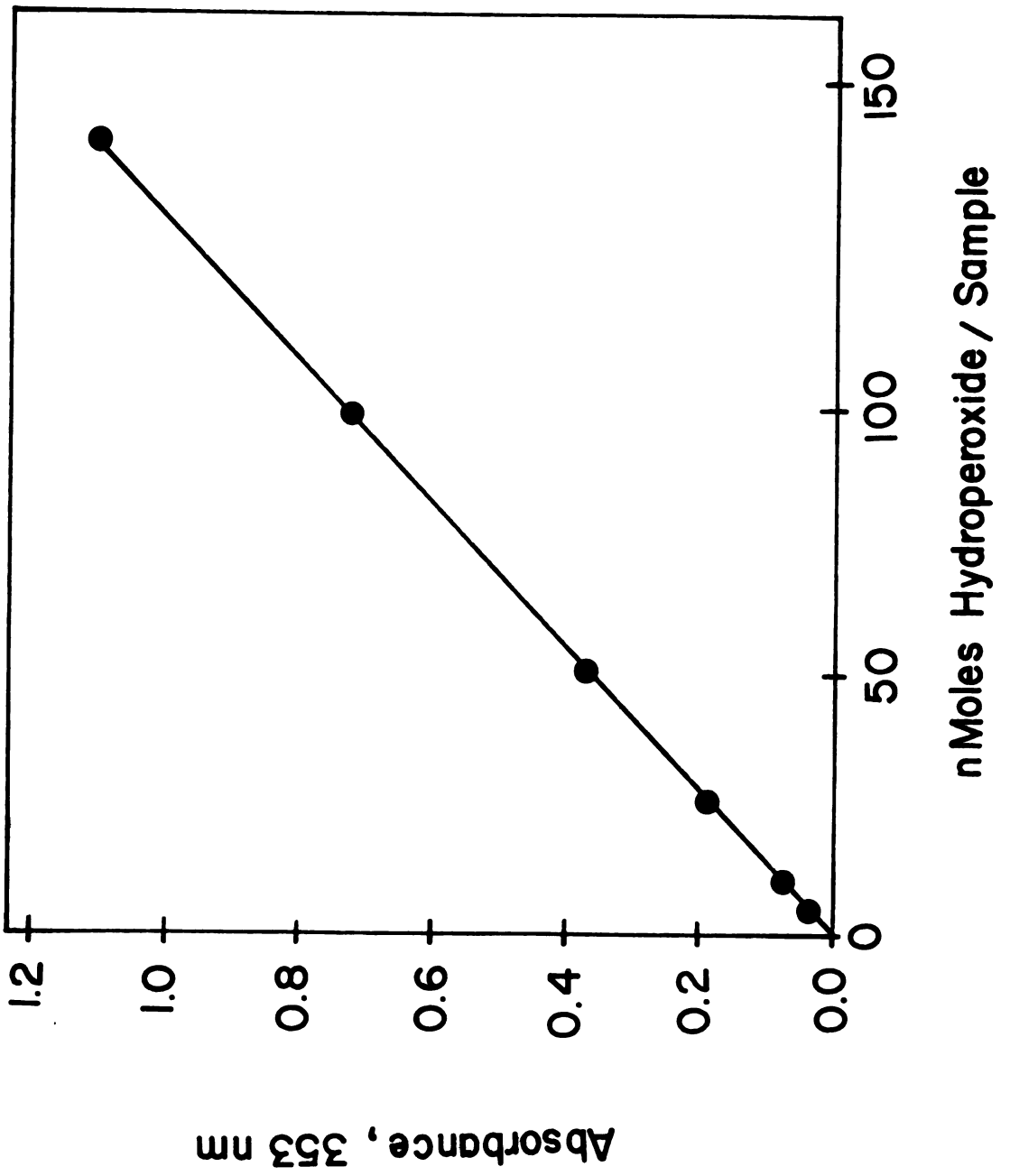
acetate was added and mixed, followed by centrifugation at 1,000 x g for ten minutes. The absorbance at 353 nm of the upper layer was determined against a reagent blank obtained in the same manner but minus lipid. Standardization of the reaction was accomplished by the use of known quantities of cumene hydroperoxide. The standard curve is shown in Figure 1. At hydroperoxide concentrations less than 150 nmoles per assay, the standard curve is linear with a slope of 0.0076 absorbance units per nmole hydroperoxide in the sample.

Isolation and Measurement of Liver and Lung Microsomal NADPH-Cytochrome c Reductase:

Liver and lung microsomes were isolated as described under "Methods." The microsomal NADPH-cytochrome c reductase activity was isolated and purified by a method employing bromelain digestion of the microsomes, followed by gel filtration on Sephadex G-100 and affinity chromatography on DEAE cellulose (87). Both liver and lung reductase were assayed for their ability to reduce cytochrome c as follows. The reaction mixture contained 0.075 mM cytochrome c, 0.1 mM NADPH, and purified liver or lung NADPH-cytochrome c reductase in 0.3 M sodium phosphate, pH 7.5 at 25° C. Absorbance measurements at 550 nm were made on a Perkin-Elmer model 124 spectrophotometer using a reagent blank missing only the reductase. The rate is expressed as nmoles of cytochrome c reduced per minute using an extinction coefficient of 2.10 x 10^4 M^{-1} cm^{-1} (180). One unit of activity is defined as one nmole of cytochrome c reduced per minute.

Figure 1. STANDARD CURVE FOR MEASURING LIPID HYDROPEROXIDES BY THE IODOMETRIC ASSAY.

The absorbance at 353 nm was determined using known quantities of cumene hydroperoxide and the method described under "Methods".


Absorbance, 353 nm
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
50
100
150
n Moles Hydroperoxide / Sample

Preparation of Antibody to Liver NADPH-Cytochrome c Reductase:

An adult, male rabbit was immunized with purified reductase in the following manner. Three, weekly, cutaneous injections of 0.85 mg reductase were administered to the rabbit in the abdomen and footpads. The reductase, in 50% Freund's complete adjuvant, was injected in 6.0, 2.0, and 1.0 ml volumes in the first, second and third weeks, respectively. A booster injection (1.0 mg) was administered one month after the third injection. Ten days after the last injection, blood was obtained by making a one-quarter inch cut into an ear vein and collecting 40 ml of blood. The rabbit was bled every five days (40 ml) for the next two weeks.

Serum was separated from whole blood by allowing the blood to clot at room temperature for four hours. The serum was collected by centrifuging the clotted blood at 1,000 x g for 20 minutes and decanting the serum.

The immunoglobulin G (IgG) fraction from both immune and preimmune serum was prepared as follows. The serum was made 1.75 M ammonium sulfate by slowly adding finely ground ammonium sulfate over a period of 30 minutes to the serum while stirring on ice. The mixture was centrifuged at 27,000 x g for ten minutes and the supernatant decanted. The pellet was resuspended to its original volume in 0.015 M sodium phosphate, pH 7.85 and the ammonium sulfate precipitation repeated. The resulting precipitate was resuspended in 0.015 M sodium phosphate pH 7.85 and dialyzed against the same buffer at 4° C for 24 hours. The dialyzed IgG fraction was applied to a DEAE affinity column (20 x 250 mm) equilibrated with 0.015 M sodium phosphate, pH 7.85 at 25° C and washed with five volumes of the same buffer. The IgG fraction elutes

from the column without being retained. The purified IgG was concentrated on a Diaflow (PM-30) filter at 4° C and dialyzed against deionized water. The IgG was lyophilyzed and stored as a powder at -20° C. The recovered IgG protein represented about 20% of the amount of starting serum protein.

Ouchterlony Double Diffusion Analysis of the Antibody-Antigen Reaction:

Ouchterlony double diffusion analysis (181) was performed in disposable plastic Petri dishes (100 x 15 mm) containing 7.0 ml of agar. The agar contained 1% special Noble agar, 5.0% glycine, and 0.45% sodium chloride in water. The agar was liquified by heating in a water bath followed by resolidification after pipetting into the Petri dish. A punch was used to make well patterns in the agar and agar was removed from the wells by suction. Each well held 0.14 ml of solution. Antibody was placed in the center well and antigen in the peripheral wells. Ouchterlony plates were developed at 4° C for 24-48 hours.

Immunoprecipitation of Liver and Lung Microsomal NADPH-Cytochrome c Reductase by Antibody:

Immunoprecipitation of NADPH-cytochrome c reductase from detergent-solubilized liver and lung microsomes was accomplished by a modification of a method used by Welton et al. (182). Lung microsomes contain less reductase than do liver microsomes. Therefore, various ratios of reductase activity to mg of IgG were tested to determine the best conditions for immunoprecipitation. The optimum ratio for the immunoprecipitation of reductase from detergent-solubilized microsomes was found to be 150 units of reductase activity per mg of IgG. Microsomes (5 mg/ml) solubilized in 1.5% sodium deoxycholate, 0.05 Tris-HCl, pH 7.5, were

diluted with IgG and 0.05 M Tris-HCl, pH 7.5, to a final sodium deoxycholate concentration of 0.75%. Immunoprecipitation occurred within 24 h at 0-4° C. Control IgG did not form precipitates with either liver or lung microsomes. The resulting immunoprecipitates were centrifuged at 500 x g for ten minutes and washed twice in 1% sodium deoxycholate, 0.05 M Tris-HCl, pH 7.5. After the final wash in distilled water, the precipitates were centrifuged and the pellet dried under a stream of air. The immunoprecipitates were prepared for sodium dodecylsulfate-polyacrylamide gel electrophoresis by resuspending the dried pellets in 1% sodium dodecylsulfate containing 7% sucrose, 1 mM EDTA, 40 mM dithiothreitol, 10 mM Tris-HCl, pH 8.1, and 10 µg per ml pyronin B. The immunoprecipitates prepared for electrophoresis were then heated to 100° C for 15 minutes.

Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis:

1% Sodium dodecylsulfate-polyacrylamide gel electrophoresis was performed according to the methods of Fairbanks et al. (183). After electrophoresis, gels were stained with Coomassie blue (184) and scanned for protein at 550 nm using a Gilford spectrophotometer. The molecular weight markers run in parallel with all membrane protein samples included phosphorylase a, bovine serum albumin, carbonic anhydrase, alcohol dehydrogenase and ribonuclease-A.

Assay of Lactoperoxidase Activity:

Lactoperoxidase catalyzes the reduction of H_2O_2 through the oxidation of a variety of electron donors, including o-dianisidine (185). The assay for lactoperoxidase activity used here employs the spectrophotometric detection of the oxidized product formed from o-dianisidine

by the action of lactoperoxidase and H_2O_2. The reaction mixture contained 1.0 mM H_2O_2 and 3.3 mM o-dianisidine in 0.01 M sodium phosphate, pH 6.0 at 25° C. The reaction was started by the addition of lactoperoxidase and the absorbance change recorded at 460 nm. The rate is expressed as the change in absorbance at 460 nm per minute. One o-dianisidine unit of activity equals a change of 0.001 O.D. per minute, according to Klebanoff et al. (186). One μg per ml of commercial lactoperoxidase equaled 152 units.

Lactoperoxidase-Catalyzed Peroxidation of Membrane Lipids:

Lactoperoxidase-catalyzed lipid peroxidation of membrane lipids was assayed in the following manner. Unless otherwise specified, the reaction mixture, totaling 5.0 ml, contained 10 μg lactoperoxidase, 1.0 mM KI, 2.5 mg microsomal protein (or 2.5 μmole lipid phosphate when liposomes were used) 0.1 mM ADP, 0.1 mM $FeCl_3$, and 0.05 M Tris-HCl, pH 7.4 at 37° C. All peroxidation reactions were performed in a 37° C shaking water bath. Peroxidation was initiated by the addition of H_2O_2. Four additions of 176 μM H_2O_2 were made at one minute intervals for the first three minutes of the reaction. Zero time peroxidation values were obtained by measuring malondialdehyde or hydroperoxide levels before H_2O_2 additions and subtracting these values from subsequent measurements. Malondialdehyde and hydroperoxide formation were measured at various time intervals during the reaction as previously described under "Methods."

Purification of Superoxide Dismutase:

Superoxide dismutase was purified from bovine erythrocytes essentially according to McCord and Fridovich (187). One liter of bovine

blood was drawn from the carotid artery into a flask containing 1.75 g of heparin. The blood was centrifuged at 4,000 x g for ten minutes and the plasma (supernatant) discarded. The pellet (red blood cells) was resuspended to the original volume in 0.9% sodium chloride and recentrifuged. The red blood cells were collected and lysed by adding 2 volumes of distilled water. Ice-cold ethanol (0.25 volumes) and ice-cold chloroform (0.1 volume) were added and the solution was mixed at 4° C for 15 minutes, followed by addition of 0.1 volume of distilled water. The mixture was centrifuged at 1,000 x g for 15 minutes and warmed to room temperature. Dibasic potassium phosphate (300 g/l) was added and mixed. The solution was allowed to stand for 15 minutes and the upper phase collected and centrifuged at 2,000 x g for 15 minutes. The resulting supernatant was mixed with 0.75 volumes of ice-cold acetone and recentrifuged at 2,000 x g for 15 minutes. The light red precipitate was resuspended in distilled water (20 ml) and dialyzed at 4° C against 2.5 mM potassium phosphate, pH 7.4 for 24 hours. The dialyzed enzyme preparation was applied to a DEAE affinity column (20 x 110 mm) equilibrated in the same buffer. The enzyme eluted from the column by washing with the same buffer, followed by dialysis against distilled water at 4° C for 24 hours. The enzyme preparation was lyophilized and stored as a powder at -20° C. Superoxide dismutase activity was assayed as the enzyme's ability to inhibit the xanthine: xanthine oxidase-catalyzed reduction of cytochrome c (187).

Preparation of Bacteria:

Trypticase soy broth (30 g/l) and trypticase soy agar (50 g/l) were prepared by mild heating of the corresponding dehydrated mixes in deionized water to facilitate dissolution. Trypticase soy agar slants

were prepared by adding 5.0 ml of agar to culture tubes sealed with cotton plugs, followed by autoclaving at 121° C for 20 minutes at 15 pound/square inch. Stock cultures of bacteria were maintained by streaking the agar slants with E. coli and storing the cotton sealed tubes at room temperature. The inoculating loop and the mouth of the culture tubes were fired over a bunsen burner before and after each inoculation.

E. coli were grown in trypticase soy broth by inoculating culture tubes containing 5.0 ml of autoclaved broth, followed by continuous shaking of the tubes in a 37° C bacteria culture room. Twenty hour broth cultures were centrifuged, washed twice with water, and the bacteria suspended in water to an absorbance at 540 nm of approximately 0.05. Bacterial growth inhibition experiments using this stock suspension of bacteria were performed as described below.

Determination of Bacterial Growth:

One ml of double strength trypticase broth and 0.1 ml of the stock suspension of bacteria were added to a small culture tube (10 x 75 mm). The bacteria killing agents to be tested were added and the volume made up to 2.0 ml with water. The tubes were sealed with cotton and incubated with continuous shaking at 37° C for four hours in an atmosphere of air. The growth of bacteria was determined turbidometrically by the increase in absorbance at 540 nm using the Elmer-Perkins model 124 spectrophotometer.

Lipid Peroxidation Catalyzed by Lipoxygenase:

Lipid peroxidation catalyzed by lipoxygenase was performed in the following manner. Lipid, either in the form of γ-linolenic acid

micelles, liposomal membranes, or microsomes, was incubated in a 25° C shaking water bath in 0.05 M sodium borate, pH 9.0, in the presence of 1.0 mM EDTA. When indicated in the text, sodium deoxycholate was included in the reaction mixtures to solubilize the membrane preparations. The reaction was initiated by the addition of 20 μg/ml lipoxygenase. Both malondialdehyde and hydroperoxide levels were determined as described earlier under "Methods."

Microsomal Drug Metabolism Assay:

The drug metabolizing activity of microsomal cytochrome P-450 was assayed as the demethylation of aminopyrine. An end product of this reaction is formaldehyde, which can be determined quantitatively by the method of Nash (188). The reaction mixture, totaling 5.0 ml, contained 10 mg microsomal protein, 20 mM aminopyrine, 1.0 mM EDTA, and 0.05 M sodium borate, pH 9.0. The reaction occurred in beakers in a 30° C shaking water bath. The reaction was initiated, as indicated in the text, by the addition of one of the following: 0.2 mM NADPH, 220 μM cumene hydroperoxide, or 100 μg/ml lipoxygenase. The reaction was terminated by the addition of 1.0 ml of 10% TCA. After mixing with TCA the solutions were allowed to stand for ten minutes to facilitate precipitation of the protein. Two ml of Nash reagent (2 M ammonium acetate, 0.05 M acetic acid, and 0.02 M 2,4 pentanedione) were added and mixed with the membrane samples, which were subsequently placed in a 60° C hot water bath for ten minutes. After cooling, the mixtures were centrifuged at 1,000 x g for ten minutes. The supernatant was recovered and the absorbance determined at 412 nm. The blank consisted of 1.0 ml 10% TCA, 1.0 ml buffer, and 2.0 ml Nash reagent. The formaldehyde

content was calculated using an extinction coefficient of 7.08×10^3 M^{-1} cm^{-1} and a dilution factor of 4 (177).

Other Methods:

Protein concentration was determined by the method of Lowry et al. (189).

CHAPTER I

CHARACTERIZATION OF NADPH-DEPENDENT MICROSOMAL LIPID PEROXIDATION

The Solubilization of NADPH-Cytochrome c Reductase:

The enzyme induced peroxidation of microsomal membrane lipids was first demonstrated when microsomes were incubated aerobically with NADPH (75). Later, it was observed that Fe^{+++} in the presence of nucleotide di-or triphosphate or pyrophosphate greatly enhanced the reaction (190). It was suggested that the peroxidative cleavage of microsomal phospholipids during NADPH oxidation was a consequence of a limited chain reaction catalyzed by a radical-like factor produced during the transport of electrons from NADPH by the membrane-bound mixed-function oxidase system (191). Masters et al. (192) demonstrated the involvement of NADPH-cytochrome c reductase in the mixed-function oxidase catalyzed hydroxylation of drugs and foreign compounds and it was suggested that NADPH-cytochrome c reductase might also be involved in NADPH-dependent lipid peroxidation (193, 194). Work had begun on the purification of NADPH-cytochrome c reductase by Dr. Thomas Pederson in an attempt to demonstrate this enzyme's involvement in NADPH-dependent lipid peroxidation.

Various surface-bound protein components of the microsomal membrane had been purified using a solubilizing technique that involved treatment of the membrane with proteolytic enzymes (195, 196). The membrane

bound NADPH-cytochrome c reductase had been solubilized from the microsomal membrane by brief incubation with trypsin (197-200), chymotrypsin (201). a Bacillus subtilis protease (201), bromelain (202) and a crude preparation of pancreatic lipase (178, 203). It was believed that NADPH-cytochrome c reductase solubilized by the action of proteases was a cleavage product of a larger protein which was anchored to the membrane surface by means of a hydrophobic peptide "tail." Such a model had been proposed for two other microsomal membrane bound proteins; cytochrome b_5 (195) and NADH-cytochrome b_5 reductase (204). The lipase-solubilized NADPH-cytochrome c reductase, however, may have represented the intact, native* enzyme which could have been released from the membrane through the lipolytic disruption of the membrane lipids catalyzed by the lipase preparation. However, the properties and reactions of the lipase-solubilized enzyme were very similar, if not identical to the protease-solubilized enzyme (86, 202). Therefore, it was necessary to determine if the lipase-solubilized enzyme was truly the native, intact enzyme potentially involved in microsomal NADPH-dependent lipid peroxidation, or a product of solubilization by a protease contaminant present in the crude pancreatic lipase preparation. Experiments with lipase 448 (Nutritional Biochemistry Co., Cleveland, OH) a crude pancreatic lipase preparation, were designed to test this possibility.

Soybean trypsin inhibitor and phenylmethylsufonylfluoride (PMSF) were tested for their ability to inhibit lipase solubilization of

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* Native NADPH-cytochrome c reductase refers to the enzyme that has not been hydrolyzed by proteolytic digestion.

NADPH-cytochrome c reductase from microsomes isolated from the livers of phenobarbital-pretreated rats. The enzyme was considered solubilized when it remains in the supernatant after centrifugation at 105,000 x g for 90 min. NADPH-cytochrome c reductase was assayed before and after centrifugation to determine the percent solubilized. Soybean trypsin inhibitor was capable of partially preventing solubilization while PMSF completely inhibit solubilization (Table 1). PMSF was found to have no effect on the ability of the lipase preparation to release fatty acids from Tween 20, and at the concentration used, had no effect on NADPH-cytochrome c reductase (PMSF was mixed with the lipase before incubation with microsomes).

The crude pancreatic lipase was subjected to gel filtration chromatography on Sephadex G-100 and each fraction was assayed for lipase and protease activity and for its ability to solubilize NADPH-cytochrome c reductase from rat liver microsomes. The lipase and protease activities separated upon exclusion chromatography and solubilization activity correlated with protease activity (Figure 2). Recovery of lipase activity was 98% while the recovery of protease activity was 70%. Some protease activity eluted with the lipase and there was a corresponding small peak of solubilization activity.

These results indicate that the ability of crude pancreatic steapsin to solubilize NADPH-cytochrome c reductase from rat liver microsomes is the result of a protease contaminant in the preparation and not the lipase. No solubilization activity could be correlated with lipase activity. Therefore, the solubilization of the catalytic portion of NADPH-cytochrome c reductase, when using both proteases and the lipase preparation, probably result in the degradation of the native, membrane bound enzyme.

TABLE 1.

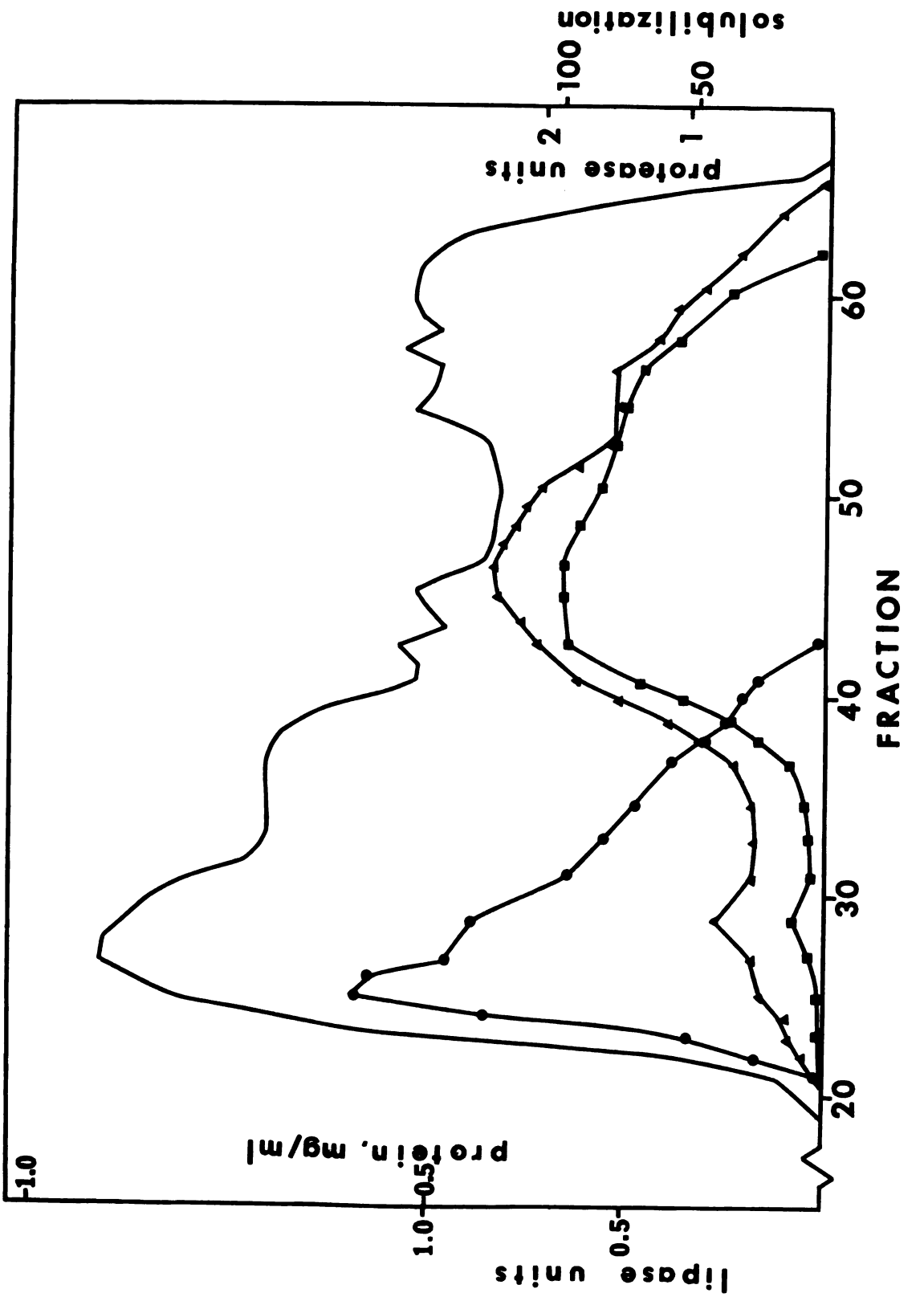
THE EFFECT OF SOYBEAN TRYPSIN INHIBITOR (STI) AND PHENYLMETHYLSULFONYL-FLUORIDE (PMSF) ON THE SOLUBILIZATION OF NADPH-CYTOCHROME c REDUCTASE BY CRUDE PANCREATIC LIPASE

Crude pancreatic lipase was incubated with rat liver microsomes (5 mg/ml in 0.05 M Tris buffer, pH 7.5) for 12 h at 5° C under nitrogen. Following incubation the preparation was diluted with ice-cold Tris buffer and centrifuged at 105 000 X g for 90 min at 0-5° C. NADPH-cytochrome c reductase was assayed as described under "Materials and Methods" before and after centrifugation to determine the per cent of the enzyme solubilized.

Lipase 448 (µg)	STI (µg)	PMSF (µg)	% solubilization	% of non-inhibited
0	-	-	0.0	-
45	-	-	22.6	100.0
45	25	-	16.3	72.2
45	12.5	-	18.1	80.0
45	5	-	19.5	86.4
45	-	33	0.0	0.0
45	-	17	1.8	8.0

Figure 2. GEL FILTRATION OF CRUDE PANCREATIC LIPASE ON A SEPHEDEX G-100 COLUMN.

Each fraction was assayed for protein (——), lipase (● —— ●) and protease (▲ —— ▲) activities and ability to solubilize NADPH-cytochrome c reductase from rat liver microsomes (■ —— ■) as described under "Methods".


lipase units
1.0
0.5
protein, mg/ml
1.0
0.5
protease units
2
1
solubilization
100
50
20
30
40
50
60
FRACTION

Preparation of an Antibody to NADPH-Cytochrome c Reductase:

Subsequently, NADPH-cytochrome c reductase was solubilized and purified to homogeneity by Dr. Thomas Pederson (87). Through the use of the purified enzyme, it was demonstrated that NADPH-cytochrome c reductase was involved in the catalysis of lipid peroxidation in a model membrane system composed of liposomes, NADPH, NADPH-cytochrome c reductase, and Fe^{+++} chelated by both ADP and EDTA (87). In order to establish that the peroxidation catalyzed by the purified enzyme in the model system was an accurate reflection of the NADPH-dependent peroxidation that occurs in intact microsomes, an antibody was raised to the purified, bromelain-solubilized NADPH-cytochrome c reductase. Antisera to the purified enzyme was prepared as described under "Methods." Dr. Thomas Pederson subsequently demonstrated that the antisera to the purified enzyme was a potent and specific inhibitor of the cytochrome c-reducing activity of the enzyme and that the immunoglobulin-G (IgG) fraction from the antisera inhibited over 90% of the NADPH-dependent microsomal lipid peroxidation (87).

Lung Microsomal Lipid Peroxidation:

Thomas et al. (205) demonstrated that rats exposed to nitrogen dioxide for four hours showed evidence of lipid peroxidation in the unsaturated fatty acids extracted from their lung tissue. The changes in lung lipids in vivo were partially prevented by a large dose of α tocopherol. Similarly, exposure to low concentrations of ozone in air produced increased lipid peroxidation in human erythrocyte suspensions, and led to speculation that lung tissue might also be affected likewise by ozone exposure. May et al. (206) demonstrated that lipid

peroxidation coupled to NADPH oxidation in the presence of ADP and ferrous ions was increased by performing the incubations in atmospheres enriched with oxygen. At five atmospheres pressure, there were extensive changes in the lipids of the endoplasmic reticulum that were characteristic of changes occurring during lipid peroxidation (207). Since lung has the highest oxygen tension in the body, it is reasonable to believe that it may be susceptible to non-enzymatic or enzymatically-induced lipid peroxidation.

Lung microsomes have an NADPH-dependent cytochrome c reductase activity that may function in the lung mixed-function oxidase system in a manner analogous to the corresponding enzyme in the liver microsomal mixed-function oxidase system (208). If this were true, then lung tissue may demonstrate a NADPH-dependent microsomal lipid peroxidation catalyzed by the lung NADPH-dependent cytochrome c reductase. In order to determine if liver and lung microsomes demonstrate similar NADPH-dependent lipid peroxidation, a comparative study of liver and lung microsomes, and the flavorprotein, NADPH-cytochrome c reductase from these two tissues, was undertaken.

Rat liver and lung microsomes were isolated simultaneously without frozen storage of the tissues. The specific activities of liver and lung microsomes, in nmoles of cytochrome c reduced/min/mg protein, were 196 and 67, respectively. Table 2 demonstrates the effect of incubating both liver and lung microsomes with ADP-Fe^{+++} and NADPH. It is apparent that lung microsomes produce much less malondialdehyde than do liver microsomes. The fact that liver microsomal peroxidation was not reduced when equal amounts of liver and lung microsomes were mixed together indicates that a soluble inhibitor of lipid peroxidation was

TABLE 2.

NADPH-DEPENDENT LIPID PEROXIDATION IN LIVER AND LUNG MICROSOMES.

The reaction mixtures contained liver or lung microsomes in the amounts indicated. Control mixtures contained no NADPH. All other conditions are the same as described for NADPH-dependent microsomal lipid peroxidation under "Methods". The reaction time equaled 15 minutes.

Description	Malondialdehyde formed nmoles/min/ml
Liver microsomes, 0.5 mg protein/ml	
Control	0.15
Plus NADPH.	1.51
Lung microsomes, 0.5 mg protein/ml	
Control	0.09
Plus NADPH.	0.15
Lung microsomes, 2.5 mg protein/ml	
Control	0.10
Plus NADPH.	0.48
Lung microsomes, 5.0 mg protein/ml	
Control	0.11
Plus NADPH.	0.67
Liver microsomes, 0.25 mg protein/ml plus lung microsomes, 0.25mg/protein/ml	
Plus NADPH.	1.40

not present in the lung microsomes. The decreased NADPH-cytochrome c reductase specific activity in lung microsomes may be responsible for the decreased lipid peroxidation. In order to increase the amount of NADPH-cytochrome c reductase present in the lung microsomal incubation mixture, the total microsomal protein concentration had to be increased. However, increasing the concentration of lung microsomes is likely to decrease the lipid peroxidation specific activity (nmoles malondialdehyde/min/mg protein). Wills (104) determined that dilute microsomal suspensions formed much more lipid peroxide/mg of protein in the presence of ascorbate than did concentrated suspensions and that this may be a result of dispersal of the reticulum in an aqueous phase so that the membranes become extended and the unsaturated lipids lie in close proximity to radicals formed in the aqueous phase. A similar effect was observed in studies of the peroxidation of emulsified tissue lipids exposed to ionizing radiation (209). This fact is demonstrated in Table 3, using liver microsomes. Increased microsomal protein concentrations decreases the amount of malondialdehyde produced/mg protein. The lower amount of malondialdehyde produced in lung microsomes may also be a function of the lipid composition of the membrane. Since only lipids possessing three methylene interrupted double bonds will produce malondialdehyde, variation in the amount of polyunsaturated fatty acids will effect malondialdehyde production. It was also possible that NADPH-cytochrome c reductase from lung microsomes was substantially different from the liver microsomal enzyme and was not capable of supporting peroxidation at the same rate or by the same mechanism as the liver enzyme. In order to determine if the reduced rate of peroxidation in lung microsomes was a function of the membrane or of a different

TABLE 3.

EFFECT OF MICROSOMAL PROTEIN CONCENTRATION OF LIPID PEROXIDATION.

The reaction mixtures contained liver microsomal protein in the amounts indicated. Reaction time equaled 8 minutes. The conditions are the same as described for NADPH-dependent microsomal lipid peroxidation under "Methods".

Description	Malondialdehyde formed	
	nmoles/min/ml	nmoles/min/mg protein
5.0 mg/ml microsomal protein	4.10	0.82
2.0 mg/ml microsomal protein	3.04	1.52
1.0 mg/ml microsomal protein	1.67	1.67
0.5 mg/ml microsomal protein	0.91	1.82
0.2 mg/ml microsomal protein	0.49	2.43

NADPH-cytochrome c reductase, the lung enzyme was isolated and compared to the corresponding liver enzyme.

Isolation and Characterization of Lung Microsomal NADPH-Cytochrome c Reductase:

NADPH-cytochrome c reductase from rat lung microsomes was solubilized by bromelain digestion in a manner identical to that already used for the liver reductase. Both liver and lung enzymes displayed a 20% enhancement of cytochrome c reducing activity following bromelain solubilization. A similar activity enhancement of NADPH-cytochrome c reductase solubilized by trypsin has been observed (198). The lung enzyme chromatographed on Sephadex G-100 and DEAE-cellulose in a manner identical to that of the liver enzyme. The specific activity of the purified liver reductase was 51,000. The corresponding lung enzyme was purified to a slightly lower specific activity of 31,000. No additional purification techniques were applied to the lung enzyme. The slightly lower specific activity of the lung enzyme may have been due to contaminating proteins found in lung microsomes that were not present in liver microsomes.

Previous reports concerning rabbit lung tissue fractionation suggested that increased microsomal protein yield was obtained by increased homogenization of the lung tissue (210). In rat lung tissue, initial microsome isolations using extensive homogenization conditions revealed a cytochrome-c reducing activity in the 105,000 x g supernatant. However, subsequent investigations showed that the appearance of this activity was not a consequence of the more extensive homogenization. The possibility that NADPH-cytochrome c reductase was being released

during microsome isolation by an endogenous protease was deemed unlikely after it was determined that time, temperature, or the addition of PMSF had no effect on the appearance of the soluble cytochrome c reducing activity. This activity which appeared to be labile during storage of the 105,000 x g supernatant, was not dependent on NADPH nor was it inhibited by the antibody directed against the purified NADPH-cytochrome c reductase. The soluble cytochrome c reducing activity therefore appears to be unrelated to the activity of microsomal NADPH-cytochrome c reductase.

Figure 3 illustrates the nearly identical activity profiles of the purified liver and lung reductases towards the reduction of cytochrome c over a wide range of ionic strengths. The optimum molar concentration of KCl appears to be 0.8 M. Both reductases undergo better than a five fold increase in activity after increasing the assay buffer from 0.01 M phosphate (μ = 0.025) to 0.8 M KCl, 0.01 M phosphate (μ = 0.825). The variation in reductase activity caused by changing the ionic strength of the assay buffer explains the discrepancies between previously published values for the specific activity of purified rat liver reductase. The molar concentrations of phosphate buffer used to assay NADPH-cytochrome c reductase from liver microsomes have varied, in the literature, from 0.05 phosphate (μ = 0.15) (203) to 0.3 M phosphate (μ = 0.85) (87). Optimal activities are achieved for both the liver and lung reductases by using 0.3 M phosphate buffer, pH 7.5 (μ = 0.85) in the assay mixture.

Figures 4 and 5 represent the resulting inhibition of both reductases upon incubation with an antibody raised against the liver microsomal enzyme. No inhibition was observed when preimmuned IgG was added

Figure 3. EFFECT OF IONIC STRENGTH ON THE ACTIVITY OF PURIFIED NADPH-CYTOCHROME c REDUCTASE FROM RAT LIVER AND LUNG MICROSOMES.

Assays for the liver (● —— ●) and lung (■ —— ■) purified reductases were performed in 0.01 M sodium phosphate, pH 7.5 (25° C), 2 mM EDTA. The ionic strength was established by the addition of various amounts of 4 M KCl.

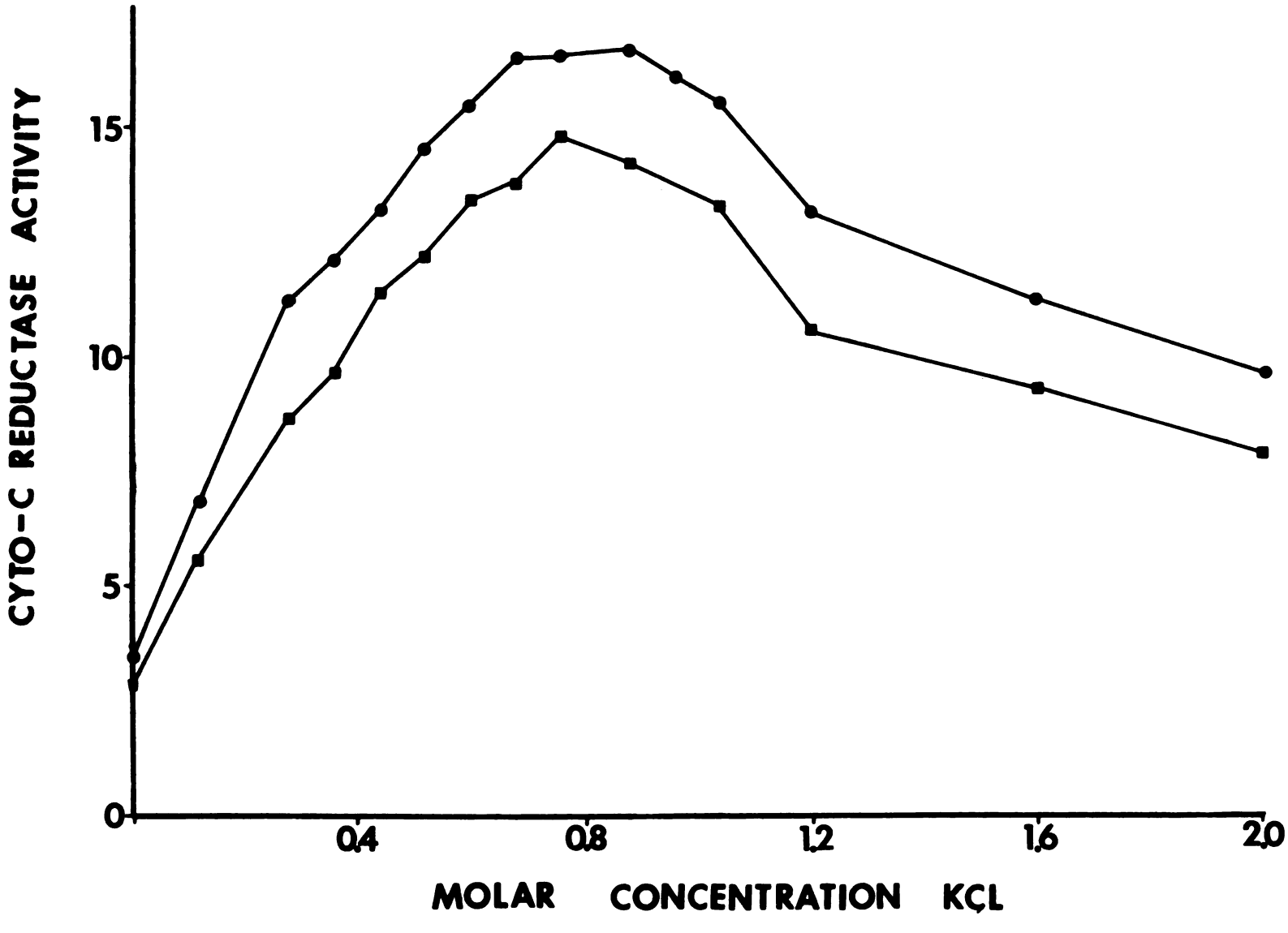

CYTO-C REDUCTASE ACTIVITY
15
10
5
0
0.4
0.8
1.2
1.6
2.0
MOLAR CONCENTRATION KCL

Figure 4. INHIBITION OF NADPH-CYTOCHROME c REDUCTASE BY ANTIBODY TO THE BROMELAIN-SOLUBILIZED LIVER REDUCTASE.

Liver (● —— ●) or lung (■ —— ■) purified reductase (0.1 µg/ml) was pre-incubated at 4° C with IgG for 10 minutes and assayed for the reduction of cytochrome c as described under "Methods".

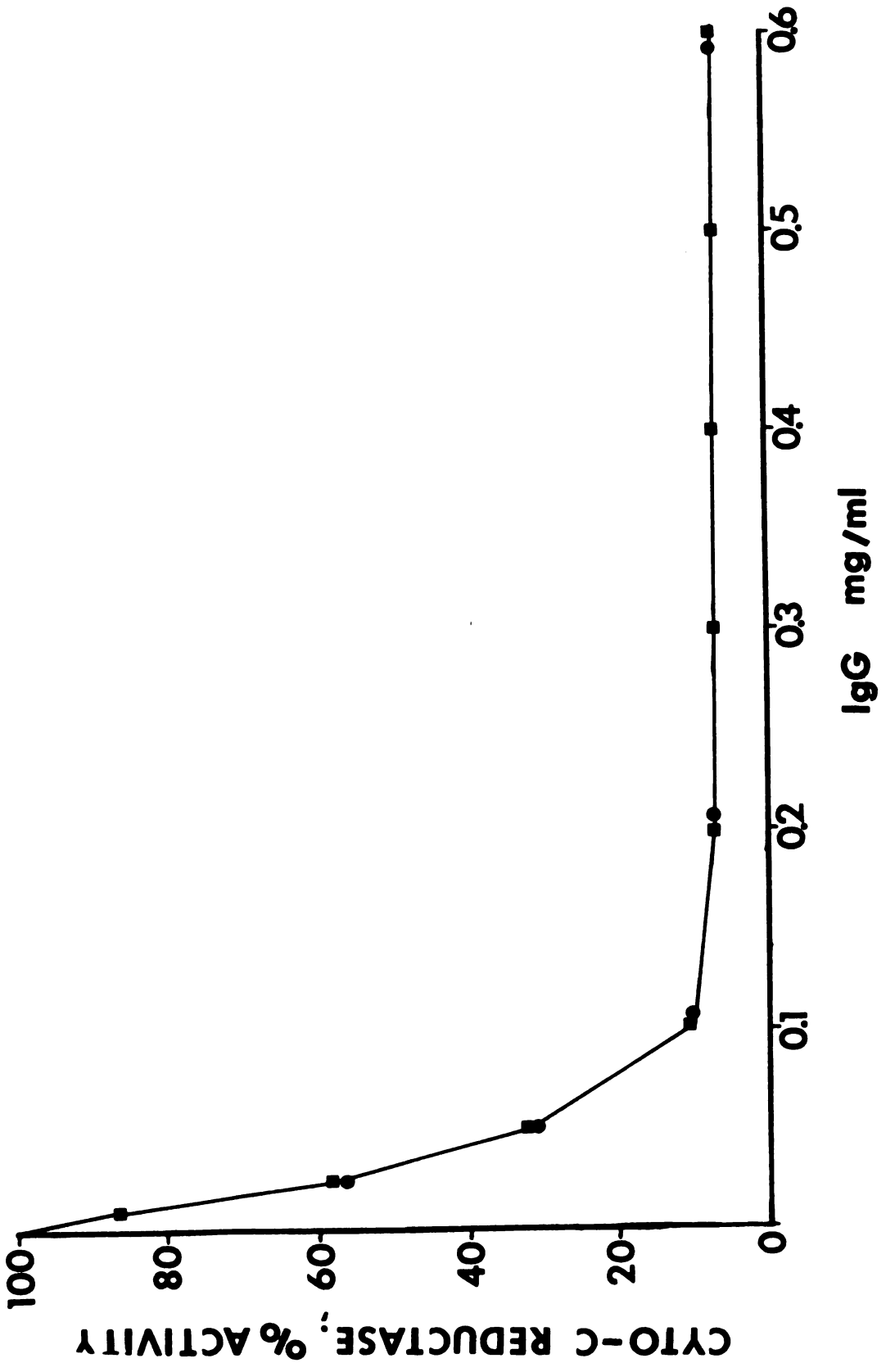

CYTO-C REDUCTASE, % ACTIVITY
100
80
60
40
20
0
0.1
0.2
0.3
0.4
0.5
0.6
IgG mg/ml

Figure 4. INHIBITION OF NADPH-CYTOCHROME c REDUCTASE BY ANTIBODY TO THE BROMELAIN-SOLUBILIZED LIVER REDUCTASE.

Liver (● —— ●) or lung (■ —— ■) purified reductase (0.1 µg/ml) was pre-incubated at 4° C with IgG for 10 minutes and assayed for the reduction of cytochrome c as described under "Methods".

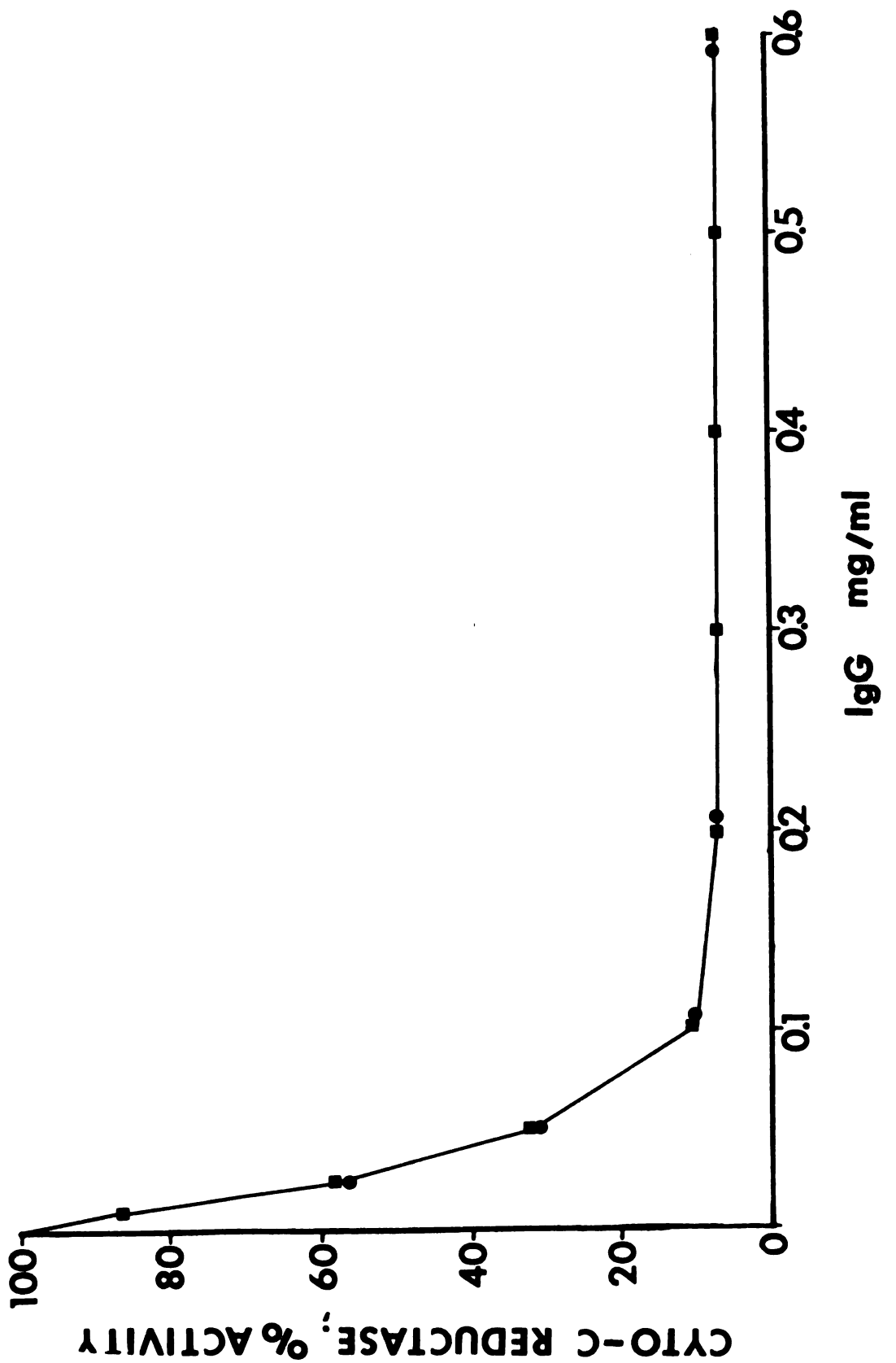
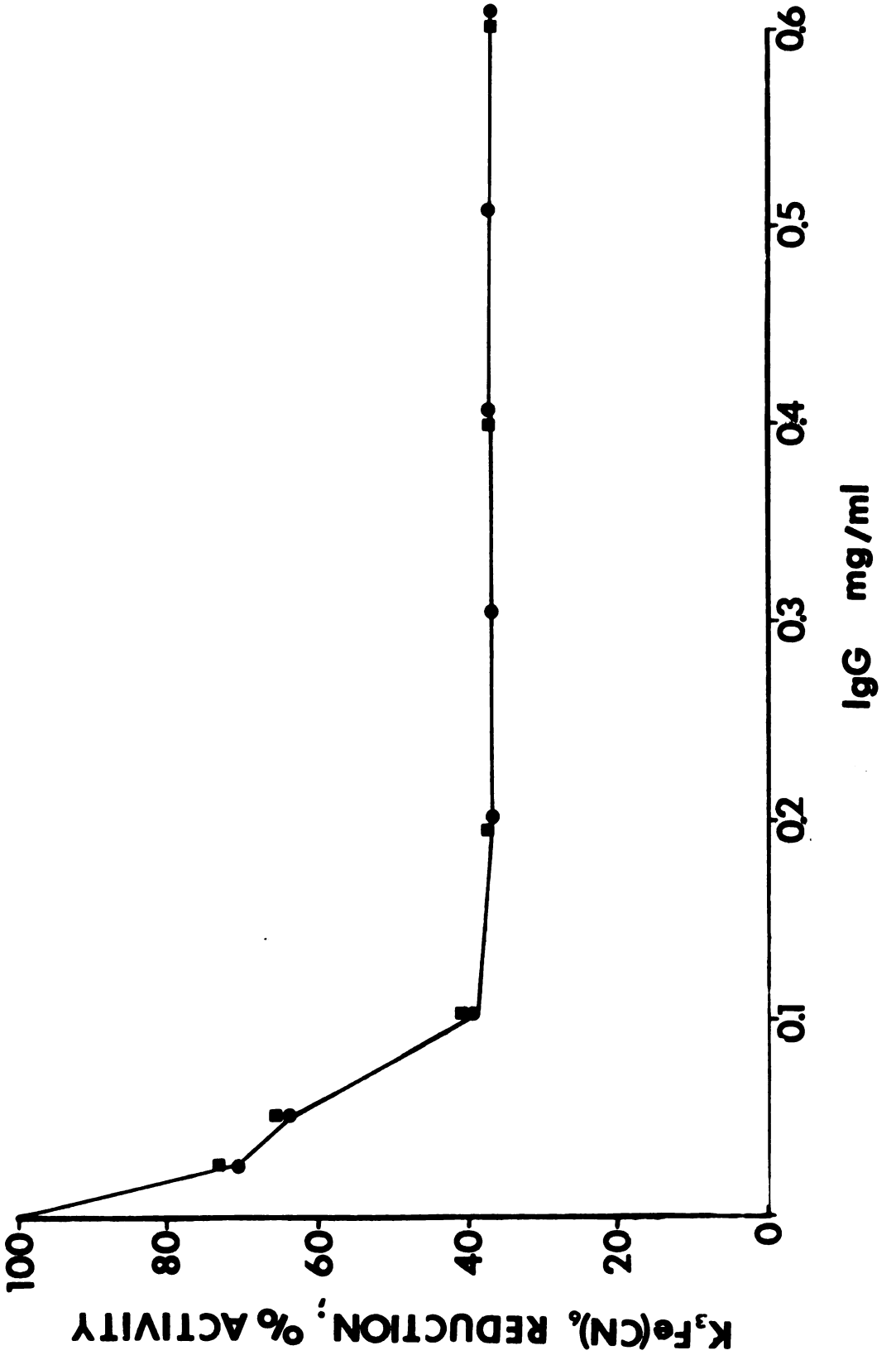

CYTO-C REDUCTASE, % ACTIVITY
100
80
60
40
20
0
0.1
0.2
0.3
0.4
0.5
0.6
IgG mg/ml

Figure 5. INHIBITION BY ANTIBODY OF THE ACTIVITY OF NADPH-CYTOCHROME c REDUCTASE WITH FERRICYANIDE AS THE TERMINAL ELECTRON ACCEPTOR.

All assays mixtures contained 0.1 μg/ml of purified liver (■ —— ■) or lung (● —— ●) reductase and 0.1 mM $K_3Fe(CN)_6$. Reduction of ferricyanide was observed as the change in absorbance at 420 nm. Enzyme and antibody were incubated for 10 minutes at 4° C before assaying ferricyanide reduction.


$K_3Fe(CN)_6$ REDUCTION, % ACTIVITY
100
80
60
40
20
0
0.1
0.2
0.3
0.4
0.5
0.6
IgG mg/ml

to either reductase. The antibody inhibits the reduction of cytochrome c by both reductases by up to 93%. The antibody is not as effective in inhibiting the reduction of ferricyanide by either enzyme, as seen in Figure 5. However, both liver and lung reductases are inhibited to the same extent.

Figure 6 shows the precipitin lines obtained after double diffusion immunoprecipitation was performed on Ouchterlony plates using both bromelain-solubilized, purified reductase and detergent-solubilized, native reductase from liver and lung microsomes. All wells containing reductase form precipitin lines that merge without forming spurs, suggesting that the antigenic sites on both liver and lung enzymes are the same.

The gel scans in Figure 7 are the protein profiles observed by scanning Coomassie blue stained sodium dodecylsulfate-polyacrylamide gels containing immunoprecipitates from detergent-solubilized liver and lung microsomes. We have previously shown that the antibody directed against the bromelain-solubilized liver reductase could precipitate the native enzyme from sodium deoxycholate-solubilized microsomes (182). When ^{125}I-labeled microsomes were solubilized in detergent and incubated with antibody, only a single protein containing radioactivity (79,000 dalton) was observed after subjecting the immunoprecipitate to sodium dodecylsulfate-polyscrylamide gel electrophoresis (182). Figure 7 (b) shows the protein profile of a gel that contained the immunoprecipitate from detergent-solubilized lung microsomes incubated with antibody. The two major bands are the heavy (52,000 dalton) and the light (25,000 dalton) chains of the reduced IgG molecules (211). The highest molecular weight band corresponds to a protein of 79,000 daltons. Only a

Figure 6. IMMUNOPRECIPITATION OF THE ANTIBODY TO PURIFIED, BROMELAIN-SOLUBILIZED NADPH-CYTOCHROME c REDUCTASE FROM RAT LIVER MICROSOMES WITH THE PURIFIED REDUCTASE AND WITH DETERGENT-SOLUBILIZED MICROSOMES FROM BOTH RAT LIVER AND LUNG ON AN OUCHTERLONY DOUBLE DIFFUSION AGAR PLATE.

The center well contains anti-NADPH-cytochrome c reductase IgG (1.4 mg protein). The outer wells are numbered counter clockwise from the top (No. 1). Well No. 1 contains 0.4 mg of liver microsomal protein (5 mg/ml in 1% sodium deoxycholate). Well No. 2 contains 1.2 mg of liver microsomal protein (15 mg/ml in 1% sodium deoxycholate). Well No. 3 contains 2 μg of purified lung reductase. Well No. 4 contains 2 μg of liver reductase. Immunoprecipitation occured in 24 h at 25°C. Ouchterlony plates were made as described under "Methods".

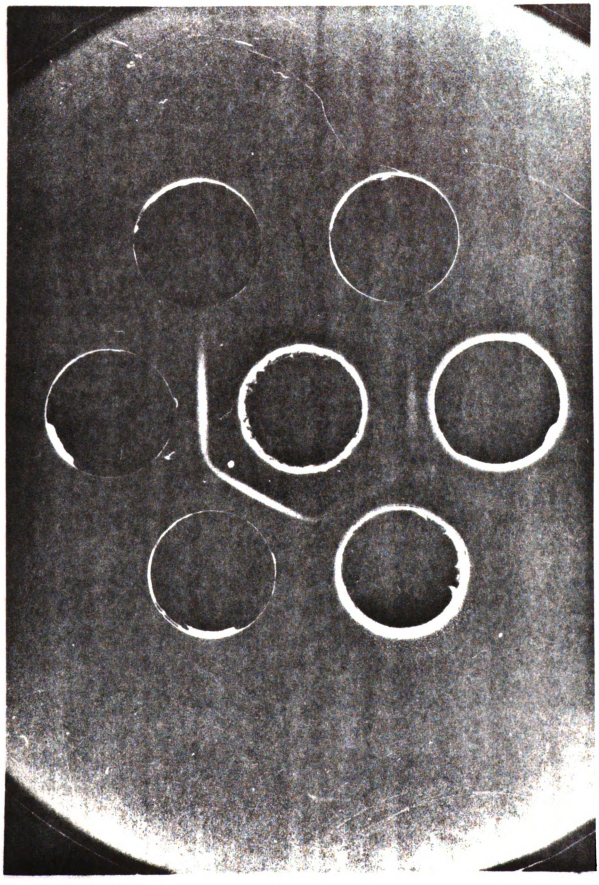
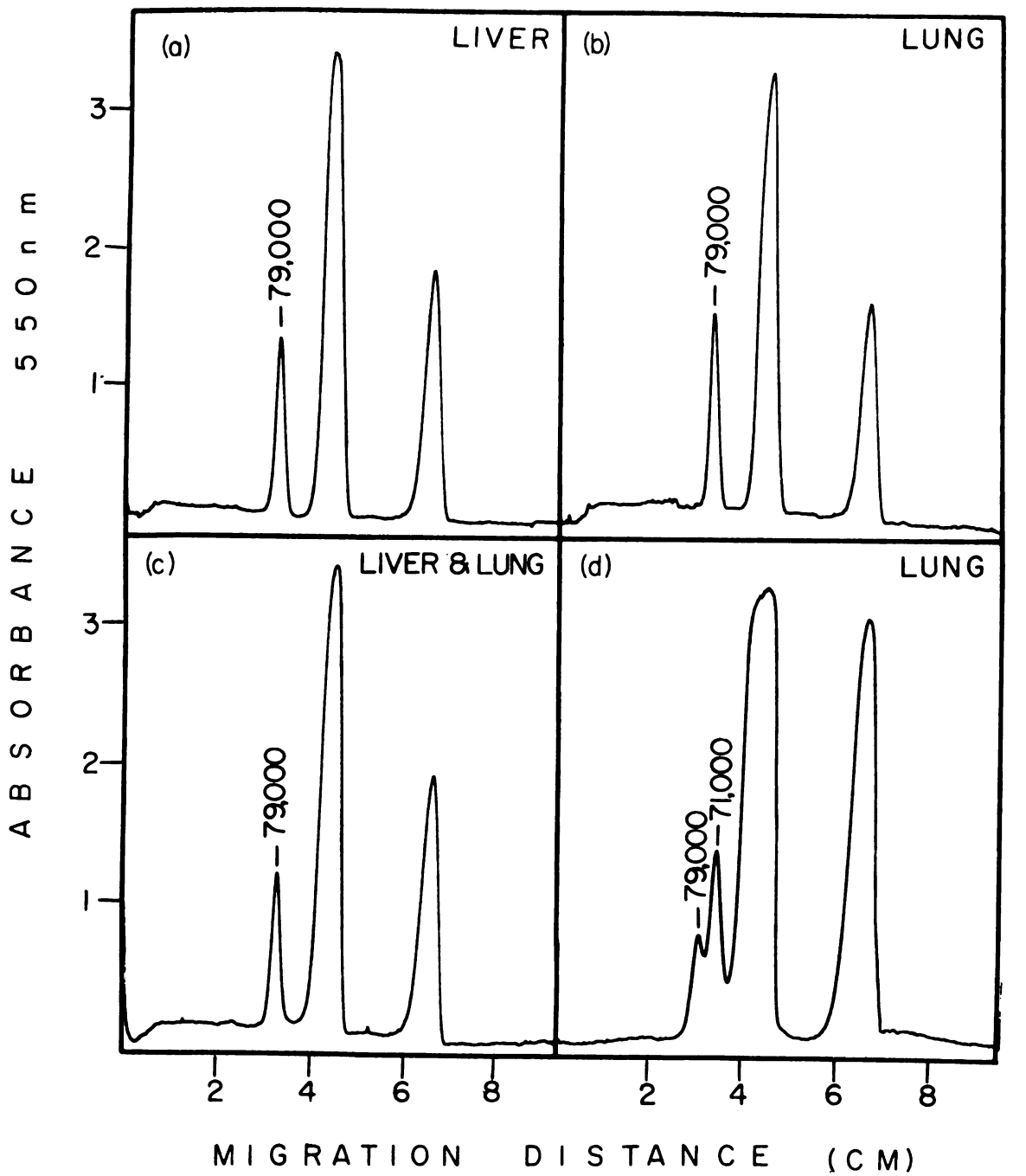


Figure 7.

SODIUM DODECYLSULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS PROTEIN PROFILES OF IMMUNOPRECIPITATES FORMED FROM SODIUM DEOXYCHOLATE-SOLUBILIZED RAT LIVER AND LUNG MICROSOMES AND THE REDUCTASE ANTIBODY.

(a) Protein scan of liver microsomal immunoprecipitate; (b) protein scan of lung microsomal immunoprecipitate; (c) protein scan of combined immunoprecipitates from both liver and lung microsomes; (d) protein scan of combined immunoprecipitates from lung microsomes and from bromelain-solubilized, purified lung reductase. Molecular weights were determined from molecular weight marker proteins run independently for each gel. Immunoprecipitation and sodium dodecylsulfate-polyacrylamide gel electrophoresis were performed as described under "Methods".


(a)
LIVER
79,000
(b)
LUNG
79,000
(c)
LIVER & LUNG
79,000
(d)
LUNG
79,000
71,000
ABSORBANCE 550nm
1
2
3
2
4
6
8
MIGRATION DISTANCE (CM)

single lung microsomal protein is precipitated by the antibody to the liver reductase. The gel scanned in Figure 7 (c) contained equal amounts of both liver and lung microsome immunoprecipitates. Only a single high molecular weight band appears, signifying that both reductases migrate together. When the immunoprecipitate from detergent-solubilized lung microsomes was combined with an immunoprecipitate of the bromelain-solubilized purified lung enzyme, two reductase bands are observed (Figure 7d). Identical results were reported for the liver reductase (182). The 71,000 dalton bromelain-solubilized lung reductase is a cleavage product of the intact, native enzyme as has been shown for the liver reductase.

The preceding evidence strongly suggests that the flavoprotein, NADPH-cytochrome c reductase, from both rat liver and lung are extremely similar, if not identical. Therefore, the lung enzyme was tested for its ability to promote lipid peroxidation in the model peroxidation system composed of liposomes, NADPH, and Fe^{+++} chelated by both ADP and EDTA. As seen in Table 4, both liver and lung NADPH-cytochrome c reductase are equally effective at promoting lipid peroxidation. This evidence would indicate that NADPH-dependent lipid peroxidation in both liver and lung microsomes is catalyzed by the same enzyme and probably by the same mechanism. The lower rate of malondialdehyde production in lung microsomes is probably a function of the membrane itself. This would prove advantageous to lung tissue since the high oxygen tension in this organ is more likely to accelerate lipid peroxidation and cause damage to the microsomal membrane.

TABLE 4.

LIPID PEROXIDATION IN LIPOSOMES CATALYZED BY PURIFIED LIVER AND LUNG NADPH-CYTOCHROME c REDUCTASE.

The reaction mixtures contained 0.5 μmoles lipid phosphate/ml, 0.2 mM NADPH and both ADP-Fe^{+++} and EDTA-Fe^{+++} as described for NADPH-dependent lipid peroxidation in liposomes under "Methods". 0.2 μg of either liver or lung purified NADPH-cytochrome c reductase were added as indicated. Control sample contained no reductase. Reaction time equaled 10 minutes.

Description	Malondialdehyde formed nmoles/min/μmoles lipid Pi
Control.....	0.19
Plus liver reductase.....	2.04
Plus lung reductase.....	1.98

The Mechanisms Involved in NADPH-Dependent Lipid Peroxidation

Among the various biological compounds most susceptible to oxidation by molecular oxygen are the unsaturated fatty acyl residues of phospholipids. This type of lipid oxidation is generally believed to involve the production and propagation of lipid radicals, which may be produced by high frequency radiation or by some naturally occurring metabolic reactions (6-9). Although some metals, including iron, have been shown to increase the amount of malondialdehyde produced by extracted biological lipids or membrane samples, it has been difficult to determine whether their effect has been to actually initiate the formation of lipid hydroperoxides or to propagate more lipid peroxides by reacting with pre-existing peroxides to form lipid radicals (101-103). It is believed that freshly isolated membrane samples may contain low levels of lipid hydroperoxides (151). The enzymatic mechanism by which lipid peroxides could be formed from peroxide-free lipid has not yet been determined. It was therefore of importance to determine exactly how the process of microsomal lipid peroxidation catalyzed by NADPH was initiated.

McCay and co-workers have studied microsomal lipid peroxidation by measuring lysis of lysosomes by radical-like agents products in microsomes during the oxidation of NADPH (91). They have suggested that O_2^- formed from the interaction of reduced NADPH-cytochrome c reductase with molecular oxygen might be involved in microsomal lipid peroxidation via the formation of hydroxy radicals:

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

$$H_2O_2 + O_2^{\cdot-} \longrightarrow O_2 + OH\cdot + OH^- \quad 2)$$

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

$$Fe^{++} + H_2O_2 \longrightarrow OH\cdot + OH^- + Fe^{+++} \quad 4)$$

NADPH-cytochrome c reductase, found either in microsomes or in its purified (bromalain digestion) state, has been shown to produce $O_2^{\cdot-}$ upon oxidation of NADPH (212-214). Their scheme called for the peroxidation of polyunsaturated lipids by the abstraction of a hydrogen from a methylene carbon atom of the lipid by the OH·. The direct extraction of a hydrogen by $O_2^{\cdot-}$ was not considered feasible since superoxide dismutase cause no inhibition of lysozomal lysis. In addition to being partially inhibited by OH· trappers, the reaction was also inhibited by catalase. However, recent studies (215) have demonstrated that catalase does not inhibit NADPH-dependent lipid peroxidation. The discrepancy between these findings is probably due to the presence of thymol, a potent antioxidant, in commercial preparation of catalase. When catalase free of thymol is added to the NADPH-dependent peroxidizing system, lipid peroxidation is no longer inhibited.

The inability of superoxide dismutase and catalase to inhibit NADPH-dependent lipid peroxidation has led to the proposal of a different reaction mechanism to explain microsomal lipid peroxidation. Pederson and Aust (215) have suggested that reduced NADPH-cytochrome c reductase acts on Fe^{+++} to produce Fe^{++}. Fe^{++} could either act on preexisting hydroperoxides present in the membrane to produce more lipid radicals, or could combine with molecular oxygen to form the perferryl ion $(FeO_2)^{++}$. Oxygen is known to combine with Fe^{++} to form the perferryl

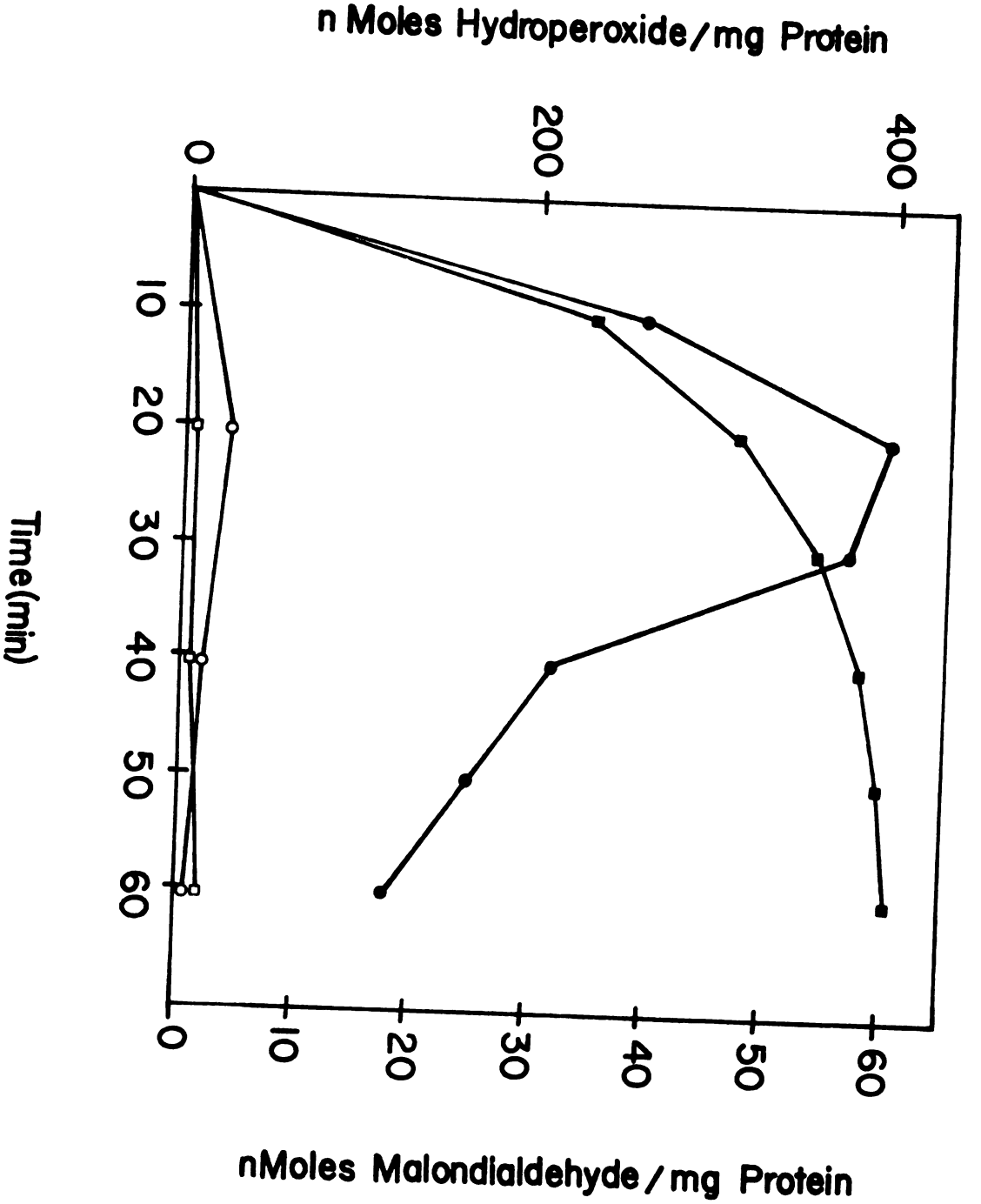
ion as an intermediate in the oxidation of Fe^{++} (216, 217). This species may be able to produce a peroxy radical or extract an electron from the lipid, yielding a lipid radical and H_2O_2. The possibility that NADPH-dependent lipid peroxidation is mediated by a reduced form of iron and molecular oxygen will now be examined.

The detectable events that occur during NADPH-dependent lipid peroxidation include an uptake of oxygen (75), the oxidation of NADPH (138), the appearance of lipid hydroperoxides (218), a perturbation of the surface structure of microsomes resulting in a change in turbidity (218), a loss of unsaturated fatty acids (1), and the appearance of lipid peroxide breakdown products, including malondialdehyde (138). Figure 8 illustrates two of these observable changes in peroxidizing microsomes over a period of time. In the presence of NADPH and ADP-chelated Fe^{+++}, microsomes produce an increasing amount of malondialdehyde over the 60 minute incubation period. May and McCay (138), have demonstrated that the continued increase in malondialdehyde depend on the availability of reducing equivalents from NADPH. When NADPH is exhausted, lipid peroxidation ceases. Subsequent additions of NADPH cause further peroxidation, as judged by oxygen uptake and malondialdehyde formation.

In addition to measuring malondialdehyde production, lipid hydroperoxide levels can also be measured through the use of the iodometric assay (6, 219) which is specific for easily reduced peroxides (peroxy acids, diacyl peroxides, and all hydroperoxides). This assay has been used extensively by nutritionist and oil and soap chemists to detect the presence of hydroperoxides in purified lipid samples (220-224). Recknagel and Ghoshal (225) have used a similar procedure on extracted

Figure 8. TIME COURSE OF HYDROPEROXIDE AND MALONDIALDEHYDE FORMATION DURING NADPH-DEPENDENT MICROSOMAL LIPID PEROXIDATION

The experimental reaction mixture contained NADPH, ADP-Fe^{+++}, and 1 mg/ml microsomal protein as described for NADPH-dependent microsomal lipid peroxidation under "Methods". The control reaction mixture contained 0.005% BHT. (● —— ●) Hydroperoxide formation and (■ —— ■) malondialdehyde formation in experimental reaction mixture. (○ —— ○) Hydroperoxide formation and (□ —— □) malondialdehyde formation in control reaction mixture. Malondialdehyde and hydroperoxide formation were assayed as described under "Methods".


n Moles Hydroperoxide / mg Protein
0
200
400
Time(min)
10
20
30
40
50
60
0
10
20
30
40
50
60
nMoles Malondialdehyde / mg Protein

lipids from the microsomal fractions of rats exposed to CCl_4 to demonstrate an increase in membrane lipid hydroperoxides during liver necrosis. Its use in detecting the appearance of hydroperoxides formed during in vitro enzymatically induced membrane lipid peroxidation is a comparatively new one. Tam and McCay (218) used a similar procedure to suggest that lipid hydroperoxides were transient species formed in the early stages of NADPH induced lipid peroxidation and that the hydroperoxides fell to low levels as their breakdown began to occur. Slater (6) demonstrated that microsomes allowed to autoxidize at 4°C had high levels of lipid hydroperoxides, but low levels of malondialdehyde. When identical microsomal samples were incubated at 37°C instead, hydroperoxide levels fell to a low level as malondialdehyde levels increased, indicating that malondialdehyde is derived from the breakdown of previously formed lipid peroxides. In the present study, it can be seen that lipid hydroperoxide and malondialdehyde levels increase simultaneously during the early phase of NADPH-catalyzed lipid peroxidation (Figure 8). However, after reaching a peak at about 20 minutes, hydroperoxide levels begin to decrease. This would suggest that the formation of lipid hydroperoxides and the breakdown of lipid peroxides to form malondialdehyde may be two consecutive and separate events. It can also be seen that the free radical trapper, BHT, abolishes both hydroperoxide and malondialdehyde formation. Therefore, at least the formation of hydroperoxides, and perhaps the breakdown of lipid peroxides to yield malondialdehyde, are dependent on the production of free radicals.

The involvement of Fe^{+++} chelated by ADP in NADPH-dependent lipid peroxidation is demonstrated in Table 5. The presence of ADP in this system is thought to prevent Fe^{+++} from precipitating from solution as

TABLE 5

NADPH-DEPENDENT MICROSOMAL LIPID PEROXIDATION AS A FUNCTION OF ADDED ADP-Fe^{+++}.

The reaction mixtures contained 1.0 mg/ml microsomal protein and 0.2 mM NADPH as described for NADPH-dependent microsomal lipid peroxidation under "Methods". ADP-Fe^{+++} or EDTA were added as indicated. NADPH was delected from the reaction mixture containing ascorbate. The reaction time equaled 5 minutes.

Description	Malondialdehyde formed nmoles/min/mg protein
Microsomes and NADPH	
Plus 0.1 mM ADP-Fe^{+++}	2.50
Plus 0.01 mM ADP-Fe^{+++}	2.41
Plus 0.001 mM ADP-Fe^{+++}	1.60
Minus ADP-Fe^{+++}	1.07
Plus 0.1 mM EDTA	.03
Microsomes	
Plus 0.5 mM ascorbate	1.60

$Fe(OH)_3$ and to prevent Fe^{+++} from binding to components on the microsomal surface (105, 218). It is apparent that small amounts of ADP-Fe^{+++} assist in NADPH-dependent lipid peroxidation as indicated by malondialdehyde production. In the absence of added ADP-Fe^{+++}, the addition of EDTA decreased the level of lipid peroxidation below the level observed when no ADP-Fe^{+++} is present. This would indicate that some metal is associated with the microsomal membrane and can be used to promote lipid peroxidation. This was confirmed by adding ascorbate to the microsomal incubation mixtures. The non-enzymatic oxidation of unsaturated membrane lipids catalyzed by ascorbate is known to be accelerated by inorganic or organic iron (226, 227). However, Table 6 demonstrates that ascorbate-catalyzed lipid peroxidation is completely dependent upon added iron in a purified liposomal system. Therefore, the peroxidation observed when microsomes and ascorbate are incubated together without exogenous Fe^{+++}, must depend on iron associated with the microsomal membrane.

If NADPH-dependent lipid peroxidation proceeds via the reduction of ADP-Fe^{+++}, then ADP-Fe^{++} must be shown to catalyze lipid peroxidation. Table 7 demonstrates the results of incubating microsomes with either ADP-Fe^{++} or ADP-Fe^{+++} in the absence of NADPH. The concentration of chelated iron is high (0.7mM) to better illustrate the enhanced catalytic activity of ADP-Fe^{++} over ADP-Fe^{+++} in promoting lipid peroxidation. In peroxidation experiments using either purified NADPH-cytochrome c reductase or ascorbate, 10 μ molar Fe^{+++} is sufficient to promote some lipid peroxidation. However, in these systems, the reduction of Fe^{+++} is continuous, thus replenishing the supply of Fe^{++}.

TABLE 6.

THE EFFECT OF ADP-Fe^{+++} ON ASCORBATE-CATALYZED LIPID PEROXIDATION IN LIPOSOMES.

The reaction mixtures contained 1.0 μmoles lipid phosphate/ml, 0.1 mM ascorbate and 0.05 M Tris-HCl, pH 7.4 at 37° C. ADP-Fe^{+++} or EDTA were added as indicated. The reaction time equaled 5 minutes.

Description	Malondialdehyde formed $\frac{\text{nmoles/min}}{\mu\text{moles lipid Pi}}$
Liposomes and ascorbate plus;	
10 μM ADP-Fe^{+++}	3.2
5 μM ADP-Fe^{+++}	3.2
1 μM ADP-Fe^{+++}	3.1
0.5 μM ADP-Fe^{+++}	2.8
0.1 μM ADP-Fe^{+++}	2.2
No ADP-Fe^{+++}	0.06
0.1 mM EDTA.	0.00

TABLE 7.

MICROSOMAL LIPID PEROXIDATION PROMOTED BY ADP-Fe^{++} OR ADP-Fe^{+++}.

The reaction mixtures contained 0.5 mg microsomal protein in 0.05 M Tris-Hcl, pH7.4 at 37° C. Iron was added as indicated. The reaction time equaled 10 minutes.

Description	Malondialdehyde formed nmoles/min/mg protein
Microsomes	
Plus 0.7 mM ADP-Fe^{++}.....	7.05
Plus 0.7 mM ADP-Fe^{+++}.....	1.90
No additions.....	0.15

When a model peroxidizing system composed of NADPH, purified NADPH-cytochrome c reductase, and ADP-Fe^{+++} was first used to promote lipid peroxidation in liposomes derived from purified microsomal lipid, it became apparent that an additional component was required for peroxidation activity (87, 215). Subsequently, others have confirmed that EDTA-chelated Fe^{+++}, in addition to ADP-Fe^{+++}, is required for peroxidation to occur in both liposomes and hemoprotein free lipoprotein particles (88, 111). Figure 9 demonstrates that both malondialdehyde and hydroperoxides are produced in the model system in the presence of EDTA-Fe^{+++}. As in microsomes, the hydroperoxide concentration rises early in the reaction and subsequently declines as hydroperoxides are apparently broken down.

However, upon close examination of the iron requirements in the model peroxidizing system, it became evident that small amounts of both malondialdehyde and hydroperoxides were being produced when EDTA-Fe^{+++} was absent from the complete reaction mixture. This led to a re-examination of the iron requirement in the model peroxidizing system. In microsomes, where ADP-Fe^{+++} alone is sufficient to catalyze NADPH-dependent lipid peroxidation, addition of EDTA-Fe^{+++} along with ADP-Fe^{+++} in equal amounts results in no more malondialdehyde production than an amount of ADP-Fe^{+++} equal to the total of the two chelated forms of iron. Yet in liposomes, deletion of EDTA-Fe^{+++} results in 60-70% decrease in malondialdehyde production. Various concentrations of ADP-Fe^{+++} and EDTA-Fe^{+++} were added to liposomes and incubated with NADPH and NADPH-cytochrome c reductase in chelex-treated 0.05m Tris-HCl buffer. Chelex treatment of 0.05 M potassium phosphate buffer reduces total iron contamination to below 10 n molar (228). As demonstrated

Figure 9. TIME COURSE OF HYDROPEROXIDE AND MALONDIALDEHYDE FORMATION DURING NADPH-DEPENDENT LIPOSOMAL LIPID PEROXIDATION.

The experimental reaction mixture contained 0.2 mM NADPH, 0.1 mM ADP-Fe^{+++}, 0.1 mM EDTA-Fe^{+++}, 0.5 μmole lipid phosphate/ml, 0.35 μg/ml purified NADPH-cytochrome c reductase in 0.05 M Tris-HCl at 37° C. Hydroperoxide formation (● —— ●) and malondialdehyde formation (■ —— ■) were assayed as described under "Methods".

n Moles Hydroperoxide / μ Mole Lipid Pi
0.0
200
400
n Moles Malondialdehyde / μ Mole Lipid Pi
0.0
15
30
45
60
75
90
10
20
30
40
50
60
Time (min)

in Table 8, EDTA-Fe^{+++} alone is not capable of supporting any lipid peroxidation in the model system, while ADP-Fe^{+++} alone will catalyze decreased levels of peroxidation. The greatest peroxidation occurs when both ADP-Fe^{+++} and EDTA-Fe^{+++} are present together. Even when the concentration of ADP-Fe^{+++} is 100 times the total iron concentration of the two combined forms of iron, less peroxidation is evidenced.

In order to verify that EDTA-Fe^{+++} undergoing reduction was not capable of supporting lipid peroxidation, ascorbate and EDTA-Fe^{+++} were incubated with liposomes. The ability of ascorbate to reduce EDTA-Fe^{+++} was tested by observing the reduction of cytochrome c by ascorbate in the absence and presence of EDTA-Fe^{+++}. A fourfold increase in the rate of cytochrome c reduction was observed in the presence of EDTA-Fe^{+++}. It appears that ascorbate reduces EDTA-Fe^{+++} which in turn reduces cytochrome c. Table 9 demonstrates that ascorbate and EDTA-Fe^{+++} do not promote lipid peroxidation, whereas ascorbate plus ADP-Fe^{+++} or the combination of ADP-Fe^{+++} and EDTA-Fe^{+++} do. If reduced EDTA-Fe^{+++} does not promote lipid peroxidation, then why does it assist so dramatically the peroxidation catalyzed by ADP-Fe^{+++} in the NADPH-dependent liposomal system (Table 8)?

Chelation by EDTA greatly effects the reduction potential of Fe^{+++} (229). The NADPH oxidase activity of the bromelain-solubilized NADPH-cytochrome c reductase with no iron, 0.1 mM ADP-Fe^{+++}, or 0.1 mM EDTA-Fe^{+++} are 0.09, 0.5, and 25,0 μ equivalents of NADPH/min/mg enzyme, respectively (230). The oxidase activity of the enzyme in the presence of the combination of ADP-Fe^{+++} and EDTA-Fe^{+++} is not appreciably different from that observed with EDTA-Fe^{+++} alone (111). The effect of EDTA chelation of Fe^{+++}, therefore, appears to be to reduce the reduction

TABLE 8.

THE EFFECT OF EDTA-Fe^{+++} ON NADPH-DEPENDENT LIPID PEROXIDATION IN LIPOSOMES.

All the reaction mixtures contained 1.0 μmoles lipid phosphate/ml, 0.2 mM NADPH, 0.12 μg/ml NADPH-cytochrome c reductase in 0.05 M Tris-HCl, pH 7.4 at 37° C. ADP-Fe^{+++} and EDTA Fe^{+++} were added in the amounts indicated. The reaction time equaled 15 minutes.

Description		
Reaction mixture plus:		Malondialdehyde formed
mM ADP-Fe^{+++}	mM EDTA-Fe^{+++}	nmoles/min/μmoles lipid Pi
0.2	-	1.70
0.01	-	1.60
0.005	-	1.21
0.001	-	0.65
-	0.2	0.05
-	0.01	0.05
-	0.005	0.05
0.1	0.1	4.33
0.005	0.005	3.43
0.001	0.001	1.92

TABLE 9.

THE EFFECT OF ADP-Fe^{+++} and EDTA-Fe^{+++} ON ASCORBATE-CATALYZED LIPID PEROXIDATION IN LIPOSOMES.

The reaction mixtures contained 1.0 μmoles lipid Pi/ml and 0.1 mM ascorbate in 0.05 M Tris-HCl, pH 7.4 at 37° C. ADP-Fe^{+++} and EDTA-Fe^{+++} were added as indicated. The reaction time equaled 10 minutes.

Description	Malondialdehde formed nmoles/min/μmoles lipid Pi
Liposomes and ascorbate	
Plus 0.1 mM ADP-Fe^{+++}.....	1.94
Plus 0.1 mM ADP-Fe^{+++} and 0.1 mM EDTA-Fe^{+++}.....	2.14
Plus 0.1 mM EDTA-Fe^{+++}.....	0.03

potential of Fe^{+++}. The NADPH oxidase activity of o-phenanthroline-chelated iron catalyzed by NADPH-cytochrome c reductase has been shown to be about four times greater than EDTA-Fe^{+++} (111). When o-phenanthroline-chelated Fe^{+++} was tested for its ability to peroxidize liposomes in the presence of NADPH, it was able to support about 30% of the peroxidation catalyzed by ADP-Fe^{+++} alone (Table 10). Therefore, it would appear that the ability to promote lipid peroxidation is directly proportional to the reduction potential of Fe^{+++}. When used alone, the more difficult to reduce forms of chelated iron initiate the greatest amount of peroxidation. Combining two forms of Fe^{+++}, one with a higher (ADP-Fe^{+++}) and one with a lower (EDTA-Fe^{+++}) reduction potential appears to be the most effective way of promoting NADPH-dependent lipid peroxidation.

Since ADP-Fe^{+++} alone is capable of initiating lipid peroxidation in the NADPH-dependent liposomal system as judged by malondialdehyde production, then the enhanced peroxidation observed in the presence of EDTA-Fe^{+++} may be due to its ability to facilitate the breakdown of hydroperoxides to further propagate more lipid radicals. Earlier work in this section indicated that there were two phases in the lifetime of lipid hydroperoxides in membranes undergoing peroxidation. The early phase was characterized by a net build-up of lipid hydroperoxides while the latter phase showed a net breakdown of hydroperoxides. If the formation of hydroperoxides and their subsequent breakdown are catalyzed in two separate events, then it seems plausible that each of the two forms of chelated iron required in liposoaml peroxidation may play a predominant role in only one of the two reactions. EDTA-Fe^{+++} may facilitate the breakdown and propagation of lipid peroxides by a mechanism similar

TABLE 10.

EFFECT OF VARIOUS Fe^{+++} CHELATORS ON NADPH-DEPENDENT LIPID PEROXIDATION IN LIPOSOMES.

The reaction mixtures contained 1.0 μmole lipid Pi/ml, 0.02 mM NADPH, 0.35 μg/ml NADPH-cytochrome c reductase in 0.05 M Tris-HCl, pH 7.4 at 37° C. Chelated Fe^{+++} was added as indicated. The reaction time equaled 15 minutes.

Description			Malondialdehyde formed
Reaction mixture plus:			$\frac{\text{nmoles/min}}{\mu\text{mole lipid Pi}}$
ADP-Fe^{+++}	EDTA-Fe^{+++}	o-phenanthroline-Fe^{+++}	
0.1 mM	-	-	0.59
0.1 mM	0.1 mM	-	2.58
-	0.1 mM	-	0.03
-	0.1 mM	0.1 mM	0.33
-	-	0.1 mM	0.19

to that proposed for the autoxidation of lipids. Lipid autoxidation is known to be facilitated by Fe^{+++} and Fe^{++} as seen below (6-9):

$$ROOH + Fe^{+++} \longrightarrow ROO\cdot + Fe^{++} + H^{+}$$

$$ROOH + Fe^{++} \longrightarrow RO\cdot + OH^{-} + Fe^{+++}$$

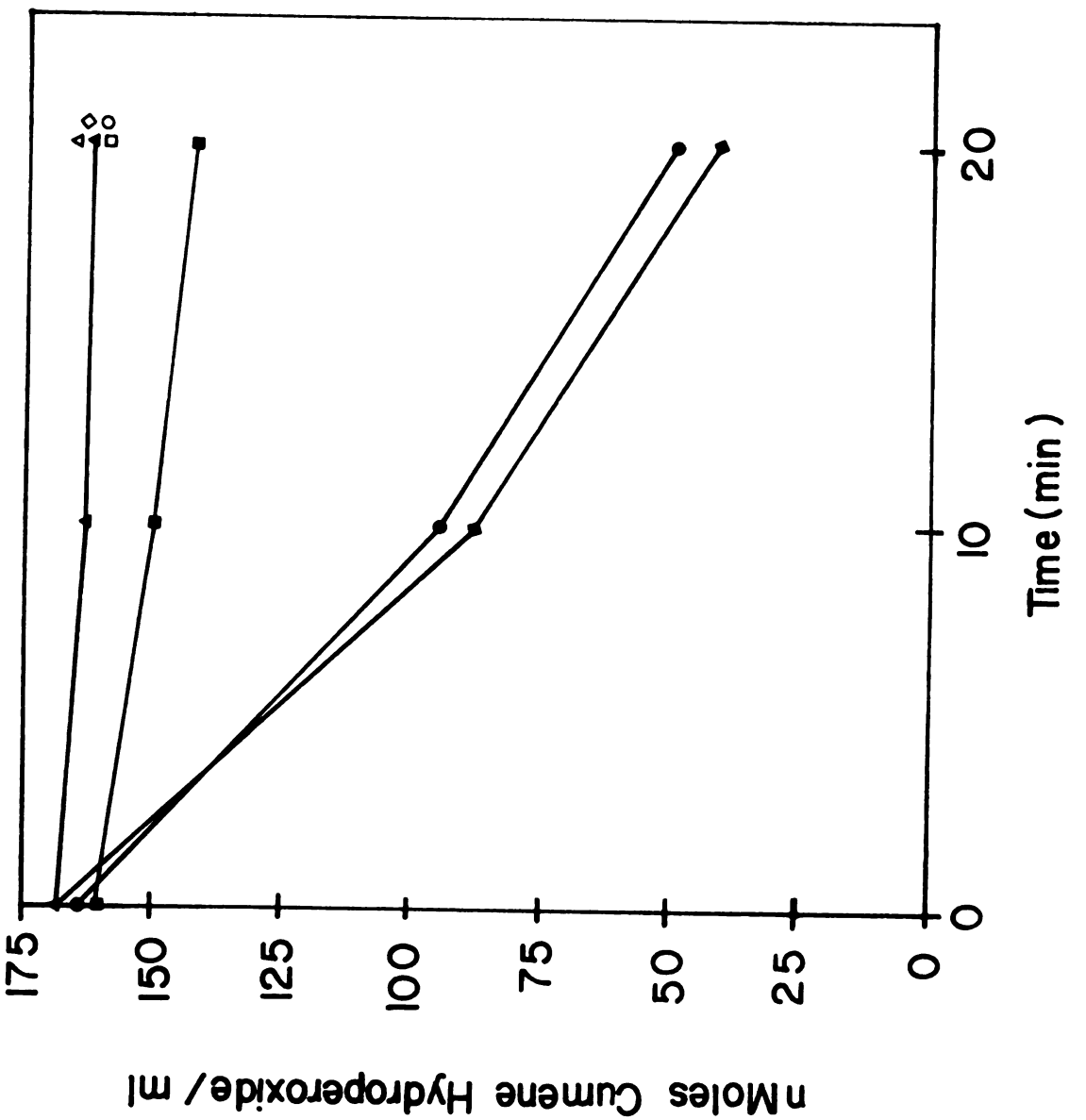
Cumene hydroperoxide was used to assess the ability of both ADP-Fe^{+++} and EDTA-Fe^{+++} to breakdown hydroperoxides. NADPH, NADPH-cytochrome c reductase, and cumene hydroperoxide were incubated in the presence of either ADP-Fe^{+++} or EDTA-Fe^{+++}. As seen in Figure 10, the ability to catalyze the breakdown of the functional group of cumene hydroperoxide is inversely proportional to the reduction potential of Fe^{+++}. EDTA-Fe^{+++} was found to be almost six times more effective than ADP-Fe^{+++} in the breakdown of cumene hydroperoxide. If the disappearance of the hydroperoxide function of cumene hydroperoxide proceeds as suggested above in the reaction of Fe^{++} with ROOH, then in membranes more lipid radicals would be formed, some leading to the formation of more lipid peroxides while others would lead to non-peroxide termination products (6-9):

$$(R\cdot, RO\cdot, ROO\cdot) + (R\cdot, RO\cdot, ROO\cdot) \longrightarrow \text{breakdown products}$$

Since EDTA-Fe^{+++} is not required in the NADPH-dependent peroxidation of microsomal membranes, it has been proposed that microsomes possess an electron transport component that is replaced by EDTA-Fe^{+++} in the model peroxidation system. This proposed component would only be reduced by NADPH-cytochrome c reductase. If NADH was used in place of NADPH, no peroxidation of microsomes was observed unless EDTA-Fe^{+++} was present in addition to ADP-Fe^{+++}. Pederson et al. (87) demonstrated

Figure 10. THE NADPH-DEPENDENT DECOMPOSITION OF CUMENE HYDROPEROXIDE IN THE PRESENCE OF CHELATED Fe^{+++}.

Experimental reaction mixtures (solid symbols) contained approximately 165 n moles cumene hydroperoxide/ml, 0.15 μg NADPH-cytochrome c reductase, and 0.2 mM NADPH in 0.05 m Tris-HCl, pH 7.4 at 37° C. Control reaction mixtures (open symbols) lacked NADPH. Chelated Fe^{+++} was present as follows: no Fe^{+++} (▲), 0.1 mM ADP-Fe^{+++} (■), 0.1mM EDTA-Fe^{+++} (●), 0.1 mM ADP-Fe^{+++} and 0.1 mM EDTA-Fe^{+++} (◆). Hydroperoxide levels were assayed by the iodometric assay as described under "Methods".


nMoles Cumene Hydroperoxide/ ml
175
150
125
100
75
50
25
0
0
10
20
Time (min)

that NADH-cytochrome b_5 reductase reduces ADP-Fe^{+++}, but not the proposed microsomal electron transport component mimiced by EDTA-Fe^{+++} in the liposomal system. Therefore, EDTA-Fe^{+++} is required in NADH-dependent peroxidation of microsomal membranes.

Table 11 demonstrates the results of washing microsomes repeatedly to remove or alter the proposed electron transport component that appears to be replaced by EDTA-Fe^{+++} in the liposomal system. Although substantial changes in the concentrations of either hydroperoxides or malondialdehyde are not observed, a trend is seen when the ratios of hydroperoxides/malondialdehyde are calculated. With each washing of the microsomes in 1.15% KCl, 1.0 mm EDTA, the ratios of hydroperoxides formed to malondialdehyde produced increases. This may indicate a shift away from the breakdown of lipid hydroperoxides. If a microsomal electron transport component similar in action to EDTA-Fe^{+++} were being removed or altered, such a shift would be reasonable.

The fact that EDTA-Fe^{+++} alone in the NADPH-dependent microsomal peroxidation system results in neither a build-up of hydroperoxides nor the appearance of the breakdown product, malondialdehyde, indicates that NADPH-dependent microsomal lipid peroxidation does not occur via the propagation of chain autoxidation from pre-existing lipid hydroperoxides. If this were true, EDTA-Fe^{+++} and NADPH would be sufficient to promote lipid peroxy and oxy radicals formation in microsomes thereby leading to both increased hydroperoxide formation and malondialdehyde production. The dependence on ADP-Fe^{+++} in both NADPH-dependent microsomal and liposomal peroxidation suggests that this species is involved in the initiation of hydroperoxide formation, probably through the formation of a perferryl iron. EDTA-Fe^{+++}, upon reduction, could both

TABLE 11.

EFFECT OF WASHING MICROSOMES ON THE FORMATION OF MALONDIALDEHYDE AND HYDROPEROXIDES IN NADPH-DEPENDENT LIPID PEROXIDATION.

Fresh rat liver microsomes were isolated by centrifuging the post-mitochondrial supernatant at 105,000 x g for 90 minutes. The pellet was resuspended in 1.15% KCl, 1.0 mM, EDTA and either used immediately (unwashes) or recentrifuged (washed). The reaction mixture contained 1.0 mg microsomal protein/ml, 0.1 mM NADPH, 0.1 mM ADP-Fe^{+++} in 0.05 M Tris-HCl, pH 7.4 at 37° C. The reaction time equaled 10 minutes. Malondialdehyde and hydroperoxides were measured as described under "Methods".

Description	Malondialdehyde formed $\frac{\text{nmoles/min}}{\text{mg protein}}$	Hydroperoxides formed $\frac{\text{nmoles/min}}{\text{mg protein}}$	$\frac{\text{Hydroperoxides}}{\text{Malondialdehyde}}$
Microsomes:			
Unwashed	2.7	11.2	4.1
Washed one time	2.6	13.6	5.2
Washed two times	2.4	13.6	5.7
Washed three times	2.1	15.2	7.2

propagate more lipid radicals from the hydroperoxides formed through the action of reduced ADP-Fe^{+++} and O_2, and could facilitate the breakdown of lipid peroxides to yield malondialdehyde in a manner analogous to the EDTA-Fe^{++} catalyzed breakdown of cumene hydroperoxide (Figure 10).

CHAPTER II

CHARACTERIZATION OF LACTOPEROXIDASE-CATALYZED LIPID PEROXIDATION

Lactoperoxidase-Catalyzed Lipid Peroxidation During Iodination of Membrane Protein:

While work was continuing on NADPH-dependent lipid peroxidation, new information became available suggesting the existence of another enzyme system capable of promoting malondialdehyde formation in microsomal membranes. The enzyme involved, lactoperoxidase, has been isolated from the salivary, lacrimal and Harderian glands as well as from bovine milk (231-233). By all parameters tested, the bovine enzyme from the lacrimal and salivary glands is identical to the enzyme isolated from milk. The enzyme is a hemoprotein and has a molecular weight of 78,000 daltons. In the presence of H_2O_2 and I^-, the enzyme catalyzes the incorporation of iodine atoms into tyrosine residues of proteins (234). Lactoperoxidase-catalyzed iodination of protein is a widely used probe for membrane surface macromolecules. This procedure has been used to investigate the arrangement of surface proteins of plasmamembranes (235), mitochondria (236), sarcoplasmic reticulum (237), chloroplasts (238), as well as the endoplasmic reticulum (239).

Previous investigations have indicated that mitochondrial membranes iodinated with this procedure undergo swelling and lysis after extensive iodine incorporation (240). Mitochondrial swelling also

results from exposure of the membrane to substances which modify sulfhydryl groups (241), fatty acids (242), phospholipase (243), lysolecthin (244), staphylococcal aphlatoxin (245), iodine-containing compounds such as thyroxine (246), and from agents that promote lipid peroxidation (247). Therefore, it was of great interest when a preliminary report was published suggesting that lipid peroxidation of microsomal membrane lipids occurred during the enzymatic iodination of microsomal surface proteins using lactoperoxidase, H_2O_2 and ^{215}I (248). Lipid peroxidation promoted during the enzymatic iodination of the microsomal surface proteins paralleled the loss of the CO-reduced difference spectra of the microsomal election transport protein, cytochrome P-450. The inactivation of enzymes and the destruction of cytochromes has previously been correlated with the occurrence of membrane lipid peroxidation (249). During iodination of the microsomal membrane using the lactoperoxidase system, both lipid peroxidation and the loss of cytochrome P-450 could be prevented by the inclusion of BHT in the reaction mixture (248). It was interesting to note that when BHT was present to inhibit lipid peroxidation, the amount of ^{125}I labeling of the surface proteins was doubled. Similar results were subsequently obtained in the ^{125}I labeling of myelin surface proteins (250). This might suggest a possible competition between lactoperoxidase-catalyzed iodination of protein and peroxidation of membrane lipids.

The peroxidation observed during lactoperoxidase-catalyzed iodination of membrane proteins raises serious questions regarding the interpretation of experimental results obtained through the use of this method. Since only surface proteins are thought to be labeled with ^{125}I during lactoperoxidase-catalyzed iodination, disruption of the membrane

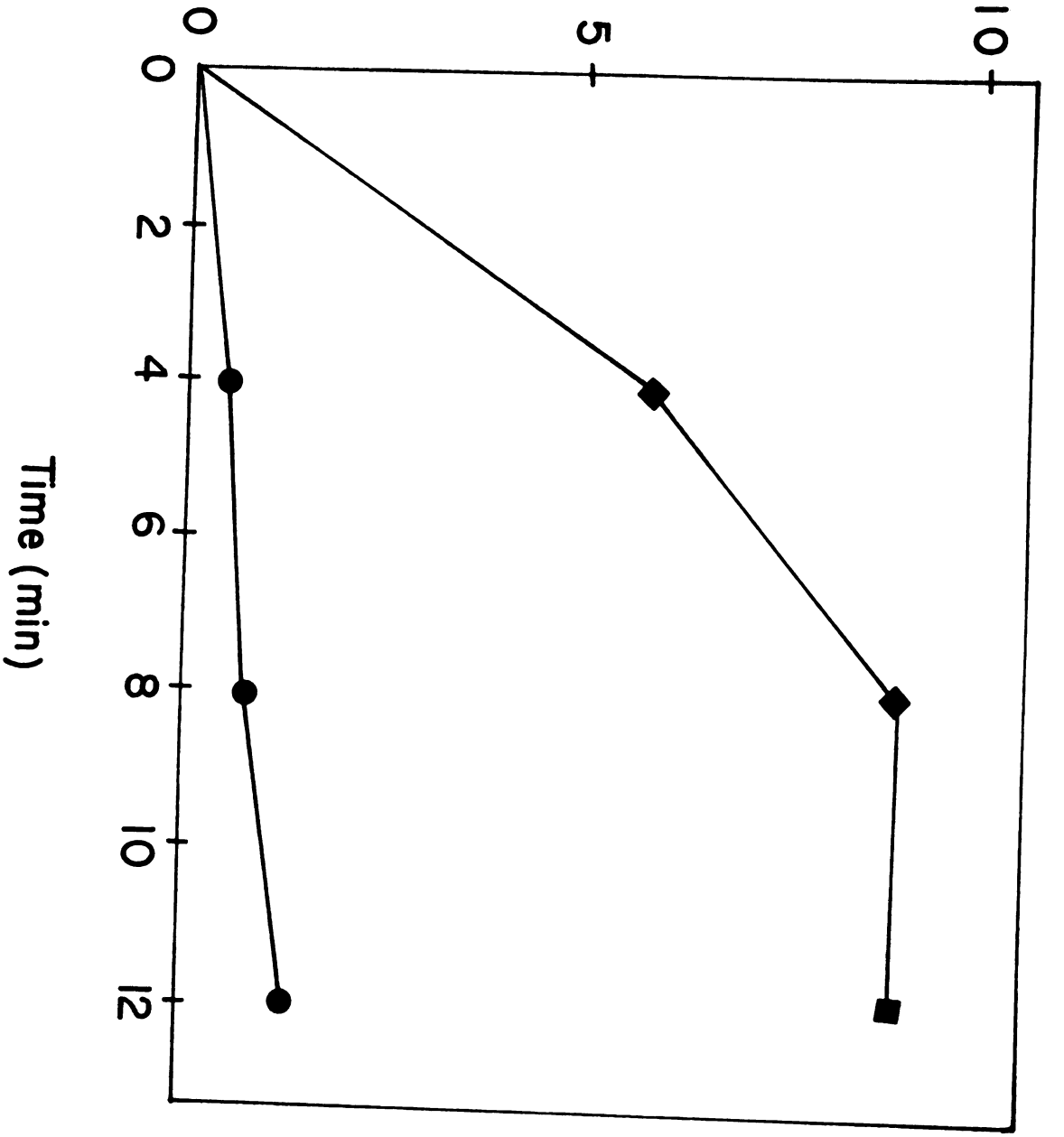
integrity through peroxide formation and distruction of the lipid moieties could result in artifactual labeling of sub-surface proteins. Tsai et al. (251), reported that proteins located at various depths of red blood cell membranes could be labeled with the lactoperoxidase system, depending on the conditions used during iodination. At increased cell concentrations or with a greater degree of enzymatic iodination, additional membrane components were labeled. It is not unreasonable to suggest that alterations of the red blood cell membrane through the formation of lipid peroxides may have been involved in the observed additional labeling of proteins. It was therefore of interest to investigate the reaction of lactoperoxidase, H_2O_2 and I^- with membrane lipids in order to establish that lipid peroxidation was indeed a consequence of the reaction. The lactoperoxidase system could then be used to further investigate membrane lipid peroxidation with particular attention to comparing it with peroxidation dependent on NADPH.

Optimum Condition for Lactoperoxidase-Catalyzed Peroxidation of Microsomal Membranes:

Lactoperoxidase, H_2O_2 and I^--catalyzed peroxidation of microsomal membranes in the presence of ADP-Fe^{+++} is illustrated in Figure 11. The reaction is enzyme mediated since peroxidation drops to low levels in the absence of lactoperoxidase. Originally, additions of H_2O_2 were made at one minute intervals over the first three minutes of the incubation period in order to duplicate the reaction conditions used previously for the enzyme mediated iodination of microsomal surface proteins (240, 248). Subsequently it could be demonstrated that multiple additions of H_2O_2 were advantageous when using microsomal membranes because of contamination of the microsomes with catalase, which competes for the exogenous

Figure 11. LACTOPEROXIDASE-CATALYZED LIPID PEROXIDATION IN MICROSOMES.

The reaction mixtures contained microsomes KI, ADP-Fe^{+++} and H_2O_2 as described under "Methods". The reaction mixtures either contained 2 μg/ml lactoperoxidase (◆——◆) or no lactoperoxidase (●——●).


n Moles Malondialdehyde/mg Protein
0
5
10
Time (min)
0
2
4
6
8
10
12

H_2O_2. It can also be shown that high concentration of H_2O_2 inhibit the peroxidation reaction. However, this problem could be circumvented by repeated addition of lower concentrations of H_2O_2 to the incubation mixture. When eight additions of H_2O_2 at one minute intervals were made instead of four additions, malondialdehyde production increased by 70%. No change was observed in controls (minus enzyme) receiving additional H_2O_2.

The use of H_2O_2 generating system is also effective in minimizing the early depletion of H_2O_2 by catalase. Table 12 demonstrates the effect of substituting a H_2O_2 generating system for exogenous H_2O_2. The aerobic oxidation of glucose by glucose oxidase results in the formation of H_2O_2 (252). As the concentration of glucose oxidase above 5 μg/ml is increased, lipid peroxidation also increases over the 15 minute time period assayed.

Optimum conditions and requirements for lactoperoxidase-catalyzed peroxidation of microsomal membrane lipids were determined assaying for malondialdehyde production as described under "Methods." Figure 12 illustrates the correlation between lactoperoxidase concentration and the amount of malondialdehyde formed over a five minute period. At lower enzyme concentrations, malondialdehyde production increased relatively linearily with lactoperoxidase.

Figure 13 and Figure 14 illustrate the effects of varying I^- and H_2O_2 concentrations, respectively, on peroxidation of microsomal lipids. Both of these substrates for the enzyme show unusual kinetic curves in that inhibition of peroxidation activity occurs at high substrate concentrations. When taken together, Figures 12-14 demonstrate that all

TABLE 12.

THE EFFECT OF H_2O_2 GENERATION ON LACTOPEROXIDASE-CATALYZED LIPID PEROXIDATION IN MICROSOMES

The reaction mixtures contained KI, lactoperoxidase, microsomes, and ADP-Fe^{+++} as described under "Methods", plus 1.0 mM glucose. Glucose oxidase was added in the amounts indicated. The reaction time equaled 10 minutes.

Description	Malondialdehyde formed nmoles/min/mg protein
No glucose oxidase	0.12
5 µg/ml glucose oxidase	0.12
25 µg/ml glucose oxidase	0.43
50 µg/ml glucose oxidase	0.85
100 µg/ml glucose oxidase	1.01

Figure 12. LACTOPEROXIDASE-CATALYZED LIPID PEROXIDATION OF MICROSOMAL LIPID AS A FUNCTION OF ENZYME CONCENTRATION.

The reaction mixtures contained microsomes, KI, ADP-Fe^{+++} and H_2O_2 as described under "Methods". Lactoperoxidase was added in the indicated amounts. The reaction time equaled 5 minutes.

nMoles Malondialdehyde/min/mg Protein
1.5
1.0
0.5
1
2
3
Lactoperoxidase (ug/ml)

Figure 13. LACTOPEROXIDASE-CATALYZED LIPID PEROXIDATION OF MICROSOMAL LIPIDS AS A FUNCTION OF KI CONCENTRATION.

The reaction mixtures contained microsomes, H_2O_2, ADP-Fe^{+++} and 2 µg/ml lactoperoxidase as described under "Methods". KI was added in the indicated amounts. The reaction time equaled 5 minutes.

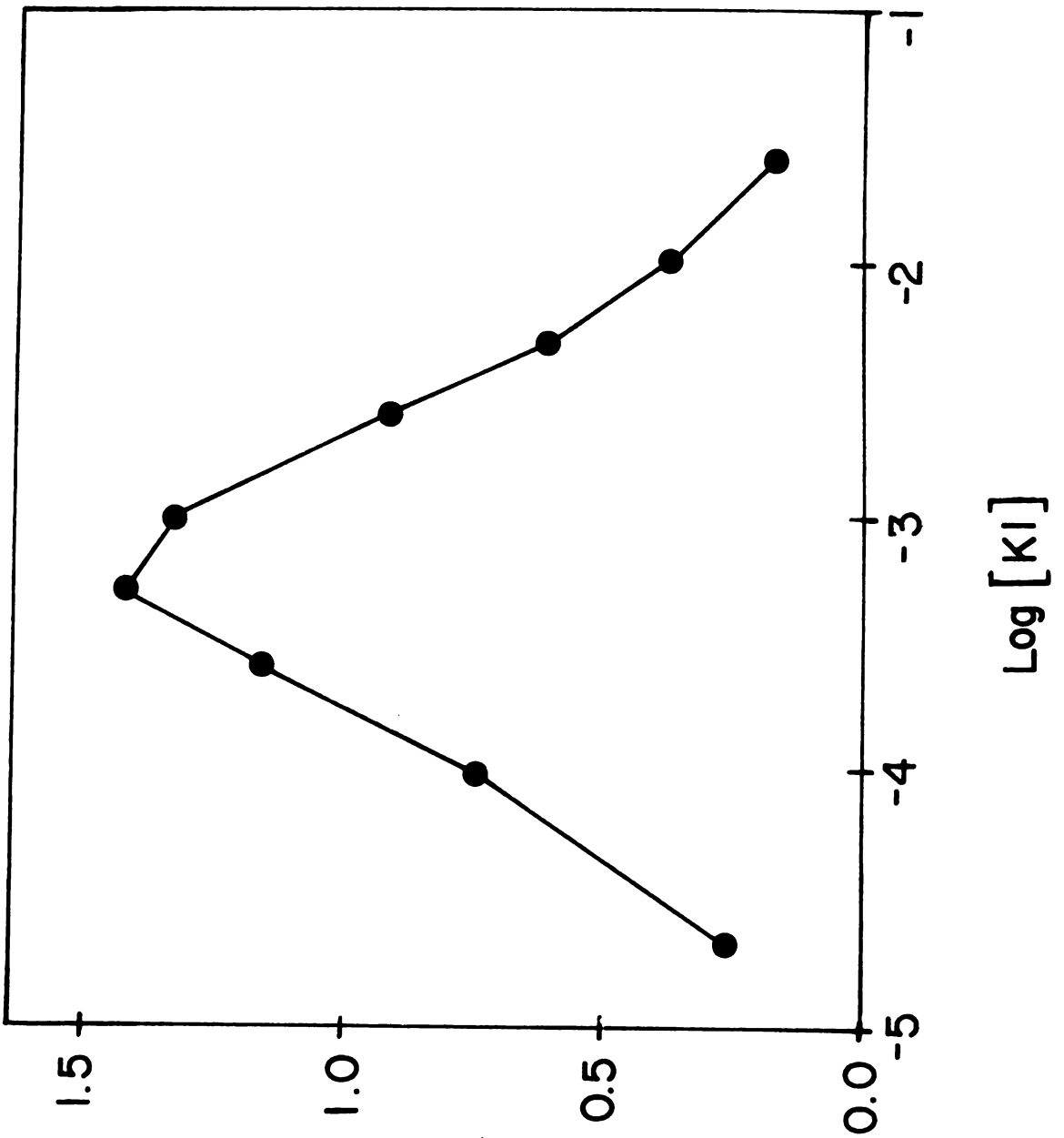
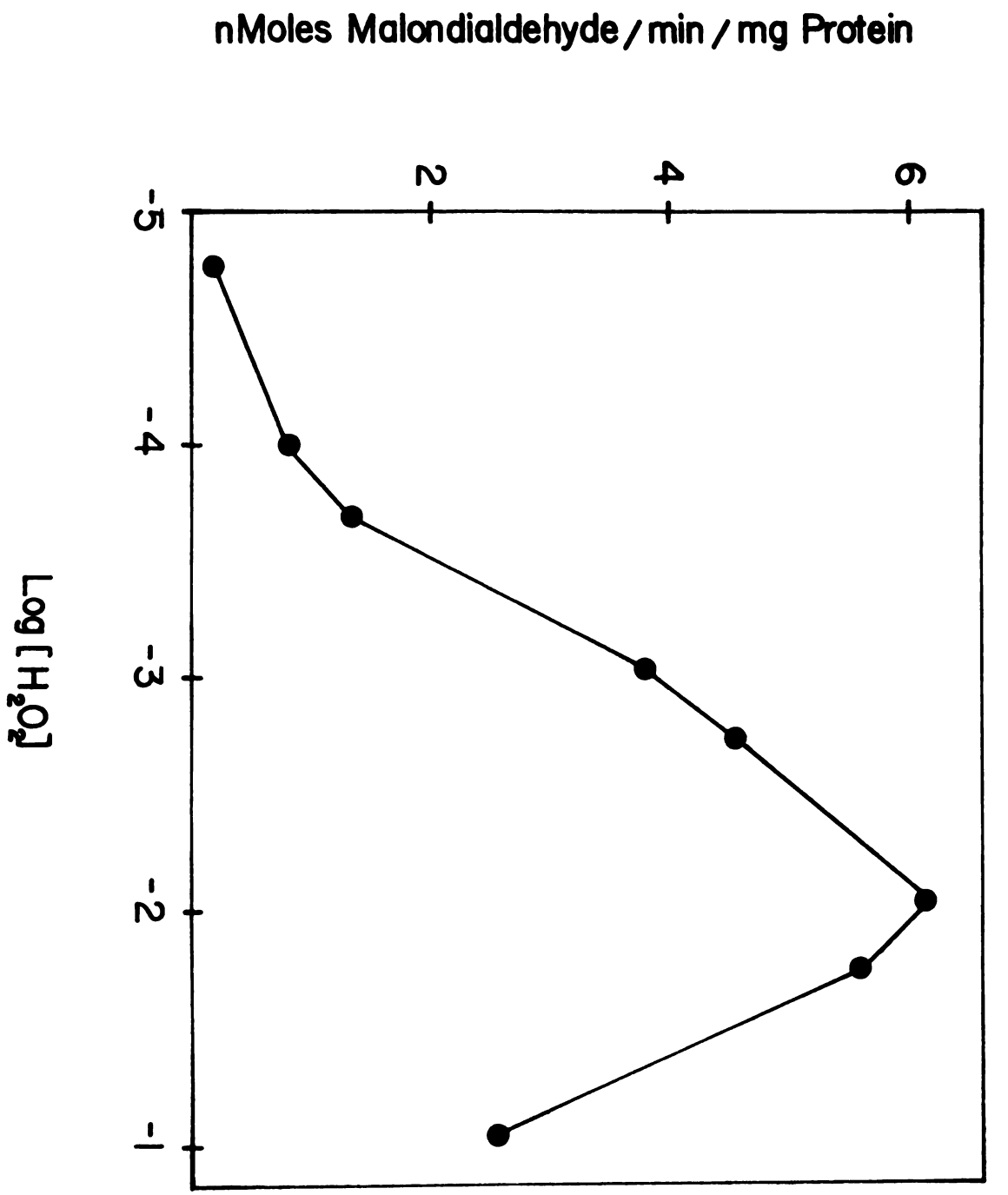

nMoles Malondialdehyde/min/mg Protein
1.5
1.0
0.5
0.0
-5
-4
-3
-2
-1
Log [KI]

Figure 14. LACTOPEROXIDASE-CATALYZED LIPID PEROXIDATION IN MICROSOMES AS A FUNCTION OF ADDED H_2O_2.

The reaction mixtures contained microsomes KI, ADP-Fe^{+++} and 2 µg/ml lactoperoxidase as described under "Methods". The concentration of H_2O_2 plotted in the graph refers to the concentration of the reaction mixture upon addition of the first of 4 equal aliquots of H_2O_2. The reaction time equaled 5 minutes.


nMoles Malondialdehyde / min / mg Protein
2
4
6
-5
-4
-3
-2
-1
Log [H2O2]

three components of the lactoperoxidase peroxidizing system are required for activity.

The unusual kinetics demonstrated at higher concentrations of both I^- and H_2O_2 may be evidence of inhibition of lactoperoxidase activity via denaturation of the enzyme. Most peroxidases are sensitive to high concentrations of H_2O_2. Morrell (253) demonstrated that lactoperoxidase has an optimum activity in the oxidation of pyrogallol at about 6.0 mM H_2O_2. Above 6.0 mM, inhibition of this reaction occurred. This value corresponds well with the present findings of an optimum H_2O_2 concentration of 8.8 mM in the microsomal lipid peroxidizing system. Previous investigations of lactoperoxidase-catalyzed iodination of L-tyrosine demonstrated that an optimum iodinating activity occurred at an I^- concentration of about 0.4 mM, using 0.1 mM H_2O_2 at pH 7.4 (254). This agrees well with an optimum of 0.5 mM I^- using 0.176 mM H_2O_2, found in this investigation. Inactivation of lactoperoxidase at high I^- concentrations would be understandable if highly reactive iodide oxidation intermediates capable of iodination proteins were being generated and destroying the enzyme.

Chloroperoxidase is similar in physical properties to the other peroxidases, including lactoperoxidase (255). In the presence of H_2O_2, chloroperoxidase is capable of catalyzing halogenation reactions using Cl^-, Br^- or I^- as the halogen anion (256). Since the lactoperoxidase-catalyzed lipid peroxidation reactions were performed in 0.05 M Tris buffer containing Cl^- anion, it was necessary to determine if Cl^- anion was involved in the peroxidation reaction. Triplicate peroxidation experiments using lactoperoxidase were performed in potassium phosphate, Tris-maleate, and Tris-HCl buffers. No change in the amount of malondialdehyde formed was observed in buffers free of Cl^- anion.

Lactoperoxidase-Catalyzed Lipid Peroxidation in the Model System:

Since the optimum substrate concentrations for lactoperoxidase-catalyzed lipid peroxidation are very similar to the optimum condition required for enzyme-catalyzed iodination of protein, two general reaction mechanisms for lactoperoxidase-catalyzed lipid peroxidation are suggested. First, the iodinated proteins or a product of protein iodination may be interacting with membrane lipids or components of the membrane which subsequently promote lipid peroxide formation in microsomes. Nakano et al. (257) has demonstrated that a relationship exists between iodinated protein (thyroxine) and microsomal lipid peroxidation. The study employed Fe^{++} or ascorbate to initiate lipid peroxidation in microsomes. When thyroxine was added to the peroxidizing microsomes, deiodination of thyroxine occurred. Although this study suggests that lipid peroxidation causes deiodination rather than the converse, it does suggest that a relationship exists in microsomes between lipid peroxidation and iodinated protein.

The second possible mechanism involved in lactoperoxidase-catalyzed lipid peroxidation involves the enzymatic generation of a peroxide forming intermediate from the oxidation of I^-. The overall reaction of lactoperoxidase with H_2O_2 and I^- can be described as follows (258):

$$2H^+ + H_2O_2 + 2I^- \xrightarrow{\text{peroxidase}} 2H_2O + I_2$$

The exact identity of the intermediates formed from the oxidation of I^- have not been clearly established. Both the iodide radical, resulting from a one electron oxidation (259, 260) and the iodonium cation, resulting from a two electron oxidation (261) have been implicated as initial, transitory reaction products of iodide oxidation. Either of

these reactive species could be involved in the initiation of membrane lipid peroxidation.

Since the first proposed reaction mechanism requires either the iodination of protein or the presence of factors in microsomes, while the second possible mechanism would be independent of microsomal protein, lactoperoxidase, H_2O_2, and I^- were incubated with liposomes derived from purified microsomal lipid in the presence of ADP Fe^{+++}. Figure 15 represents the results of this experiment. The resulting peroxidation indicates that proteins or other non-lipid material from intact microsomal membranes are not required for lactoperoxidase-catalyzed lipid peroxidation to occur. Therefore, it is more likely that a reactive intermediate produced through the oxidation of I^- is involved in lactoperoxidase-catalyzed lipid peroxidation. This possibility will be considered later.

The pH optimum of the reaction was determined using liposomes as the lipid source. As demonstrated in Figure 16, the pH optimum occurs around pH 7.4 and decreases rapidly at lower pH. Morrison et al. (240) demonstrated that glutamic acid dehydrogenase, a soluble enzyme located in the inner matrix space of mitochondria, was released from within the mitochondria by 72% during the enzymatic iodination of the membrane at pH 7.4. At pH 6.5, only 2% of the enzyme was released. The increased ability of the lactoperoxidase system to form lipid peroxides in the mitochondrial membrane at pH 7.4 would easily account for this result.

The use of liposomes in the lactoperoxidase system also affords an opportunity to access the amount of competition for H_2O_2 caused by the catalase contaminant present in microsomes but absent in the liposomal system. It was previously demonstrated that multiple additions

Figure 15. LACTOPEROXIDASE-CATALYZED LIPID PEROXIDATION IN LIPOSOMES.

The reaction mixture contained liposomes (0.5 μmole lipid Pi/ml), lactoperoxidase, ADP-Fe^{+++}, and KI as described under "Methods". The reaction mixtures contained H_2O_2 (● —— ●) or lacked H_2O_2 (◆ —— ◆).

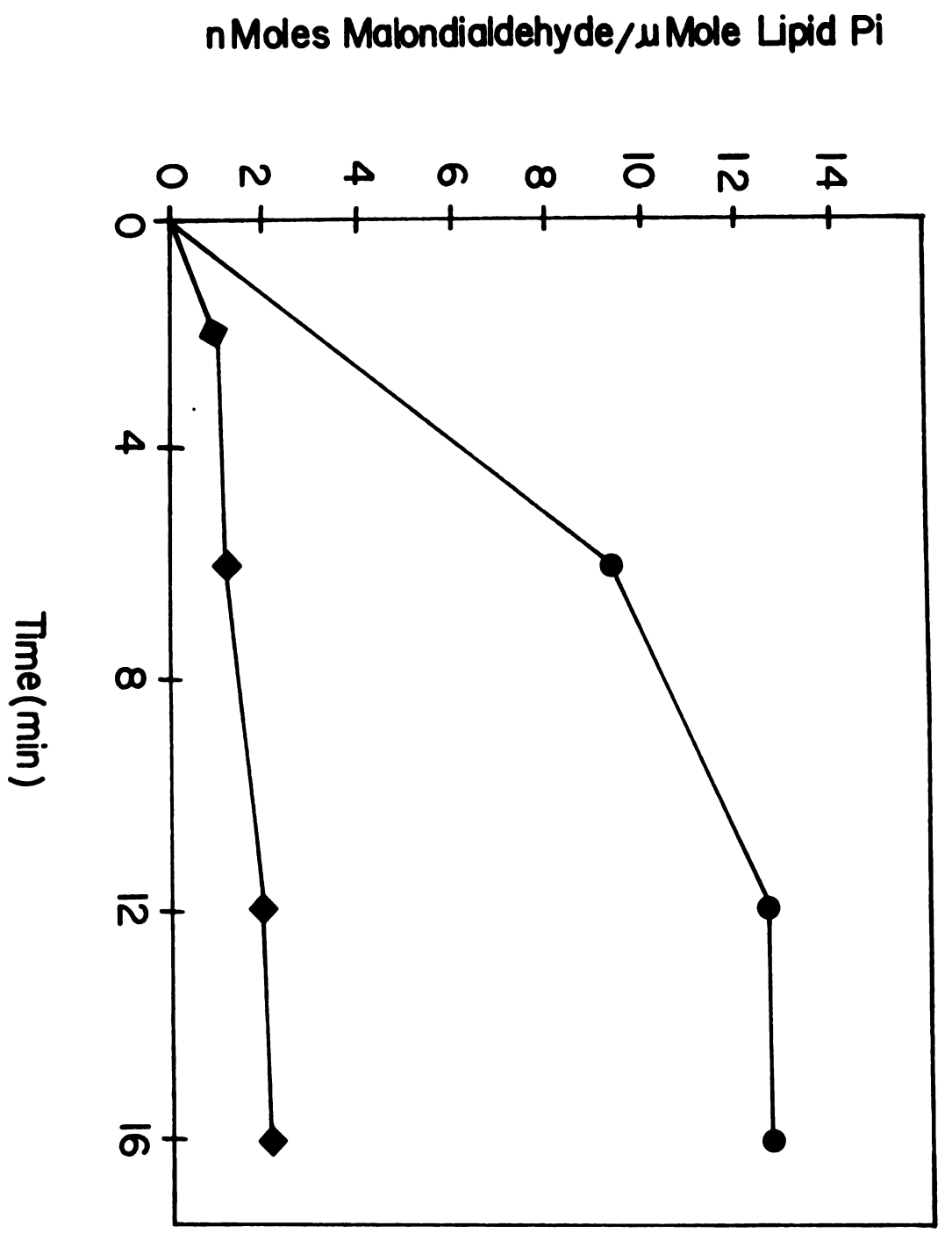

n Moles Malondialdehyde/μ Mole Lipid Pi
0
2
4
6
8
10
12
14
Time(min)
0
4
8
12
16

Figure 16. LACTOPEROXIDASE-CATALYZED LIPID PEROXIDATION OF LIPOSOMES AS A FUNCTION OF pH.

The reaction mixtures contained 0.5 μmoles lipid Pi/ml, KI, H_2O_2, ADP-Fe^{+++} and lactoperoxidase as described under "Methods". The pH was established with 0.05 M Tris-HCl, at 37° C. The reaction time equaled 5 minutes.

nMoles Malondialdehyde/ min /μMole Lipid Pi
1.5
1.0
0.5
0.0
6.0
7.0
8.0
pH

of H_2O_2, bringing the reaction mixture to 8.8 mM (assuming complete consumption of the previous aliquot) produced the greatest amount of lipid peroxidation in the lactoperoxidase-catalyzed microsomal system. If microsomal catalase was reducing the effective concentration of H_2O_2, then liposomes free of catalase should require less H_2O_2. Figure 17 demonstrates that lower concentration of H_2O_2 are required for maximum peroxidation in the liposomal system. In liposomes, a single addition of H_2O_2 bringing the reaction mixture to 0.176 mM produces the greatest amount of enzymatic lipid peroxidation.

The Effect of Fe^{+++} on Lactoperoxidase-Catalyzed Lipid Peroxidation

During the course of the study of lactoperoxidase-catalyzed lipid peroxidation in liposomes, it became evident that H_2O_2 might be contributing to the net production of lipid peroxides via a non-enzymatic reaction with Fe^{+++}. Both Fe^{+++} and Fe^{++} may react with H_2O_2 to generate radicals (98-100):

$$Fe^{+++} + H_2O_2 \longrightarrow Fe^{++} + HOO\cdot + H^+$$

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

Since the reaction mixtures contain both ADP-Fe^{+++} and H_2O_2, it is possible that a radical-generating reaction of this type may be occurring. When liposomes were incubated with ADP-Fe^{+++}, KI^-, and H_2O_2 (no lactoperoxidase) a significant amount of malondialdehyde was produced (Table 13). It was evident that the non-enzymatic production of lipid peroxidation was dependent on the presence of H_2O_2, since the complete reaction mixture minus H_2O_2 produced little malondialdehyde. When the time course of the non-enzymatic reaction was accessed, it was evident that preincubation of lipid, H_2O_2, and Fe^{+++} was not necessary. It would

Figure 17. LACTOPEROXIDASE-CATALYZED LIPID PEROXIDATION IN LIPOSOMES AS A FUNCTION OF H_2O_2 CONCENTRATION.

The reaction mixtures contained 0.5 μmole lipid Pi/ml, KI, ADP-Fe^{+++} and lactoperoxidase as described under "Methods". The reaction mixtures received a single addition of H_2O_2. The reaction time equaled 10 minutes.

nMoles Malondialdehyde/min/μMole Lipid Pi
1.75
1.0
0.0
-4
-3
-2
-1
Log [H2O2]

TABLE 13.

THE EFFECT OF ADP-Fe^{+++} AND H_2O_2 ON LIPOSOMES.

The complete reaction mixture contained liposomes (0.5 μmoles lipid Pi/ml), lactoperoxidase, KI, ADP-Fe^{+++}, and H_2O_2 as described under "Methods". The reaction time equaled 10 minutes.

Description	Malondialdehyde formed nmoles/min/μmoles lipid Pi
Complete.....	1.04
Minus lactoperoxidase.....	0.53
Minus H_2O_2.....	0.19

therefore appear that the production of lipid peroxides and subsequently malondialdehyde was occurring during the heating of the lipid with the trichloroacetic acid-thiobarbituric acid (TCA-TBA) reagent used to develop the chromophore produced by malondialdehyde. Since this non-enzymatic production of lipid peroxidation had not been observed to this extent in microsomes, the possibility that the catalase contaminant present in microsomes was reducing the concentration of H_2O_2 to a level too low to effectively promote non-enzymatic lipid peroxidation during the heating step was tested. Table 14 demonstrates the effect of adding thymol-free catalase to both microsomal and liposomal membrane samples that have been incubated with lactoperoxidase, H_2O_2, and I^-. 50 µg of catalase was mixed with 1 ml aliquots from the reaction mixtures for 30 seconds prior to inactivation of the reactions with TCA-TBA reagent. It can be seen that in microsomes, addition of catalase at the end of the incubation period had little effect on either the complete reaction mixture or on the sample missing lactoperoxidase. It would appear that the catalase contaminant of microsomes was reducing the H_2O_2 concentrations to levels too low to promote non-enzymatic peroxidation. In liposomes, addition of catalase to the sample missing lactoperoxidase greatly reduced the amount of apparent lipid peroxidation. The complete liposome reaction mixture containing lactoperoxidase was not significantly effected by the addition of catalase. Therefore, lactoperoxidase and I^- in the complete liposomal reaction mixture must be reducing the H_2O_2 concentration during the course of the reaction to a level too low to contribute non-enzymatically to the overall production of malondialdehyde. This fact was confirmed by incubating lactoperoxidase, H_2O_2, and I^- in the absence of liposomes and assaying the

TABLE 14.

EFFECT OF CATALASE ON NON-ENZYMATIC LIPID PEROXIDATION IN MICROSOMES AND LIPOSOMES.

The complete reactions contain either 1.0 mg microsomal protein/ml or 1 μmole lipid Pi/ml as indicated. Both microsomal and liposomal systems contained 1.0 mM KI, 0.1 mM ADP-Fe^{+++} and 4 additions of 0.176 mM H_2O_2. 2 μg/ml lactoperoxidase was present in the complete reaction mixtures. When indicated, 1 ml aliquots of the reaction mixture were incubated with 50 μg thymol-free catalase for 30 seconds before adding TCA-TBA reagent. The reaction time equaled 10 minutes.

Description	Malondialdehyde formed nmoles/min/ml	
	no catalase	50 μg/ml catalase
Complete microsomal system. . .	1.30	1.18
Minus lactoperoxidase . .	0.13	0.00
Complete liposomal system . . .	1.41	1.31
minus lactoperoxidase . .	0.72	0.02

disappearance of H_2O_2 by the iodometric assay. In a five minute incubation period, the H_2O_2 (0.176 mM) concentration fell below the detectable limit of the assay (5 μmolar). Only when liposomes were incubated with H_2O_2 in the absence of lactoperoxidase did H_2O_2 levels remain high enough to catalyze non-enzymatic lipid peroxidation. Peroxidation induced in liposomes in the absence of lactoperoxidase is therefore an artifact not related to the enzymatic reaction.

The appearance of the non-enzymatically induced lipid peroxidation in the liposomal samples proved to be of significant important in characterizing the peroxidation reaction catalyzed by lactoperoxidase. Since H_2O_2 and ADP-Fe^{+++} were shown to catalyze lipid peroxidation during the heating of lipid with TCA-TBA reagent, it was possible that ADP-Fe^{+++} was catalyzing the same reaction in the heating step of the malondialdehyde assay with lipid peroxides forming through the action of lactoperoxidase, H_2O_2, and I^- on membrane lipids.

In lipid peroxidation catalyzed by NADPH-cytochrome c reductase, peroxidation depends upon the enzymatic reduction of ADP-Fe^{+++}. In the absence of ADP-Fe^{+++} in the liposomal system, no NADPH-dependent peroxidation is observed. Therefore, it was not surprising that lactoperoxidase-catalyzed lipid peroxidation appeared to require ADP-Fe^{+++}. However, the discovery of artifactual, ADP-Fe^{+++} mediated lipid peroxidation in the liposomal control (non-enzymatic) samples in the lactoperoxidase system led to a reevaluation of the ADP-Fe^{+++} requirement. Since the reduction of ADP-Fe^{+++} by lactoperoxidase would not be a normal reaction for this enzyme, ADP-Fe^{+++} might be functioning only during the heating of the lactoperoxidase-treated membrane sample, to form radicals by a mechanism analogous to the reaction of Fe^{+++} with

H_2O_2. Table 15 verifies this conclusion. In liposomes, addition of ADP-Fe^{+++} (or $FeCl_3$), either during or after the incubation of the lipid with lactoperoxidase, H_2O_2 and I^-, had the same net result on the formation of malondialdehyde. The fact that ADP-Fe^{+++} assists in the production of malondialdehyde becomes evident when EDTA is present throughout the reaction. In liposomes, EDTA decreases malondialdehyde production by 73%, while its effect in microsomes is somewhat less. This is probably true owing to the presence of Fe^{+++} in microsomes not accessible to EDTA or already complex with membrane components. When EDTA is present during the incubation of lipid with the lactoperoxidase system, followed by the addition of excess Fe^{+++} at the end of the incubation, malondialdehyde levels are returned to high levels. This data clearly indicates that Fe^{+++} is not involved in the initiation of lactoperoxidase-catalyzed lipid peroxidation, but does assist in the production of malondialdehyde during the heating step, probably by reacting with a peroxide or peroxide-like product formed by the lactoperoxidase system during the incubation phase to propagate more lipid peroxidation.

The Effect of BHT on Lactoperoxidase-Catalyzed Lipid Peroxidation:

BHT is a potent inhibitor of lipid peroxidation owing to its ability to trap free radicals (6-9). In the present study, it was used to determine if the production of malondialdehyde during the heating of lactoperoxidase treated membranes with TCA-TBA reagent was radical mediated. The breakdown of lipid hydroperoxides to yield malondialdehyde is likely to be mediated by radical intermediates and accelerated by Fe^{+++}:

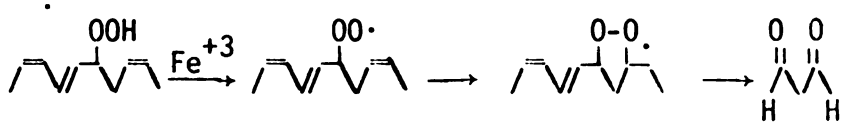


TABLE 15.

THE EFFECT OF ADDING ADP-Fe^{+++} DURING OR AFTER INCUBATION OF MEMBRANES WITH THE LACTOPEROXIDASE LIPID PEROXIDATION SYSTEM.

The reaction mixtures contained microsomes (1.0 mg protein/ml) or liposomes (1.0 μmole lipid Pi/ml), KI, H_2O_2 and lactoperoxidase as described in the legend for Table 14. ADP-Fe^{+++} or EDTA, in the amounts indicated, were added to the reaction mixture either before initiation of the reaction (during) or immediately prior to termination of the reaction by addition of TCA-TBA reagent (after). The reaction time equaled 10 minutes.

Description	Malondialdehyde formed	% Control
	nmoles/min/ml	
Microsomes;		
Plus 0.1 mM ADP-Fe^{+++} during. . . .	1.34	100
Plus 0.1 mM ADP-Fe^{+++} after	1.27	95
Plus 0.1 mM EDTA during	0.72	53
Plus 0.1 mM EDTA during and 0.2 mM ADP-Fe^{+++} after.	1.26	94
Liposomes;		
Plus 0.1 mM ADP-Fe^{+++} during. . . .	1.19	100
Plus 0.1 mM ADP-Fe^{+++} after	1.18	99
Plus 0.1 mM EDTA during	0.33	27
Plus 0.1 mM EDTA during and 0.2 mM ADP-Fe^{+++} after.	1.30	109

Therefore, BHT would be likely to trap the radical intermediate formed during the conversion of lipid hydroperoxides to malondialdehyde.

BHT was added to the TCA-TBA reagent used to terminate the lactoperoxidase-catalyzed peroxidation reaction. Table 16 demonstrates that when BHT is mixed with the membrane samples at the end of the incubation period, malondialdehyde production in liposomes is completely abolished and is significantly decreased in microsomes. It is apparent that in liposomes, all of the malondialdehyde production is occurring during the heating of the membrane sample with the TCA-TBA reagent. Since malondialdehyde production in liposomes has been shown to depend on the incubation of the membrane with the complete lactoperoxidase system, it appears that a species capable of promoting lipid peroxidation when exposed to heat is being produced during the incubation of the membrane with the complete enzyme system. ADP-Fe^{+++} probably accelerates the formation of malondialdehyde from this species during the heating step. In microsomes, however, about 60% of the total malondialdehyde produced from the reaction of microsomal membrane with the lactoperoxidase system must be produced prior to the heating of the membrane with TCA-TBA reagent, since BHT added at the end of the reaction does not block the formation of this amount of malondialdehyde. The apparent difference between liposomes and microsomes in their ability to produce malondialdehyde during the incubation period with the lactoperoxidase system may be related to either iron or some other peroxide reacting factor present in microsomes but absent in liposomes. The endogenous iron previously demonstrated to be present in microsomes may react with the peroxide forming species produced by the lactoperoxidase system during the incubation period to begin the production of malondialdehyde prior to the

TABLE 16.

THE EFFECT OF BHT ON MEMBRANE LIPID PEROXIDATION CATALYZED BY LACTOPEROXIDASE WHEN ADDED AFTER THE REACTION PERIOD.

The reaction mixture contained microsomes (or liposomes) KI, H_2O_2 and lactoperoxidase as described in the legend for Table 14. ADP-Fe^{+++} were present in the reaction mixtures as indicated. The TCA-TBA reagent either contained no BHT or 0.005% BHT as indicated. The reaction time equaled 10 minutes.

Description	Malondialdehyde formed nmoles/min/ml	
	no BHT	0.005% BHT
Microsomes;		
Plus ADP-Fe^{+++}	1.07	0.64
Liposomes;		
Plus ADP-Fe^{+++}	2.14	0.00
Plus EDTA-Fe^{+++}	1.98	0.00

heating stage. This microsomal factor is not duplicated in liposomes by either ADP-Fe^{+++} or EDTA-Fe^{+++} (Table 16).

It was of interest to note that lipid hydroperoxide formation (iodometric assay) catalyzed by the lactoperoxidase system paralleled the apparent production of malondialdehyde during the incubation period but prior to heating with TCA-TBA (Table 16). When no malondialdehyde was produced in liposomes prior to heating, no hydroperoxides were detected. In microsomes, where 60% of the total malondialdehyde was produced before heating, lipid hydroperoxides were detected in the incubation mixture. Therefore, the lactoperoxidase system may be forming a "pre-peroxide" species that subsequently reacts with Fe^{+++} during heating (or a microsomal component before heating) to produce lipid hydroperoxides and ultimately, malondialdehyde. Alternately, the lactoperoxidase system may be forming levels of lipid hydroperoxides below the detectible limit of the iodometric assay, that react with Fe^{+++} during heating (or a microsomal component before heating) to promote more lipid peroxides and malondialdehyde via a rapid, radical mediated chain autoxidation. These two possibilities will be considered further in the Discussion.

Reaction Mechanisms Capable of Promoting Lactoperoxidase-Catalyzed Lipid Peroxidation:

Although it has been demonstrated that malondialdehyde production in the lactoperoxidase system occurs entirely during the heating stage in liposomes, it is apparent that initiation of lipid peroxidation occurs during the incubation of membrane with lactoperoxidase, I^-, and H_2O_2. Several lines of evidence argue in favor of a build-up of a

species in microsomes or liposomes that produces part or all of the lipid peroxidation breakdown product, malondialdehyde, during the heating step. The appearance of both malondialdehyde and hydroperoxides in microsomes before heating with TCA-TBA reagent strongly suggest that initiation of peroxidation occurs during incubation of the membrane with the lactoperoxidase system. The initiating of peroxidation is not a non-enzymatic reaction between H_2O_2 and microsomal endogenous iron since lactoperoxidase and I^- are both required in addition to H_2O_2. Also, the addition of BHT to microsomes undergoing lactoperoxidase-catalyzed iodination blocked the destruction of cytochrome P-450, indicating that peroxidation is occurring as a consequence of treatment with lactoperoxidase, H_2O_2 and I^- (248). Finally, the time-dependent increase in malondialdehyde production in liposomes exposed to the lactoperoxidase system suggests a build-up of the peroxide producing species. Therefore, a study was undertaken to determine the mechanism of initiation of peroxidation by the lactoperoxidase system.

Crude preparations of lactoperoxidase from bovine milk are contaminated with a protein known as "red protein" or lactoferrin (262). This iron containing protein has a molecular weight of 93,000 daltons and has proved difficult to separate from lactoperoxidase during purification (262). Since Fe^{++} catalyzes non-enzymatic peroxidation and Fe^{+++} has been shown to be involved in peroxidation catalyzed by NADPH-cytochrome c reductase, ascorbate, and now lactoperoxidase, it was necessary to demonstrate whether the iron containing "red protein" was present as a contaminant of commercially obtained lactoperoxidase. Therefore, SDS-polyacrylamide gel electrophoresis was performed on samples of the commercial preparation of lactoperoxidase. Figure 18

Figure 18. SODIUM DODECYLSULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS OF COMMERCIAL LACTOPEROXIDASE.

The sample (25 μg) in 1% sodium dodecylsulfate, was heated in a boiling water bath for 15 minutes prior to electrophoresis. The scan of the Coomaaise blue stained gel was made at 550 nm.

ABSORBANCE 550 nm
2
4
6
8
MIGRATION DISTANCE (cm)

demonstrates that a single, Coomassie blue stained band was detected. This band corresponded to a molecular weight of 79,000 daltons. Lactoperoxidase has a reported molecular weight of 78,000 daltons (234). The iron containing "red protein" does not appear to be a detectible contaminant of the lactoperoxidase sample and therefore does not play a role in lactoperoxidase-catalyzed lipid peroxidation.

Various inhibitors of lipid peroxidation catalyzed by other peroxidizing systems were tested in order to determine the mechanism involved in lactoperoxidase-catalyzed lipid peroxidation. The possibility that hydroxyl radicals were involved in peroxidation catalyzed by lactoperoxidase was considered. Superoxide, produced by either the lactoperoxidase system or by microsomes may interact with H_2O_2 to produce hydroxyl radicals. Hydroxyl radicals produced by such a mechanism have been suggested as the killing agent in leukocytes of phagocytized bacteria (264). Therefore, hydroxyl radical trappers were tested for their ability to inhibit lactoperoxidase-catalyzed lipid peroxidation. The effects of t-butyl alcohol, mannitol, and sodium formate, all known scavengers of hydroxyl radicals (265-267), were investigated. Table 17 demonstrates that none of these hydroxyl radical scavengers were effective in inhibiting lactoperoxidase-catalyzed lipid peroxidation.

Singlet oxygen was also considered as a potential initiator of lipid peroxidation. The reaction of singlet oxygen with unsaturated fatty acids to form hydroperoxides has been demonstrated directly using both singlet oxygen generated by a radiofrequency gas discharge source and by photoactivation using sensitizers known to generate singlet oxygen (268). Recent findings by Allen (269) and Krinsky (270) indicate that myeloperoxidase is capable of producing singlet oxygen via the

TABLE 17.

THE EFFECTS OF HYDROXYL RADICAL SCAVENGERS ON LACTOPEROXIDASE-CALTALYZED LIPID PEROXIDATION.

The reaction mixture contained microsomes, KI, H_2O_2, and ADP-Fe^{+++} as described under "Methods". Inhibitors of hydroxyl radicals were added as indicated. The reaction time equaled 6 minutes.

Description	Malondialdehyde formed nmoles/min mg protein	% Control
Control	1.01	100
Plus 0.1 M t-butyl alcohol.	1.01	100
Plus 0.25 M mannitol.	1.01	100
Plus 0.5 M sodium formate	1.14	112
Minus lactoperoxidase	0.12	12

oxidation of chloride anion. The resulting chemiluminescence can also be obtained when iodide is substituted for chloride (269). Therefore, efforts were made to determine if singlet oxygen functioned in lactoperoxidase-catalyzed lipid peroxidation. 1, 3-Diphenylisobenzofuran (DPIF) reacts readily with singlet oxygen to form o-dibenzoylbenzene (DBB) (271). Table 18 represents the results obtained when microsomes were incubated with the lactoperoxidase system in the presence of this singlet oxygen trapper. The addition of DPIF had no effect on the appearance of malondialdehyde. The reactions were performed in a dark room under a safe light to prevent the generation of singlet oxygen by photosensitization and to rule out the possible effect of photoexcited DPIF. Since acetone was used as a vehicle for added DPIF, an equivalent amount of acetone was added to one incubation mixture to determine its effect on lipid peroxidation. Acetone, in the amount added, had no effect on lactoperoxidase-catalyzed lipid peroxidation.

In order to determine whether the lactoperoxidase, H_2O_2, I^- system was producing superoxide or using superoxide produced elsewhere, superoxide dismutase was included in the reaction mixtures. Superoxide dismutase catalyzes the dismutation of O_2^-, yielding H_2O_2 and molecular oxygen (187). Previous studies with horseradish peroxidase (272) and myeloperoxidase (273) regarding the aerobic oxidation of indoleacetic acid and NADH, respectively, have implicated O_2^- formation in these reactions, probably resulting from the interaction of molecular oxygen and the substrate's free radical form. Therefore, the possible production of O_2^- by the lactoperoxidase system was tested by the addition of superoxide dismutase to the peroxidizing system. When superoxide

TABLE 18

THE EFFECT OF SINGLET OXYGEN SCAVENGERS ON LACTOPEROXIDASE-CATALYZED LIPID PEROXIDATION.

The reaction mixtures contained microsomes, KI, H_2O_2 and ADP-Fe^{+++} as described under Methods". DPIF, in acetone, was added as indicated. The reaction time equaled 10 minutes.

Description	Malondialdehyde formed $\frac{\text{nmoles/min}}{\text{mg protein}}$	% Control
Control.	1.00	100%
Plus 0.2 µmoles DPIF/mg microsomal protein.	0.98	98
Plus 1.0 µmoles DPIF/mg microsomal protein.	0.99	99
Plus 50 µl acetone	1.01	101
Minus lactoperoxidase.	0.12	12

dismutase was added at concentrations ranging from 30-150 μg/ml of the reaction mixture essentially no inhibition of lipid peroxidation was observed. Therefore, superoxide does not appear to be involved in lactoperoxidase-catalyzed lipid peroxidation.

Cadmium acetate was added to the reaction mixture in order to determine if I_3^- may be involved in any way with peroxidation catalyzed by lactoperoxidase. I_3^- can be formed from the combination of molecular iodine and iodide and was routinely observed (as a species absorbing at 353 nm) in the initial (up to 3 min) phase of lipid peroxidation in this system. Cadmium acetate forms a complex with I_3^- and stabilizes the anion (274). No inhibition was observed when cadmium acetate was added to the peroxidizing microsomes. In addition, an equimolar solution of I_2 and I^- was added to microsomes not containing lactoperoxidase. No additional peroxidation was observed over the non-enzymatic control level. Morrison and Bayse (254) have also demonstrated that at neutral pH, neither I_2 nor I_3^- function with lactoperoxidase in the absence of H_2O_2 to iodinate tyrosine. Therefore, I_3^- does not appear to participate in lipid peroxidation catalyzed by lactoperoxidase.

The possibility that a transitory intermediate of I^- oxidation may be involved in initiating peroxidation cannot be ignored. Either the one electron or two electron oxidation of I^- could result in the formation of a species capable of initiating lipid peroxidation. The mechanism of lactoperoxidase-catalyzed protein iodination is thought to involve the two electron oxidation of I^- to yield I^+ (259-261). This electrophilic species may be capable of extracting an electron from a membrane lipid resulting in a lipid radical which would readily react with molecular oxygen to form a peroxy radical. If oxidation of I^- were to

proceed via a one electron oxidation, an iodide radical would result. This reactive species could readily react with an unsaturated lipid to form a lipid radical. In the case of phenol oxidation by lactoperoxidase and H_2O_2, the existing evidence indicates that such a one electron oxidation occurs, resulting in a phenoxy radical (261). Additional evidence implicating a transitory state of iodide oxidation as a reactive species responsible for peroxidation comes from the fact that SCN^-, which can replace I^- as an oxidizable anion for lactoperoxidase and H_2O_2, is not apparently capable of supporting lipid peroxidation in this system. SCN^- has also been shown to inhibit iodination of protein by lactoperoxidase, H_2O_2 and I^-.

The Antimicrobial Activity of the Lactoperoxidase Lipid Peroxidation System:

Previous investigations have shown that cow's milk inhibits the growth of certain bacteria (275, 276). Hansen (277) first suggested that the antimicrobial activity of milk might be due in part to "oxidizing enzymes" since both the peroxidase activity and the antimicrobial activity of milk were inhibited by identical heat treatment. This was confirmed and extended by Wright and Tramen (278), who found a high correlation between the inhibition of the peroxidase activity and the inhibition of the antimicrobial activity of milk by variations in temperature, pH, sodium azide concentration and H_2O_2 concentrations. Portmann and Auclair (279) observed during the purification of lactoperoxidase that there is a direct relationship between the peroxidase activity of the fractions obtained and their antimicrobial activity. Stadhouder and Veninga (280) and Jago and Morrison (281) confirmed that a highly

purified preparation of lactoperoxidase had antimicrobial activity. Reiter et al. (282) reported that the antimicrobial system of milk requires halide ions in addition to lactoperoxidase and suggested that the milk and saliva antimicrobial systems may be similar. Subsequently, Klebanoff et al. (186) showed that the addition of H_2O_2 greatly increases the spectrum of organisms inhibited by the peroxidase-halide antimicrobial system.

Myeloperoxidase from polymorphonuclear leukocytes also possesses antimicrobial activity in the presence of halide and H_2O_2 (283). This potent antimicrobial system is effective against bacteria (284), fungi (285), viruses (285), and mycoplasma (286). It has been demonstrated that a chemiluminescent signal is produced by the myeloperoxidase, halide, H_2O_2 system and that the pH optimum for the enzyme mediated chemiluminescence is identical to the pH optimum observed for antimicrobial activity (269). It was noted earlier that chemiluminescence is also associated with peroxidizing membrane lipids, probably through the interaction of lipid hydroperoxides during their breakdown (111-113). The fact that both the antimicrobial peroxidase system and peroxidizing membranes produce chemiluminescence suggest a potential correlation.

The mechanism by which milk kills bacteria has not yet been resolved. However, the present finding that lactoperoxidase is associated with lipid peroxidation suggests that lipid peroxidation of bacterial membranes may be responsible for the antibacterial activity of milk.

In order to test the possible relationship between lactoerpxoidase-catalyzed bacterial killing and peroxidation of membrane lipids, a stock Escherichia coli culture was obtained and maintained as described under

"Methods." Optimum growth of bacteria was found to occur in trypto-case soy broth. Thioglycolate broth was unacceptable for growth inhibition studies since lactoperoxidase is inhibited by some sulfur containing compounds including thiourea, glutathione, and thioglycolate (186).

Table 19 demonstrates the antibacterial activity of the complete lactoperoxidase system. Catalase inhibits the reaction by reducing the H_2O_2 concentration. Glucose and glucose oxidase will substitute for exogenous H_2O_2. All three components of the lactoperoxidase system are required for bacterial killing.

If bacterial killing is mediated by lactoperoxidase-catalyzed lipid peroxidation, then the antioxidant BHT should prevent bacterial killing by this system. Table 19 demonstrates that addition of BHT to the bacterial growth system was unable to reverse the killing of bacteria by lactoperoxidase. This would indicate that lactoperoxidase-catalyzing killing of bacteria was not mediated by lipid peroxidation.

In order to verify that lipid peroxidation was not causing bacterial killing, bacteria were grown with two other lipid peroxide forming systems. Table 20 demonstrates the effect of the NADPH-cytochrome c reductase and ascorbate peroxide forming systems of bacterial growth. Not only were these two peroxidizing systems unable to prevent bacterial growth, but growth enhancement occurred. The growth enhancement appeared to be caused by the iron suppliments required by these two peroxidizing systems, since ADP-Fe^{+++} and EDTA-Fe^{+++} caused growth enhancement by themselves.

Bacterial lipids were extracted with chloroform: methanol and sonicated to make liposomes by the same methods used for microsomal

TABLE 19.

INHIBITION OF BACTERIAL GROWTH BY LACTOPEROXIDASE, KI, AND H_2O_2.

The reaction mixture contained tryptocase broth, E. coli. and the suppliments as follows: lactoperoxidase, 240 o-dianisidine units; KI, 1.25 mM, H_2O_2, 0.45 mM; glucose 1.25 mM; glucose oxidase, 1 µg/ml; BHT, 0.005%. Growth conditions and determination of growth are described under "Methods".

Suppliments	Increase in absorbance at 540 nm in 4 hours
None	0.59
Lactoperoxidase	0.61
H_2O_2	0.47
KI	0.59
Lactoperoxidase + KI + H_2O_2	0.00
Lactoperoxidase + KI + H_2O_2 + catalase	0.59
Lactoperoxidase + KI + glucose + glucose oxidase	0.01
Lactoperoxidase + KI + glucose + glucose oxidase + BHT	0.01

TABLE 20.

THE EFFECT OF PEROXIDE FORMING SYSTEMS ON BACTERIAL GROWTH.

The reaction mixtures contained tryptocase broth, E. coli, and the following suppliments: lactoperoxidase, 120 o-dianisidine units; glucose, 1.25 mM; glucose oxidase, 1 μg/ml; ADP-Fe^{+++} or EDTA-Fe^{+++}, 0.1 mM; NADPH-cytochrome c reductase, 0.2 μg/ml; ascorbate, 0.1 mM; NADPH, 0.2 mM. Growth conditions and determination of growth are described under "Methods".

Suppliments	Increase in absorbance at 540 nm in 4 hours
None	0.55
Lactoperoxidase + glucose + glucose oxidase + KI	0.01
NADPH- cytochrome c reductase + ADP-Fe^{+++} + EDTA-Fe^{+++}	1.10
NADPH-cytochrome c reductase + ADP-Fe^{+++} + EDTA-Fe^{+++} + NADPH . . .	1.03
Ascorbate.	0.56
Ascorbate + ADP-Fe^{+++}.	1.01
ADP-Fe^{+++} + EDTA-Fe^{+++}	1.10

lipid. When the liposomes from bacteria were incubated with the lactoperoxidase and ascorbate lipid peroxidation systems, no hydroperoxides or malondialdehyde were produced. It appears that lipids from E. coli are not susceptible to lipid peroxidation. The presence of a lipid associated antioxidant in bacterial membranes may account for this finding.

Since BHT does not inhibit lactoperoxidase-catalyzed iodination of protein, while it does inhibit lipid peroxidation, the lactoperoxidase system is catalyzing the production of at least two reaction intermediates at the same time. Therefore, the reaction intermediate involved in bacterial killing (and perhaps iodination of protein) is not the same as the one promoting lipid peroxidation.

CHAPTER III

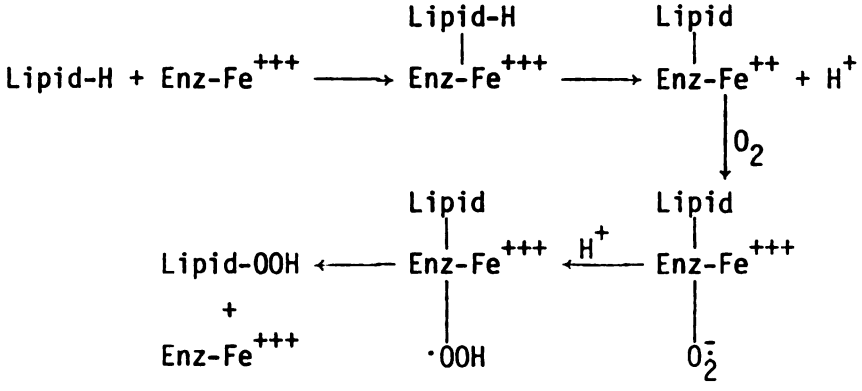
CHARACTERIZATION OF LIPID PEROXIDATION CATALYZED BY LIPOXYGENASE

Lipid Peroxidation Catalyzed by Lipoxygenase:

The results presented in Chapters I and II concerning enzyme-catalyzed lipid peroxidation in liposomes indicate that NADPH-dependent peroxidation results in a build up and subsequent breakdown of lipid hydroperoxides during the incubation phase of the reaction. The lactoperoxidase system, however, does not appear to build up hydroperoxides during the incubation phase when liposomes are used as the lipid source. It was suggested that a peroxide forming species was produced by the lactoperoxidase system. This species, in the presence of Fe^{+++} and heat, catalyzed the production of malondialdehyde. Much could be contributed to resolving the differences between these two peroxide forming enzyme systems if a method were available that would permit the build up of lipid hydroperoxides in membranes without their subsequent breakdown to yield malondialdehyde. This would permit the separate analysis of the factors responsible for the build up of hydroperoxides versus those factors catalyzing the breakdown of lipid hydroperoxides. Lipid hydroperoxide formation catalyzed by the plant dioxygenase, lipoxygenase, was considered as a potential means to accomplishing this goal.

Soybean lipoxygenase catalyzes the aerobic formation of conjugated cis, trans diene hydroperoxides in emulsions of purified, unsaturated

fatty acids containing methylene interrupted, cis double bonds (287). Suitable substrates for this enzyme include linoleic, linolenic, and arachidonic fatty acids (287). Lipid peroxidation catalyzed by lipoxygenase differs from all other peroxides forming systems discussed so far, in that lipoxygenase has a high degree of specificity with respect to the type of lipid substrate utilized, as well as the position within the lipid chain where the hydroperoxide group is introduced (289). It has been demonstrated that soybean lipoxygenase-catalyzed oxygenation of lipids occurs only at the w-6 position of the lipid substrate. Although the factors unique to the enzyme that determine this specificity are yet unresolved, it is now believed that lipoxygenase forms a complex with both the lipid substrate and molecular oxygen. The proposed mechanism of action for this non-heme, iron containing dioxygenase is shown below (136):



The reaction proceeds in the presence of EDTA. Therefore, metal-catalyzed autoxidation and breakdown of hydroperoxides is not expected to occur. Experiments were designed that would permit analysis of the conditions required for the breakdown or autoxidation of the enzyme formed hydroperoxides.

Soybean lipoxygenase was obtained commercially and used without further purification. Porter et al. (93) reported that high yields of

hydroperoxides from γ-linolenic and arachidonic acids were achieved with the commercial preparation of soybean lipoxygenase. Table 21 represents the results obtained from incubating sonicated, γ-linolenic acid with lipoyxgenase. When the enzyme was omitted or heat treated at 100° C for five minutes, no build up of hydroperoxides was observed. However, the native enzyme produced a substantial amount of hydroperoxides within a five minute period. The appearance of malondialdehyde was unexpected since EDTA had been included in the reaction mixture to prevent metal catalyzed decomposition of the hydroperoxides. However, the amount of malondialdehyde produced was only 3% of the amount of hydroperoxides present. Since γ-linolenic acid possesses the three methylene-interrupted double bonds necessary for the formation of malondialdehyde, very little of the potential amount of malondialdehyde was formed.

Initial attempts to peroxidize both liposomal and microsomal membrane lipids using lipoxygenase failed. This was not unexpected since the proposed reaction mechanism for lipoxygenase requires the binding of the lipid substrate to the enzyme. Both liposomal and microsomal membranes should be impermeable to the enzyme. Therefore, the membranes were partially solubilized by treatment with the detergent, sodium deoxycholate (DOC). Table 22 represents the results of treating both microsomes and liposomes with DOC in the presence of lipoxygenase. In both liposomes and microsomes, increasing the concentration of DOC causes a corresponding increase in the enzyme catalyzed production of lipid hydroperoxides. The effect of the detergent is to disrupt the integrety of the membrane, thus exposing lipid molecules to attack by lipoxygenase.

Detergent-solubilized microsomes were incubated with lipoxygenase under varying conditions. Table 23 demonstrates the correlation between

TABLE 21.

LIPOXYGENASE-CATALYZED LIPID PEROXIDATION OF LINOLENIC ACID

The complete reaction mixture contained 0.5 mM sonicated γ-linolenic acid, 1.0 mM EDTA, 20 μg/ml lipoxygenase and 0.05 M Sodium borate, pH 9.0 at 25° C. Malondialdehyde and hydroperoxide formation were assayed as described under "Methods". The reaction time equaled 5 minutes.

Description	Hydroperoxides formed nmoles/min/ml	Malondialdehyde formed nmoles/min/ml
Complete reaction mixture.	24.0	0.74
Minus lipoxygenase	0.0	0.00
Heat killed lipoxygenase	0.0	0.00

TABLE 22.

THE EFFECT OF DETERGENT OF LIPOXYGENASE-CATALYZED LIPID PEROXIDATION IN MICROSOMES AND LIPOSOMES.

The reaction mixtures contained microsomes (1 mg protein/ml) or liposomes (1 μmole lipid Pi/ml), 1.0 mM EDTA, 20 μg/ml lipoxygenase in 0.05 M sodium borate pH 9.0 at 25° C. DOC was present as indicated. The reaction time equaled 5 minutes.

Description	Hydroperoxides formed nmoles/min/ml
Micorosmes:	
Minus enzyme.	2.0
Plus enzyme	4.0
Plus enzyme + 0.04% DOC	4.6
Plus enzyme + 0.16% DOC	5.4
Plus enzyme + 0.32% DOC	11.3
Plus enzyme + 0.64% DOC	14.0
Liposomes:	
Minus enzyme.	2.0
Plus enzyme	2.0
Plus enzyme + 0.16% DOC	2.2
Plus enzyme + 0.32% DOC	8.4
Plus enzyme + 0.64% DOC	11.2

TABLE 23.

CONDITIONS FOR LIPOXYGENASE-CATALYZED LIPID PEROXIDATION IN MICROSOMES.

The reaction mixture contained microsomes (1.0 mg protein/ml), 1.0 mM EDTA, and the indicated buffer at 25° C. Lipoxygenase was included as indicated. The reaction time equaled 5 minutes.

Description	Hydroperoxide formed nmoles/min mg protein	Malondialdehyde formed nmoles/min mg protein
Microsomes in 0.05 M sodium borate, pH 9.0:		
Minus enzyme.	1.6	0.00
Plus heat killed enzyme . . .	1.4	0.00
Plus 20 µg/ml enzyme.	12.3	0.73
Plus 80 µg/ml enzyme.	40.0	1.20
Plus 200 µg/ml enzyme	84.0	1.90
Microsomes, in 0.05 M Tris-HCl, pH 7.4:		
Plus 20 µg/ml enzyme.	1.9	0.00

enzyme concentration and the amount of hydroperoxides formed. The reaction is dependent on pH and does not produce hydroperoxides in 0.05 M Tris-HCl, pH 7.4. Some breakdown of lipids hydroperoxides to yield malondialdehyde was observed in microsomes. The breakdown of hydroperoxides to yield malondialdehyde in microsomes was occurring to a greater extent than in γ-linolenic acid, since only about 24% of the lipids present in microsomes have the necessary double bonds required to produce malondialdehyde (1).

When ADP-Fe^{+++} was added to the reaction mixture, no change in the amount of hydroperoxides produced was observed (Table 24). This may indicate that Fe^{+++} does not interact with lipid hydroperoxides during the incubation period to catalyze breakdown of the hydroperoxides. This is consistent with the fact that ADP-Fe^{+++} and EDTA-Fe^{+++}, in the absence of reducing agents or heat, were unable to catalyze the decomposition of cumene hydroperoxide at 37° C (Figure 10). The 25% enhancement of malondialdehyde production observed when ADP-Fe^{+++} was present in the reaction mixture probably represents the interaction of Fe^{+++} with lipid hydroperoxides during heating with TCA-TBA reagent, in a manner analogous to the reaction of Fe^{+++} and H_2O_2 (Table 13).

BHT was added to the reaction mixture both during and after the incubation period. The presence of BHT during the incubation period completely blocks lipid peroxidation. This is consistent with the proposed, radical mediated reaction mechanism for lipoxygenase (136), and the inhibition of peroxidation observed by others when the antioxidant, nordihydroquaiaretic acid, was added to the lipoxygenase system (289). When BHT was added after the completion of the incubation of enzyme with microsomes, malondialdehyde production was inhibited by only 7%. It is

TABLE 24

THE EFFECT OF Fe^{+++} AND BHT ON THE BREAKDOWN OF LIPID HYDROPEROXIDES FORMED BY LIPOXYGENASE IN MICROSOMES.

The complete reaction mixtures contained microsomes (1.0 mg protein/ml) 20 µg/ml lipoxygenase, and 0.05 M sodium borate. EDTA, ADP-Fe , and BHT were added as indicated. The reaction time equaled 5 minutes.

Description	Hydroperoxides formed nmoles/min mg protein	Malondialdehyde formed nmoles/min mg protein
Complete reaction mixture:		
Plus 1.0 mM EDTA.	12.3	0.73
Plus 0.1 mM ADP-FE^{+++} during the reaction . .	12.2	0.97
Plus 0.005% BHT during the reaction . .	1.6	0.00
Plus 0.005% BHT after the reaction. . .	12.3	0.68

apparent that the conversion of lipid hydroperoxides to malondialdehyde is occurring during the incubation period. This is consistent with the proposal of a microsomal component responsible for the breakdown of lipid peroxides. This component has functioned in both the NADPH-dependent and lactoperoxidase-dependent microsomal peroxidation systems.

Lipoxygenase-catalyzed lipid peroxidation of detergent-solubilized liposomes provides additional evidence supporting the existence of this microsomal component (Table 25). Although lipid hydroperoxides build up during the incubation period, very little malondialdehyde production is observed. When BHT is added after the reaction period, malondialdehyde production is completely abolished. When Fe^{+++} is included in the incubation mixture, malondialdehyde values increase. Therefore, it appears evident that in liposomes, malondialdehyde production from the breakdown of lipid hydroperoxides occurs only after the reaction period is ended. Both Fe^{+++} and heat assist in the conversion of hydroperoxides to malondialdehyde.

The results presented in this section indicate that lipoxygenase acts on detergent solubilized membrane lipids to produce lipid hydroperoxides. In microsomes, a proposed membrane component catalyzes the breakdown of lipid hydroperoxides to yield malondialdehyde. In liposomes, breakdown of hydroperoxides occurs only during the heating of membrane with TCA-TBA reagent and is accelerated by the presence of Fe^{+++}.

Lipid Hydroperoxide Mediated Drug Metabolism:

The liver mixed-function oxidase system metabolizes a variety of drugs, steroids, fatty acids, alkanes, and other foreign compounds (290). This system has been resolved into three components, including

TABLE 25.

THE EFFECT OF Fe^{+++} AND BHT ON THE BREAKDOWN OF HYDROPEROXIDES FORMED BY LIPOXYGENASE IN LIPOSOMES.

The complete reaction mixture contained liposomes, (1.0 μmole lipid Pi/ml), 20 μg lipoxygenase, 1.0 mM EDTA, and 0.05 M sodium borate, pH 9.0 at 25° C. ADP-Fe^{+++} and BHT were added as indicated. The reaction time equaled 5 minutes.

Description	Hydroperoxides formed nmoles/min / μmole lipid Pi	Malondialdehyde formed nmoles/min / μmole lipid Pi
Complete reaction mixture.	11.0	0.19
Minus enzyme	2.0	0.00
Plus ADP-Fe^{+++} (without EDTA).	11.2	0.87
Plus BHT after the incubation period. . . .	11.0	0.00

NADPH-cytochrome c reductase, cytochrome P-450, and a lipid component (291). Rahimtula and O'Brien (292) demonstrated that cumene hydroperoxide will support P-450 catalyzed aromatic hydroxylations in the absence of NADPH and molecular oxygen. Recently, others have increased the list of drugs metabolized by the cumene hydroperoxide-cytochrome P-450 system (293, 294). This may not be difficult to accept since the reduced, oxygen containing active site of P-450 would, in theory, be very similar to peroxidase Compound I (295). Peroxidase Compound I results from the interaction of H_2O_2 with the Fe^{+++} form of the enzyme. A major question left unanswered concerning peroxide supported drug metabolism was whether endogenous microsomal lipid hydroperoxides could support cytochrome P-450 catalyzed drug metabolism. If this were true, then membrane lipid hydroperoxides may act as a reserve source of energy to metabolize foreign compounds in the absence of NADPH. The present use of lipoxygenase on detergent-solubilized microsomes indicates that lipid hydroperoxides can be produced in microsomes without the use of NADPH. It was therefore of interest to determine whether endogenous, microsomal lipid hydroperoxides, resulting from the action of lipoxygenase, could support cytochrome P-450-catalyzed drug metabolism.

Since detergent solubilization of microsomes is necessary for lipoxygenase-catalyzed lipid peroxidation to occur, it was necessary to determine if cumene hydroperoxide supported drug metabolism was affected by treatment of the microsomes with DOC (Table 26). Although NADPH-dependent drug metabolism is almost entirely abolished by 0.16% DOC, cumene hydroperoxide supported drug metabolism still retains some drug metabolizing activity at 0.32% DOC. Therefore, lipoxygenase was incubated with aminopyrine in the presence of detergent-solubilized

TABLE 26.

THE EFFECT OF DETERGENT-SOLUBILIZATION OF MICROSOMES ON BOTH NADPH AND HYDROPEROXIDE-DEPENDENT DRUG METABOLISM.

The control reaction mixture contained microsomes, aminopyrine, EDTA, and buffer as described under "Methods". 0.2 mM NADPH or 220 nmoles/ml cumene hydroperoxide (CHP) were included as indicated. DOC was present in the amounts indicated. Formaldehyde production was assayed as described under "Methods". The reaction time equaled 5 minutes.

Description	Formaldehyde formed nmoles/min/ml
Control	0.5
Plus NADPH.	7.6
Plus NADPH + 0.16% DOC. . . .	0.6
Plus CHP.	8.8
Plus CHP + 0.04% DOC.	7.0
Plus CHP + 0.08% DOC.	4.9
Plus CHP + 0.16% DOC.	3.0
Plus CHP + 0.32% DOC.	2.9

microsomes to determine if microsomal lipid hydroperoxides would support cytochrome P-450 catalyzed drug metabolism. The demethylation of aminopyrine is a reaction catalyzed by cytochrome P-450 (188). Table 27 demonstrates that lipid hydroperoxides, formed through the action of lipoxygenase on microsomal lipid, support the cytochrome P-450 linked demethylation of aminopyrine. It was of particular interest to note that the specific activity of lipid hydroperoxide-catalyzed drug metabolism was 0.028 nmoles formaldehyde formed per nmole lipid hydroperoxide formed versus 0.013 nmoles formaldehyde formed per nmole cumene hydroperoxide. The amount of lipid hydroperoxide present in the reaction mixture was based on the lipoxidase catalyzed yield of hydroperoxide during a five minute incubation in microsomes solubilized in 0.32% DOC. This would indicate that endogenous lipid hydroperoxides may be more effective in catalyzing cytochrome P-450 linked drug metabolism than exogenous, artificial organic peroxides.

The significance of peroxide-catalyzed drug metabolism has not been established at this time. However, the fact that microsomal membrane lipid hydroperoxides are suitable substrates for this reaction has been established. Whether this bears any significance to the <u>in vivo</u> reactions of cytochrome P-450 remains unanswered.

TABLE 27

MICROSOMAL DRUG METABOLISM CATALYZED BY MEMBRANE LIPID HYDROPEROXIDES.

The control reaction mixture contained microsomes, aminopyrine, EDTA, and buffer as described under "Methods". Lipoxygenase (100 µg/ml) was present or absent as indicated. DOC was included in the amounts indicated. Formaldehyde production was assayed as described under "Methods". The reaction time equaled 5 minutes.

Description	Formaldehyde formed nmoles/min/ml	
	- lipoxygenase	+ lipoxygenase
Control + 0.08% DOC	0.6	2.7
Control + 0.16% DOC	0.6	3.6
Control + 0.32% DOC	0.6	7.3

DISCUSSION

The Prooxidant Activity of Cytochrome P-450:

Results presented in Chapters I, II, and III show that enzyme-mediated lipid peroxidation in intact microsomes differs in one important respect from peroxidation catalyzed in liposomes. Lipid peroxidation in liposomes that is catalyzed by either the lipoxygenase or the lactoperoxidase systems does not appear to produce the hydroperoxide breakdown product, malondialdehyde, until the membrane lipids are heated with TCA-TBA reagent in the presence of Fe^{+++}. This fact is indicated by the studies in which the free radical trapper, BHT, was added to the reaction mixture upon completion of the incubation period. BHT abolished the appearance of malondialdehyde when present during the heating of the membrane with TCA-TBA reagent. In microsomes, however, addition of BHT to the reaction mixture after the incubation period did not substantially decrease the appearance of malondialdehyde. This fact suggested the existence of a factor in microsomes which facilitates the breakdown of enzymatically produced lipid hydroperoxides. Since hydroperoxide breakdown usually occurs through a radical producing mechanism, it is likely that the factor responsible for hydroperoxide breakdown also increases the production of lipid free radicals.

Results obtained from the study of NADPH-dependent lipid peroxidation in microsomes and liposomes further supports the concept of a microsomal factor which functions to facilitate the breakdown of lipid

hydroperoxides. It was demonstrated that EDTA-Fe^{+++} was required, in addition to ADP-Fe^{+++}, for maximum NADPH-dependent lipid peroxidation in liposomes, while microsomal peroxidation required only ADP-Fe^{+++}. Subsequently, it was demonstrated that EDTA-Fe^{+++}, in the presence of NADPH and NADPH-cytochrome c reductase, greatly facilitated the breakdown of cumene hydroperoxide. The iron catalyzed breakdown of cumene hydroperoxide paralleled the ability of NADPH-cytochrome c reductase to reduce chelated forms of Fe^{+++}. It was suggested that enzymatically reduced EDTA-chelated iron replaces the factor present in microsmes which facilitates hydroperoxide breakdown. Since neither the lactoperoxidase nor the lipoxygenase lipid peroxidation systems are capable of reducing EDTA-Fe^{+++}, addition of EDTA-Fe^{+++} to liposomes during incubation with either of these two enzyme systems did not appear to facilitate the breakdown of hydroperoxides, as judged by malondialdehyde production.

Recent reports indicate that cytochrome P-450 may be the component present in microsomes that catalyzes the breakdown of lipid hydroperoxides. O'Brien and Rahimtula (296) have demonstrated that the addition of cumene hydroperoxide to microsomes results in the rapid uptake of oxygen by the microsomes. A rancid odor characteristic of peroxidized lipid was detected concurrent with the production of the lipid hydroperoxide breakdown product, malondialdehyde. The rate of oxygen uptake with 1.5 mM cumene hydroperoxide was 150 nmoles of O_2 per minute per mg microsomal protein. Since cumene hydroperoxide is not structurally similar to unsaturated fatty acids and lacks the molecular configuration necessary for the breakdown formation of endoperoxides and subsequently, malondialdehyde, cumene hydroperoxide must be promoting the peroxidation of endogenous, microsomal lipids, which subsequently breakdown to

produce malondialdehyde. Inhibitors of cytochrome P-450 abolished the cumene hydroperoxide promoted peroxidation of endogenous microsomal lipids. When purified cytochrome P-450 was incubated with egg yolk lecithin in the presence of cumene hydroperoxide, oxygen uptake was again observed, indicating the peroxidation of lecithin was occurring. This fact clearly indicates that cytochrome P-450 interacts with hydroperoxide to promote the peroxidation of unsaturated lipid substrates.

Previous studies concerning the metal catalyzed breakdown of lipid hydroperoxides at 60° C indicated that hemoproteins like cytochrome P-450 were the most effective metal containing compounds tested (20, 136). The order of effectiveness was: hemoproteins and hematin > Fe^{++} > Fe^{+++} > Cu^{++}. Decomposition of hydroperoxides by metals in their higher valence state was greatly facilitated by reducing agents, such as ascorbate and cysteine. Therefore, the reduction of EDTA-Fe^{+++} by NADPH-cytochrome c reductase in the liposomal peroxidation system must duplicate the decomposition of hydroperoxides and the propagation of lipid radicals promoted by cytochrome P-450 in intact microsomes.

It has previously been reported that the decomposition of lipid hydroperoxides catalyzed by heme containing compounds results in the destruction of the heme group (102). The incubation of hydroperoxides with microsomes also resulted in the destruction of cytochrome P-450, as indicated by the loss of drug metabolizing activity in microsomes (297). It is interesting to note that peroxidation of microsomal lipid catalyzed by NADPH (298, 299) and the lactoperoxidase protein iodinating system (248) both result in the destruction of cytochrome P-450. Therefore, the destruction of cytochrome P-450 during enzyme mediated lipid

peroxidation probably results from the catalytic interaction of cytochrome P-450 with lipid hydroperoxides during hydroperoxide breakdown.

The Peroxidase Activity of Cytochrome P-450:

In a second series of papers, Hrycay and O'Brien (297, 300), observed another cytochrome P-450 catalyzed reaction with lipid hydroperoxides. In the presence of a hydrogen donor, such as N, N, N', N'-tetramethyl-p-phenylenediamine, (TMPD), cytochrome P-450 acts as a peroxidase to decompose linoleic acid hydroperoxide. A peroxidase reaction results in the reduction of a peroxide with the simultaneous oxidation of a hydrogen donor (295). When microsomal "P-450 particles," containing cytochrome P-450 as the sole protoheme containing constituent, were incubated with linoleic acid hydroperoxide, in the presence of TMPD, TMPD was oxidized as the linoleic acid hydroperoxide disappeared. During the course of the reaction, cytochrome P-450 was not destroyed. Therefore, in the presence of an oxidizable substrate and a lipid hydroperoxide, cytochrome P-450 acts as a peroxidase without undergoing destruction. If the interaction of lipid hydroperoxides with cytochrome P-450 results in the formation of a peroxidase Compound I type species, (295), then the peroxidase activity of cytochrome P-450 is chemically reasonable. A peroxidase Compound I type species was also proposed for cytochrome P-450 as an intermediate in the lipid hydroperoxide-dependent hydroxylation of drugs. Of particular interest was the finding that linoleic acid hydroperoxide was three times more effective than cumene hydroperoxide in the cytochrome P-450 mediated oxidation of TMPD. It was demonstrated in Chapter III that endogenous, microsomal lipid hydroperoxides formed by lipoxygenase were more effective in promoting cytochrome P-450 mediated drug metabolism than cumene hydroperoxide.

It may be true that drug substrates, like peroxidase hydrogen donors, protect cytochrome P-450 from destruction. If lipid hydroperoxides do not destroy cytochrome P-450 in the presence of hydrogen donors, then drug substrates may also afford protection. If this were not true, then lipid hydroperoxides would destroy cytochrome P-450 and hydroperoxide-dependent drug metabolism would not be possible.

Mixed type reactions in which monooxygenases, peroxidases, and oxidases interchange activities under defined conditions have been observed (295). If peroxides are intermediate products of oxygen reduction, then it may be said in many cases that peroxide metabolism is involved as a part of the overall metabolism of oxygen.

Based on the evidence presented here, the following three reactions can be proposed for cytochrome P-450 in the presence of lipid hydroperoxides.

$$LOOH + AH_2 \xrightarrow{\text{Cyt. P-450}} LOH + H_2O + A \qquad 1)$$

$$LOOH + \text{drug substrate} \xrightarrow{\text{Cyt. P-450}} \text{drug-OH} + \text{lipid products} \qquad 2)$$

$$LOOH \xrightarrow{\text{Cyt. P-450}} \text{lipid radicals (degraded Cyt. P-450)} \qquad 3)$$

Reactions 1) and 2) probably do not result in the degradation of cytochrome P-450 and may not be radical producing. Reaction 3) is of interest in the present study, and available evidence indicates that both breakdown of hydroperoxides and promotion of lipid peroxidation occur via this reaction (297, 300).

A Model for the Enzyme-Catalyzed Peroxidation of Membrane Lipids:

Based on reaction 3), the following model can be proposed for the mechanisms involved in lipid peroxidation catalyzed by the NADPH-

cytochrome c reductase, lactoperoxidase, and lipoxygenase systems (Figure 19).

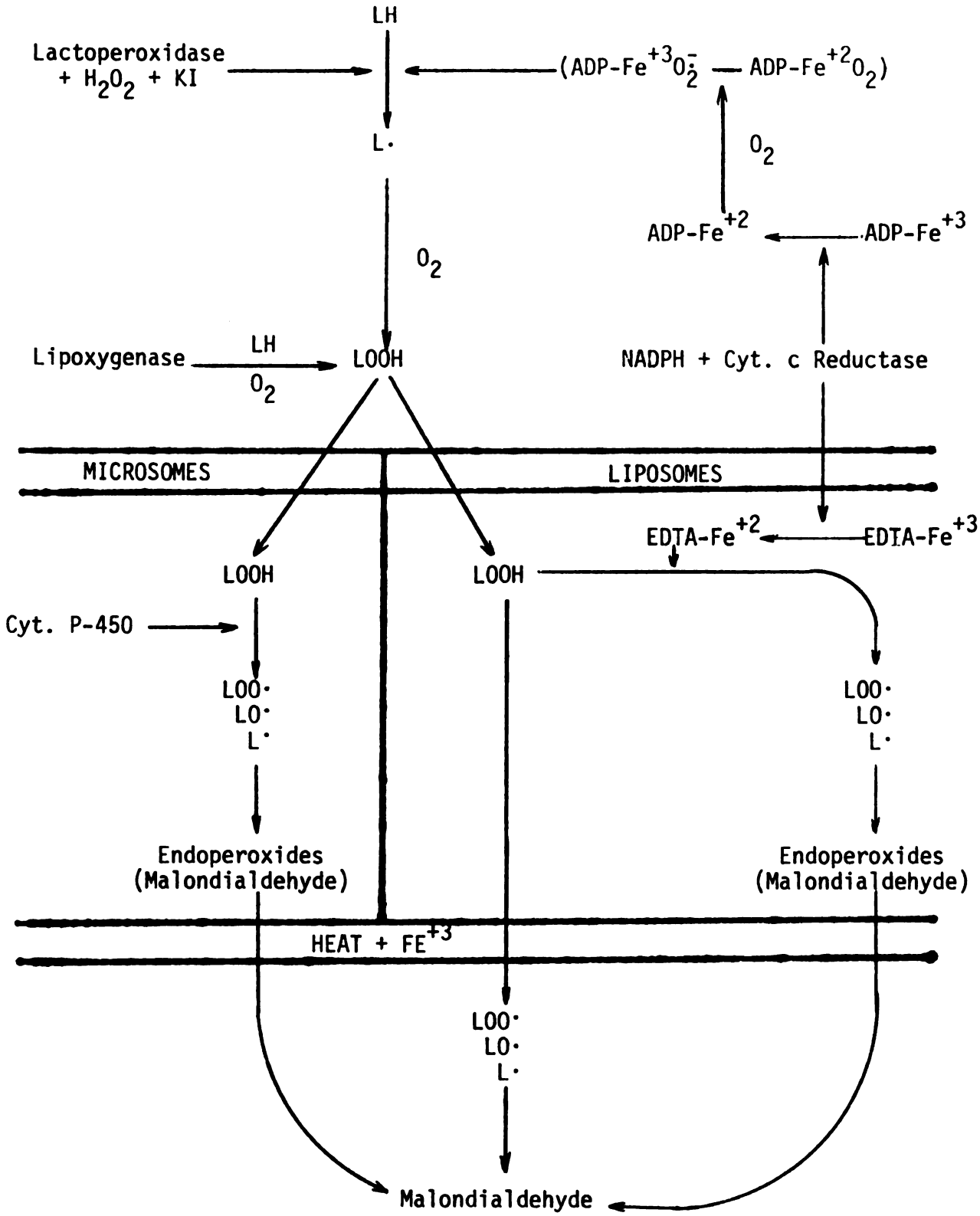
NADPH-dependent lipid peroxidation is probably initiated by the interaction of reduced, ADP-chelated iron with molecular oxygen to produce the perferryl ion. This species probably acts very much like the superoxide anion to abstract a hydrogen from unsaturated lipids. Lipid hydroperoxides subsequently result from the interaction of molecular oxygen with newly formed lipid radical. In microsomes, cytochrome P-450 acts to further propagate more free radicals via the catalytic breakdown of lipid hydroperoxides. Cytochrome P-450 is destroyed in the process. In liposomes, enzymatically reduced EDTA-chelated iron replaces cytochrome P-450 to catalyze the breakdown of lipid hydroperoxides and to propagate more free radicals. In both microsomes and liposomes, lipid endoperoxides (precursors to malondialdehyde) are formed during the incubation period.

Lipoxygenase-catalyzed lipid peroxidation results in the formation of lipid hydroperoxides without the intermediate production of free, lipid radicals. In microsomes, cytochrome P-450 catalyzes the breakdown of hydroperoxides and the propagation of more lipid radicals. Lipid endoperoxides (and malondialdehyde) are formed during the incubation period. In liposomes, hydroperoxides are produced by the enzyme without their subsequent breakdown to form malondialdehyde. Only when lipid hydroperoxides are heated under mild acid conditions with Fe^{+++}, do the hydroperoxides undergo breakdown. Malondialdehyde is formed only during the heating step.

In lactoperoxidase-catalyzed lipid peroxidation, initiation of peroxidation probably occurs via the formation of intermediate oxidation

Figure 19.

REACTION MECHANISMS INVOLVED IN MICROSOMAL AND LIPOSOMAL LIPID PEROXIDATION CATALYZED BY THE NADPH-CYTOCHROME c REDUCTASE, LIPOXYGENASE, AND LACTOPEROXIDASE SYSTEMS.


LH
Lactoperoxidase
+ H_2O_2 + KI
(ADP-$Fe^{+3}O_2^-$ — ADP-$Fe^{+2}O_2$)
L·
O_2
ADP-Fe^{+2}
ADP-Fe^{+3}
O_2
Lipoxygenase
LH
O_2
LOOH
NADPH + Cyt. c Reductase
MICROSOMES
LIPOSOMES
EDTA-Fe^{+2}
EDTA-Fe^{+3}
LOOH
LOOH
Cyt. P-450
LOO·
LO·
L·
LOO·
LO·
L·
Endoperoxides
(Malondialdehyde)
Endoperoxides
(Malondialdehyde)
HEAT + FE^{+3}
LOO·
LO·
L·
Malondialdehyde

products of I^-. Either $I\cdot$ or I^+ would be capable of interacting with unsaturated lipids to effect lipid radical formation. Lactoperoxidase-catalyzed lipid peroxidation in microsomes is facilitated by lipid radicals produced through the interaction of lipid hydroperoxides with cytochrome P-450. In liposomes, neither lipid hydroperoxides nor malondialdehyde are detected during the incubation period. This probably indicates that the rate of initiation of hydroperoxide formation is slow in the lactoperoxidase system. Without a catalyst like cytochrome P-450 or reduced iron, peroxidation does not proceed very fast. Only under the influence of heat and mild acid does Fe^{+++} act catalytically to promote increased hydroperoxide formation and breakdown.

The involvement of cytochrome P-450 in the metabolism of membrane lipid hydroperoxides has some important implications on the regulation and control of the destructive processes associated with lipid peroxidation. If cytochrome P-450 acts as a true peroxidase in the presence of hydrogen donors, then lipid hydroperoxides should be reduced to lipid hydroxides and water. If this reaction does not proceed via the production of free lipid radicals, then lipid hydroperoxides would effectively be neutralized. Therefore, if suitable hydrogen donors are present in the endoplasmic reticulum, lipid peroxidation (either NADPH-dependent or non-enzymatically induced) would be inhibited by the peroxidase catalyzed reduction of hydroperoxides by cytochrome P-450. O'Brien has demonstrated that suitable hydrogen donors for the peroxidase activity of cytochrome P-450 include ascorbate, cysteine, ubiquinol, α tocopherol, reduced glutathione, tyrosine, and tryptophan (301). In the absence of suitable hydrogen donors, however, cytochrome P-450 would act as a prooxidant, resulting in increased peroxidation and its own destruction.

SUMMARY

1) The solubilization of NADPH-cytochrome c reductase from the microsomal membrane by either crude pancreatic lipase or protease results in the proteolytic cleavage and release of a catalytically active protion of the native enzyme. Antibody to the purified, liver microsomal reductase inhibits the cytochrome c and ferricyanide reducing activity of both liver and lung microsomal NADPH-cytochrome c reductase equally. Ouchterlony analysis and SDS-gel electrophoresis of the immunoprecipitates obtained from detergent-solubilized liver and lung microsomes indicate that the liver and lung NADPH-cytochrome c reductase are extremely similar, if not identical.

2) Purified NADPH-cytochrome c reductase from lung microsomes is as effective as the corresponding liver enzyme in catalyzing lipid peroxidation in model liposomal system. NADPH-dependent peroxidation in lung microsomes, however, occurs much slower than NADPH-dependent peroxidation in liver microsomes. The decreased rate of malondialdehyde production in lung microsomes is probably a function of the structure and composition of the lung microsomal membrane.

3) NADPH-dependent peroxidation of microsomes and liposomes requires ADP-chelated ferric ions. ADP-Fe^{++} is more effective in catalyzing lipid peroxidation than ADP-Fe^{+++}. Therefore, the enzymatic reduction of ADP-Fe^{+++} by NADPH-cytochrome c reductase is probably responsible for the initiation of lipid peroxidation, presumably through

the interaction of a perferryl ion with unsaturated microsomal lipid. Neither the enzymatic nor non-enzymatic reduction of EDTA-Fe^{+++} results in the lipid peroxidation of microsomal lipid.

4) NADPH-dependent peroxidation in microsomes and liposomes results in an early build-up of lipid hydroperoxides followed by a net breakdown of hydroperoxides. Enzymatically reduced EDTA-Fe^{+++} is very effective in catalyzing the breakdown of cumene hydroperoxide and probably functions in the model, liposomal system to breakdown lipid hydroperoxide. The breakdown of lipid hydroperoxides by EDTA-Fe^{++} probably results in the production of peroxide forming lipid radicals. Neither ADP-Fe^{+++} nor EDTA-Fe^{+++}, in their oxidized states, catalyze lipid peroxide breakdown at 37° C. Since intact microsomes do not require EDTA-Fe^{+++} in NADPH-dependent peroxidation, EDTA-Fe^{+++} probably functions in the model liposomal system to replace a component of the microsomal membrane which catalyzes lipid hydroperoxide breakdown and radical propagation.

5) Lactoperoxidase, H_2O_2 and I^- catalyze both the iodination of protein and the peroxidation of microsomal and liposomal lipid membranes. Optimum concentrations of I^- and H_2O_2 for lactoperoxidase-catalyzed peroxidation of microsomes are 0.4 mM and 8.8 mM, respectively. The optimum concentration of H_2O_2 decreases for peroxidation of liposomes owing to elimination of the catalase contaminant present in microsomes.

6) Fe^{+++} is not required for the initiation of lactoperoxidase-catalyzed lipid peroxidation, but does assist in the propagation of lipid radicals via the breakdown of lipid hydroperoxides during heating of the membrane with TCA-TBA reagent. BHT added at the completion of the incubation period completely abolishes malondialdehyde production in liposomes and causes a small decrease in malondialdehyde production in

microsomes. Therefore, in liposomes, malondialdehyde produced from the breakdown of lipid peroxides, does not occur in appreciable amounts until the membrane lipids are heated in the presence of Fe^{+++}.

7) Initiation of lipid peroxidation by the lactoperoxidase system is probably catalyzed by an oxidation product of I^-. Singlet oxygen, superoxide, hydroxyl radicals and I_3^- are not involved in initiation of lactoperoxidase-catalyzed lipid peroxidation.

8) The antimicrobial activity of the lactoperoxidase, H_2O_2, I^- system is not apparently mediated by the peroxidation of bacterial membrane lipids. BHT does not block lactoperoxidase-catalyzed bacterial killing. Other peroxide forming systems do not cause killing of bacteria.

9) Lipoxygenase catalyzes the production of lipid hydroperoxides in γ-linolenic micelles and in detergent-solubilized microsomes and liposomes. The reaction is dependent on pH and enzyme concentration.

10) Hydroperoxides formed by lipoxygenase undergo breakdown to produce malondialdehyde in microsomes during the incubation period. In liposomes, breakdown of membrane lipid hydroperoxides occurs only upon heating of the membranes with Fe^{+++} and is radical mediated.

11) Lipoxygenase-catalyzed hydroperoxide formation in detergent-solubilized microsomes supports the cytochrome P-450 mediated metabolism of aminopyrine. Endogenous microsomal lipid hydroperoxides are more effective in cytochrome P-450 mediated drug metabolishm than exogenous cumene hydroperoxide.

12) Recent evidence suggests that cytochrome P-450 in microsomes is the factor responsible for the radical mediated breakdown of lipid hydroperoxides.

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APPENDIX

APPENDIX

List of Publications

Buege, J. A. and Aust, S. D. (1972) "On the Solubilization of NADPH-Cytochrome c Reductase from Rat Liver Microsomes with Crude Pancreatic Lipase," Biochem. Biophys. Acta, 286, 433-436.

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Buege, J. A. and Aust, S. D. (1976) "Lactoperoxidase-Catalyzed Lipid Peroxidation of Microsomal and Artificial Membranes," Biochem. Biophys. Acta, 444, 192-200.

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