ROLE OF PARTIALLY REDUCED OXYGEN, POLYUNSATURATED FATTY ACIDS, AND VITAMIN E IN *IN VITRO* LIPID PEROXIDATION.

By

Robert Arthur Leedle

AN ABSTRACT OF A DISSERTATION

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

DOCTOR OF PHILOSOPHY

Department of Pathology

1987

ABSTRACT

ROLE OF PARTIALLY REDUCED OXYGEN, POLYUNSATURATED FATTY ACIDS, AND VITAMIN E IN *IN VITRO* LIPID PEROXIDATION.

By

Robert Arthur Leedle

Factors affecting peroxidation of membrane lipids include the partially reduced oxygen species, superoxide ($O_2^{\cdot}$) and hydrogen peroxide (H_2O_2), as well as the polyunsaturated fatty acid, and vitamin E content of the lipid. This work was undertaken to furthur investigate the influence of these factors on initiation and inhibition of *in vitro* lipid peroxidation.

Rat lung microsomes were found to be more resistant to NADPH-dependent lipid peroxidation than were liver microsomes. Lung microsome-derived lipids contained a lower percentage of polyunsaturated fatty acids and a higher vitamin E concentration than those from liver. Resistance to peroxidation was attributed to a low polyunsaturated fatty acid to vitamin E ratio.

Addition of glutathione to liver microsomal lipid peroxidation reactions containing NADPH and ADP-Fe^{+3} partially inhibited peroxidation. With addition of vitamin

E to microsomes, inhibition increased. Inhibition was characterized by a vitamin E concentration-dependent lag phase followed by rapid peroxidation. Vitamin E concentrations were constant until the end of the lag phase. Progressively less inhibition occurred with lower iron concentrations or higher ADP:Fe^{+3} ratios. Lipid hydroperoxide concentrations in the peroxidation mixtures increased with increasing ADP:Fe^{+3} ratios. Results indicated that glutathione inhibited peroxidation by maintenance of vitamin E concentrations and by an interaction with iron to maintain low lipid hydroperoxide concentrations.

The contribution of $O_2^{\cdot}$ and H_2O_2 to NADPH-dependent lipid peroxidation was examined using lung microsomes which were susceptible to peroxidation. These microsomes originated from rats fed a vitamin E deficient diet. Superoxide and H_2O_2 production was similar in susceptible and resistant microsomes indicating that these factors did not influence peroxidation. Lung microsomes from rats fed the vitamin E deficient diet supplemented with vitamin E peroxidized even though their polyunsaturated fatty acid and vitamin E content was similar to that of resistant microsomes. The peroxidizable microsomes contained more arachidonic acid. Thus, polyunsaturated fatty acid content as well as vitamin E concentration should be considered in predicting microsomal resistance to peroxidation.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADP	-	adenosine diphosphate
ARDS	-	adult respiratory distress syndrome
EDTA	-	ethylenediaminetetraacetate
GC	-	gas chromatography
GSH	-	reduced glutathione
L·	-	lipid alkyl radical
LH	-	polyunsaturated fatty acid
LO·	-	lipid alkoxyl radical
LOH	-	lipid alcohol
LOO·	-	lipid peroxyl radical
LOOH	-	lipid hydroperoxide
MDA	-	malondialdehyde
NADPH	-	reduced nicotinamide adenine dinucleotide phosphate
PUFA	-	polyunsaturated fatty acid
TBA	-	thiobarbituric acid

INTRODUCTION

Partially reduced oxygen is thought to be important in many processes resulting in cellular injury. The partially reduced species, superoxide and hydrogen peroxide, can be produced within cells and there is good evidence that low levels of production occur normally. These species originate from the mitochondrial and microsomal electron transport systems and peroxisomal enzymes such as glucose oxidase. Normally, superoxide and hydrogen peroxide production does not result in overwhelming cellular damage, presumably because cells maintain reduced oxygen concentrations at very low levels via superoxide dismutase and catalase.

Toxicity is thought to occur when superoxide or hydrogen peroxide production exceeds the ability of cellular defenses to deal with them. Peroxidation of membrane lipids is a potentially important mechanism for cellular damage from these species. Presumably the mechanism for partially reduced oxygen mediated lipid peroxidation is via reactions with cellular free iron to produce a species capable of initiating lipid peroxidation.

Oxidative damage to lung has received increased interest recently, because certain clinical conditions, such as hyperoxic pulmonary damage in premature infants, point toward oxygen radical mediated damage. Toward understanding these processes, partially reduced oxygen production and lipid peroxidation were investigated in microsomal and microsomally derived lipid vesicle (liposomes) systems. Two general results came out of this work. The first is that lung microsomes are resistant to lipid peroxidation because of high vitamin E and low polyunsaturated fatty acid contents. The second is that production of partially reduced oxygen by lung microsomes is insufficient to support lipid peroxidation.

Chapter I deals with the resistance of rat lung microsomes to lipid peroxidation. Data are presented showing that resistance is due to both a low percentage of polyunsaturated fatty acids and a high vitamin E content. The levels of both polyunsaturated fatty acids and vitamin E have been determined previously by several investigators, and an interaction between them has been suspected for many years. By reconstituting lipid membranes with varying percentages of polyunsaturated fatty acids and vitamin E concentrations, the interaction between these two factors was demonstrated.

In liver microsomally derived liposomes, vitamin E is known to inhibit lipid peroxidation, but inhibition requires concentrations that are relatively high compared

to those normally found in microsomes. In recent years, ascorbic acid and glutathione have been shown to complement vitamin E dependent inhibition of lipid peroxidation and lower the amount of vitamin E required to inhibit peroxidation. In Chapter II data are presented showing that glutathione allows vitamin E concentration dependent lowering of rates of peroxidation rather than inhibition. However, the effect of glutathione on microsomal lipid peroxidation is not straight forward. Glutathione partially inhibits peroxidation without participation of vitamin E via an interaction with iron to maintain low lipid hydroperoxide concentrations.

The resistance of rat lung microsomes to lipid peroxidation can be removed by isolating microsomes from rats fed a vitamin E deficient diet. With lung microsomes that undergo peroxidation, the potential contribution of superoxide and hydrogen peroxide to peroxidation can be evaluated. In Chapter III the contribution of partially reduced oxygen to lung microsomal lipid peroxidation was investigated. It was shown that O_2^- and H_2O_2 production did not correlate to differences in peroxidation.

This dissertation is divided into three chapters similar in format to individual scientific journal articles. Each chapter contains an abstract, an introduction, a materials and methods section, a results section, a discussion, and a list of references. In addition to the chapters, there is at the beginning of this

dissertation, an in depth review of the recent literature pertinent to the chapters.

REVIEW OF LITERATURE

Lipid peroxidation has been proposed as a mechanism for cell toxicity from exposure to a wide variety of chemicals and in some types of non-chemical injury such as ischemia (Comporti, 1985). The process of lipid peroxidation is proposed to occur by a free radical mechanism, i.e., intermediates in this reaction contain unpaired electron(s). Lipid peroxidation has been divided into three phases, initiation, propagation and termination (Figure 1). In the initiation phase, a hydrogen atom is abstracted from a methylene carbon of a polyunsaturated fatty acid (LH) to form a lipid alkyl radical ($L^{\cdot}$). Following abstraction of a hydrogen atom, the radical center and the double bonds in the fatty acid chain rapidly rearrange to form a conjugated diene. Molecular oxygen adds to this lipid radical forming a lipid peroxyl radical ($LOO^{\cdot}$). Once the peroxyl radical is formed, it can undergo two fates. One fate is for it to abstract a methylene hydrogen from another PUFA to form a lipid alkyl radical and a lipid hydroperoxide (LOOH). This is a propagation reaction. Termination reactions, the other common fate of lipid peroxyl radicals, are those in which lipid radicals

Figure 1.

Lipid Peroxidation Scheme: Initiation, Propagation, and Termination Reactions.

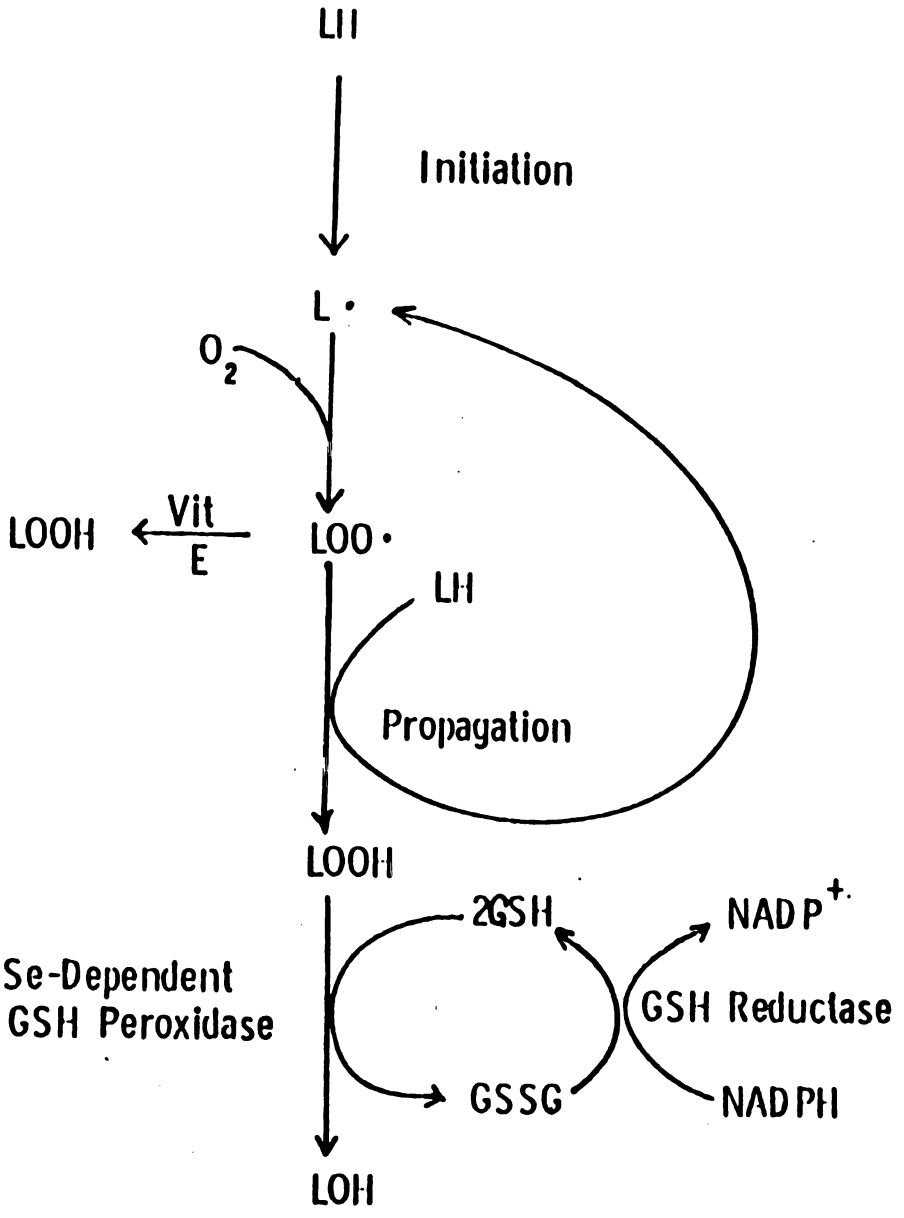

LH
Initiation
L·
O_2
LOOH
Vit
E
LOO·
LH
Propagation
LOOH
2GSH
$NADP^+$
Se-Dependent
GSH Peroxidase
GSH Reductase
GSSG
NADPH
LOH

Figure 1

are converted to non-reactive compounds without generation of additional lipid radicals. Vitamin E is regarded as a terminator of lipid peroxidation because it can donate a hydrogen atom to a lipid peroxyl radical to form a lipid hydroperoxide without formation of another PUFA radical (Kappus, 1985). Lipid hydroperoxides, however, can reenter lipid peroxidation via a reaction with ferrous ion to produce an intermediate capable of abstracting a methylene hydrogen from another PUFA. In order for the termination reaction to be complete, lipid hydroperoxides must be converted to products which are non-reactive in lipid peroxidation. Conversion of lipid hydroperoxides to the corresponding lipid alcohols (LOH) by the glutathione peroxidase system is an example of such a reaction (Kappus, 1985).

In recent years there has been intense debate on the mechanism of initiation of lipid peroxidation. Numerous authors (reviewed in Halliwell and Gutteridge, 1986) believe that partially reduced oxygen, specifically the hydroxyl radical ($OH\cdot$), is responsible for initiation. Generation of the hydroxyl radical requires a three electron reduction of molecular oxygen. Sequential one electron reductions of molecular oxygen produce superoxide, hydrogen peroxide, hydroxyl radical, and finally water. Production of hydroxyl radicals is thought to occur via Fenton's reaction in which hydrogen peroxide reacts with the ferrous ion (Halliwell and Gutteridge, 1986).

$$H_2O_2 + Fe^{+2} \text{-------} > OH\cdot + OH^- + Fe^{+3} \quad (1)$$

One mechanism for generation of hydrogen peroxide and ferrous ion is the reaction of superoxide with ferric ion. Generation of hydroxyl radical in this way can occur by a series of three reactions collectively referred to as the iron catalyzed Haber-Weiss reaction. (Note: The last reaction is Fenton's reaction.)

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

$$O_2\cdot^- + O_2\cdot^- \text{---------} > O_2 + H_2O_2 \quad (3)$$

$$H_2O_2 + Fe^{+2} \text{-----} > OH\cdot + OH^- + Fe^{+3} \quad (1)$$

The iron catalyzed Haber-Weiss reaction is a plausible explanation for cellular injury via the hydroxyl radical because superoxide and hydrogen peroxide are produced within cells. Mitochondrial and microsomal electron transport systems and enzymes such as glucose oxidase are potential cellular sources of superoxide and hydrogen peroxide (Halliwell and Gutteridge, 1985).

Ferrous ion autoxidation is another possible mechanism for generation of reduced oxygen beginning with molecular oxygen (Aust et al., 1985).

$$Fe^{+2} + O_2 \text{------} > Fe^{+3} + O_2\cdot^- \quad (4)$$

$$Fe^{+2} + O_2\cdot^- \text{------} > Fe^{+3} + H_2O_2 \quad (5)$$

$$Fe^{+2} + H_2O_2 \text{----} > Fe^{+3} + OH\cdot + OH^- \quad (1)$$

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

The extent to which autoxidation contributes to the reduced oxygen concentration depends on many factors such as the nature of the ferrous chelate and pH. In general, an iron

chelate with oxygen donor atoms tends to increase autoxidation rates, perhaps because of their greater affinity for ferric ions (Harris and Aisen, 1973). EDTA is an example of a chelate that causes ferrous to autoxidize rapidly. Chelates with nitrogen donor atoms, on the other hand, cause ferrous to autoxidize slowly (Bumbry and Massey, 1967; Beinert, 1978). Slowly autoxidizing chelators, such as bathophenanthroline are often used in assays for ferrous ion. The rate of ferrous ion autoxidation increases with increasing pH (Goto et al., 1970).

In in vitro lipid peroxidation systems, inhibition of peroxidation by superoxide dismutase, catalase, or hydroxyl radical scavengers is used as proof for the participation of superoxide, hydrogen peroxide, and hydroxyl radical, respectively. Superoxide dismutase catalyzes the destruction of superoxide producing hydrogen peroxide. Catalase catalyzes the dismutation of hydrogen peroxide to water and molecular oxygen. Hydroxyl radical scavengers are compounds such as mannitol, ethanol, and benzoate which react with few compounds except the hydroxyl radical. Reactions of the hydroxyl radical scavengers with hydroxyl radicals produce non-reactive products. Insensitivity of some lipid peroxidation systems to superoxide dismutase, catalase, and/or various hydroxyl radical scavengers has led several investigators to reevaluate the role of reduced oxygen in the initiation of lipid peroxidation. Systems

which depend on the microsomal electron transport system, and those systems which use thiols as sources of reducing equivalents are not affected by addition of superoxide dismutase (Tien et al., 1982a ;Morehouse et al., 1984). The microsomal electron transport system is composed of cytochrome P450 and NADPH cytochrome P450 reductase. (Cytochrome P450 is a family of at least 10 closely related monooxygenases with distinct and sometimes overlapping substrate specificities.) Catalase had little effect on EDTA-Fe^{+2} catalyzed lipid peroxidation in detergent dispersed lipid (Tien et al., 1982b). (Commercial preparations of catalase contain an antioxidant which may have been responsible for the small amount of observed inhibition [Morehouse et al., 1983].) In other reports, catalase stimulated superoxide-dependent lipid peroxidation (Tyler, 1975; Thomas et al., 1985). Mannitol, a hydroxyl radical scavenger, did not have any effect on lipid peroxidation in a system employing ferrous and ferric ions (Bucher et al., 1983; Braughler et al., 1986). Mannitol had inhibitory activity in the system of Tien et al. (1982b). This result would seem to support involvement of the hydroxyl radical, but alternatively, mannitol slows the oxidation of ferrous ion by hydrogen peroxide (Braughler et al., 1986; Minotti and Aust, 1987). In addition, microsomes, which are used in a large portion of the in vitro lipid peroxidation work, contain relatively high catalase and superoxide dismutase activities (Thomas and

Aust, 1985). (These activities are found in microsomes isolated by deferential centrifugation and are removed by ultrafiltration chromatography.)

The extreme reactivity of the hydroxyl radical has also led to skepticism of its role in lipid peroxidation. The hydroxyl radical is capable of reacting with most cellular constituents, and thus, in order for lipid peroxidation to occur, the radical must be generated close to its target (Halliwell and Gutteridge, 1986). Site specific generation of hydroxyl radicals implies that the entire iron catalyzed Haber-Weiss reaction takes place where superoxide dismutase, catalase, and hydroxyl radical scavengers are not present (Czapski and Goldstein, 1986). Without site specific reactions the intermediates would be destroyed by the superoxide dismutase and catalase which remain in microsomal preparations. Using ultrafiltration chromatography and inhibitors, the activities of superoxide dismutase and catalase can be decreased to low levels. Lowering superoxide dismutase and catalase activities, however, does not affect lipid peroxidation as might be expected if the site specific Haber-Weiss reaction were occurring. An effect on peroxidation would be expected because superoxide and hydrogen peroxide cannot react directly with lipid, and thus, some diffusion of $O_2^{\cdot}$ and H_2O_2 to locations accessible to degradative enzymes could be expected, and as such, there should be partial inhibition by superoxide dismutase and catalase.

Inhibition of lipid peroxidation by superoxide dismutase occurs in some systems, such as the xanthine oxidase-xanthine driven system, in which the reducing equivalents for iron reduction originate from superoxide. In other systems in which iron is reduced by other means, superoxide dismutase has no effect. In these systems which are not susceptible to inhibition by superoxide dismutase, it appears that iron may play a direct role in lipid peroxidation.

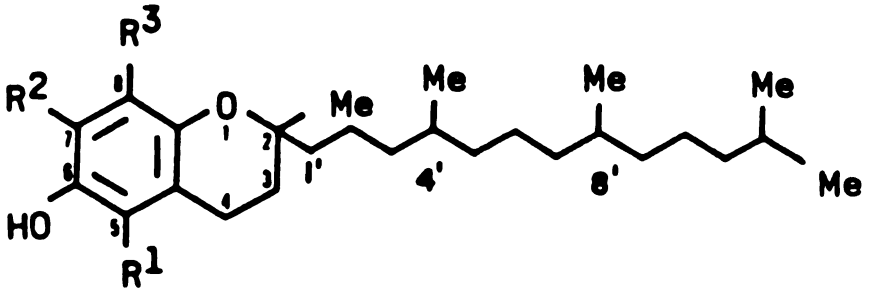
In a system containing ferrous ion, superoxide could be generated at the proposed site of the Haber-Weiss reaction by autoxidation. The autoxidation reaction with unchelated ferrous iron is thermodynamically unfavorable (Koppenol and Butler, 1985), however, chelates which lower the ferric/ferrous redox potential favor superoxide generation from ferrous ion and molecular oxygen. EDTA is an example of a ferrous chelate which generates superoxide, but the effect of EDTA chelation on lipid peroxidation indicates that superoxide production may not contribute significantly to lipid peroxidation. Evidence for this contention is that maximal rates of peroxidation are obtained with EDTA:ferrous ratios of 1:1. Higher ratios result in inhibition (reviewed in Minotti and Aust, 1987). In addition, systems containing only ferrous ion experience a lag phase before peroxidation begins; the duration depends on the rate of ferrous autoxidation (Bucher *et al.*, 1983). Abolition of the lag phase by addition of ferric

ion cannot be explained by a reaction between ferrous ion and molecular oxygen. The abolition can be explained if initiation of lipid peroxidation requires both ferrous and ferric ions.

Investigators focusing on the role of iron in the initiation of lipid peroxidation report that maximal rates of lipid peroxidation are obtained with a ferric to ferrous ion ratios of approximately 1:1 (Bucher et al., 1983; Braughler et al., 1986; Minotti and Aust, 1987). These authors believe that depending on whether the iron is mostly in the ferric or ferrous form any oxidant or reductant, including partially reduced oxygen, may either promote or inhibit lipid peroxidation (Aust et al., 1986). Thomas et al. (1985) reported that catalase stimulated xanthine oxidase-dependent lipid peroxidation when the added iron was in the ferric form. (Xanthine oxidase produces superoxide and hydrogen peroxide.) They explained the stimulation by hypothesizing that removal of hydrogen peroxide from the system slowed oxidation of ferrous ion. According to the hypothesis, superoxide promoted lipid peroxidation by reducing ferric to ferrous ion (reaction 2) at a rate that led to the presence of both ferric and ferrous ion in the reaction mixture. Hydrogen peroxide reacts much faster to oxidize ferrous to ferric ion (reactions 1 and 6) than superoxide does to reduce ferric to ferrous, thus, inclusion of catalase resulted in a higher ferrous ion concentration which could then catalyze

lipid peroxidation at a higher rate. Morehouse et al. (1984) reported that superoxide dismutase slightly stimulated peroxidation in a liposomal system utilizing cytochrome P450 reductase and NADPH as the source of reducing equivalents. This result provides further support for superoxide-independent lipid peroxidation since the reductase was present in the aqueous phase where any superoxide produced by it would be easily accessible to superoxide dismutase.

Regardless of the identity of the species responsible for initiation of lipid peroxidation, the generation of the initiator does not insure that lipid peroxidation will occur. The occurrence of peroxidation also depends on the concentration of membrane antioxidants and the fatty acid composition of the membrane. Most membranes contain vitamin E, which may inhibit peroxidation under some circumstances. The term vitamin E refers to 4 closely related tocopherols; all of which are derived from plants (Draper, 1980). The tocopherols, designated as alpha, beta, gamma, and delta, differ in the number and location of methyl groups on the aromatic ring of the chromanol core of the molecule (Kasparek, 1980).



alpha-tocopherol: $R^1 = R_2 = R_3 = CH_3$

beta-tocopherol: $R_1 = R_3 = CH_3$; $R_2 = H$

gamma-tocopherol: $R_1 = R_2 = CH_3$; $R_3 = H$

delta-tocopherol: $R_1 = CH_3$; $R_2 = R_3 = H$

The antioxidant activities of the tocopherols are alpha > beta > gamma > delta (Burton and Ingold, 1981). Further evidence on the relative reactivities was provided by Niki *et al*. (1986) who showed that in membranes containing mixtures of tocopherols, a-tocopherol disappeared at the highest rate during peroxidative challenge.

It appears that the hydroxyl group at the 6 position of the aromatic ring is responsible for the antioxidant activity of the molecule since tocopherol esters formed at this position are not active antioxidants. However, tocopherol esters are often used as dietary supplements due to their stability and because they are hydrolyzed in the gut to the respective alcohols (Bauernfeind, 1980; Gallo-Torres, 1980). Tocopherol reacts with a lipid peroxyl radical to form a lipid hydroperoxide and a relatively stable chromanoxyl (tocopherol) radical (Figure 2).

Figure 2.

Lipid Peroxidation Scheme: Hydroperoxide Dependent Lipid Peroxidation.

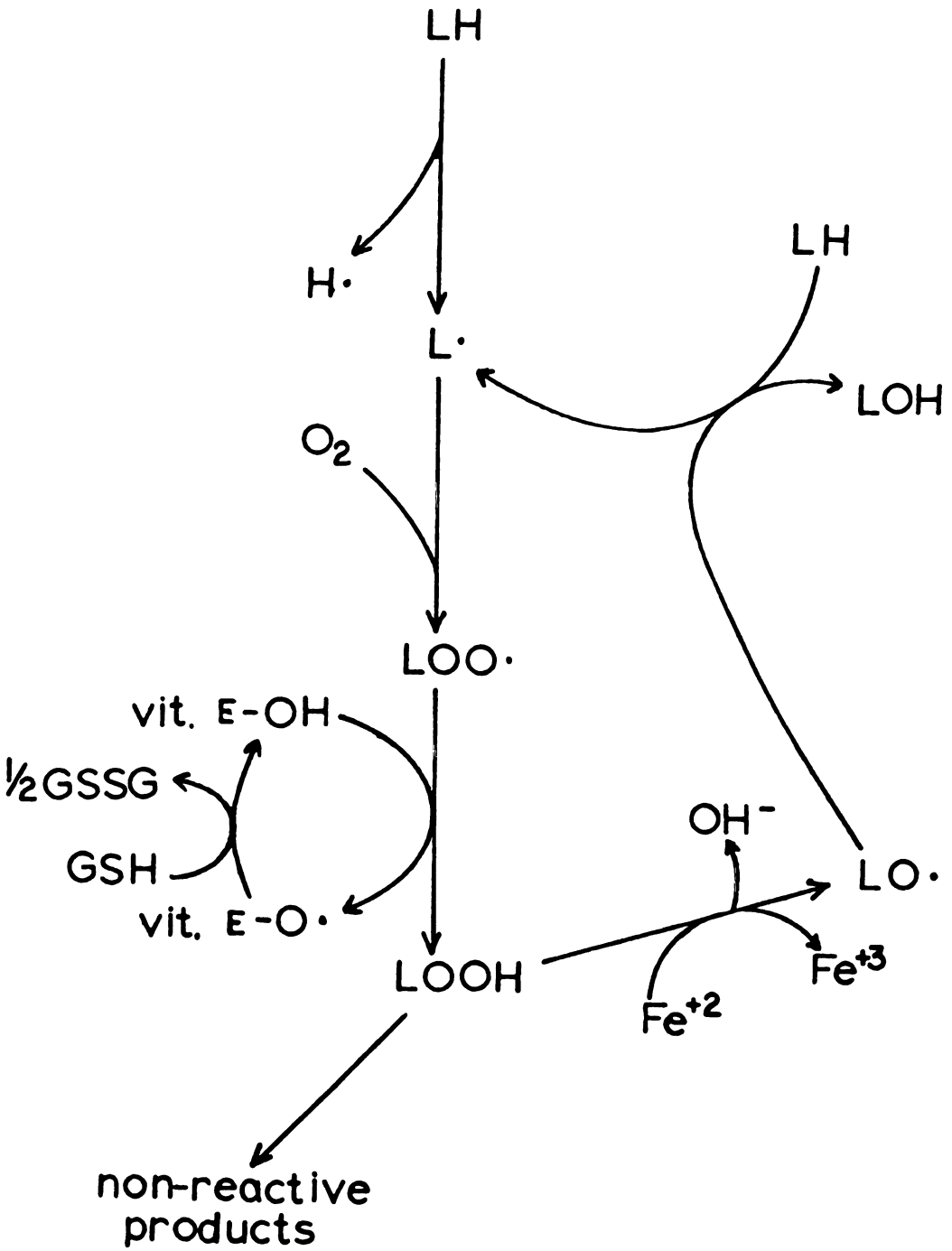

LH
H·
L·
LH
LOH
O2
LOO·
vit. E-OH
½GSSG
GSH
vit. E-O·
OH-
LO·
LOOH
Fe+3
Fe+2
non-reactive
products

Figure 2

(Chromanoxyl radical production has been demonstrated by electron spin resonance and is widely believed to occur *in vivo* [Boguth and Niemann, 1971; Niki *et al.*, 1982; Scarpa *et al.*, 1984]).

The chromanoxyl radical is stabilized by dislocation of the unpaired electron among the ring structures. Specifically, the stability of the chromanoxyl radical depends on the extent of orbital overlap between the 2p type electron pair on the para oxygen atom and the aromatic pi orbitals, and on the electron-donating or withdrawing character of the group bound to the para oxygen atom (Burton and Ingold, 1981; Burton *et al.*, 1985; Burton and Ingold, 1986). Orbital overlap is maximized when the 2p orbitals of the para oxygen atom are perpendicular to the plane of the aromatic ring. In a-tocopherol the oxygen orbitals are only 17° from perpendicular. The methyl group placements on the aromatic ring cause the other tocopherols to have greater deviations from perpendicular (reviewed in Burton and Ingold, 1986). Burton *et al.* (1985) reported that an a-tocopherol analogue which had a 5 membered ring attached to the aromatic ring instead of a 6-membered ring had less deviation from perpendicular than did a-tocopherol. Predicably, this synthetic analogue was more active than a-tocopherol in a rat myopathy bioassay (Ingold *et al.*, 1986).

The function of vitamin E in inhibition of lipid peroxidation is generally well accepted, but its fate after

conversion to a radical is not certain. Since the tocopherol radical is relatively stable, it can be reduced back to the active compound. At present there is interest in ascorbic acid as a direct reductant of tocopherol radicals (McCay, 1985), and in the reduced form of glutathione in the presence of a microsomal membrane protein (Reddy et al., 1982; Burk, 1983). If a reductant is not available for regeneration of reduced vitamin E, the fate of the radical is uncertain. Several possible reactions have been proposed and demonstrated under specific conditions. In general three reaction sequences have been proposed. In the first a quinone is produced from the chromanoxyl radical, perhaps followed by conversion to a quinol and cleavage of the non-aromatic ring. Numerous investigators have assayed for the quinone, but it does not appear to be a major metabolite (Csallany et al., 1962; McCay et al., 1972; Pascoe and Reed, 1987). Alternatively, rearrangement of the oxygen centered radical (bound to the carbon at position 6) to a methyl carbon centered radical and subsequent dimerization has been proposed (reviewed in Gallo-Torres, 1980). Lastly, McCay et al. (1972) showed in in vivo studies in rats that most of the oxidized tocopherol was associated with a highly polar fraction rather than as the quinone or dimer. The identity of the polar vitamin E metabolite(s) is not known but it is believed to be a chromanoxyl radical-lipid hydroperoxide adduct (McCay, 1985).

Reduction of the chromanoxyl radical to the functional molecule by ascorbic acid was proposed by Tappel (1968). Since then, numerous investigators have demonstrated that the reaction occurs, but others are not convinced that inhibition of lipid peroxidation by ascorbate involves an interaction with the chromanoxyl radical (reviewed in McCay, 1985). Based on thermodynamic considerations, ascorbate reacts preferentially with lipid peroxyl radicals before it reacts with vitamin E radicals. Predicted reactions based solely on thermodynamics may not be accurate in this case, because ascorbate and lipid peroxyl radicals are localized in different fractions. Ascorbate probably remains in the aqueous phase while lipid peroxyl radicals probably remain in the lipid phase (McCay, 1985). If the ascorbate and peroxyl radicals are in different phases, then vitamin E might function as an intermediate.

Some of the skepticism on the reduction of vitamin E by ascorbate is because ascorbic acid does not affect lipid peroxidation in a straightforward manner. In the absence of vitamin E, ascorbate can have pro-oxidant or antioxidant effects depending on the concentration (Halliwell and Gutteridge, 1985; McCay, 1985). The complexity of the ascorbate effect may be attributed to its direct reduction of ferric to ferrous ion. Ascorbate at concentrations below ferric concentrations promotes peroxidation, but when ascorbate exceeds ferric concentrations an antioxidant effect is seen presumably because all the iron is converted

to ferrous ion (Braughler et al., 1986). (When all the iron in a peroxidation system is ferrous, peroxidation does not occur unless preformed lipid hydroperoxides are present.) Since many of the compounds added to lipid peroxidation mixtures can interact with iron, the antioxidant or pro-oxidant character of ascorbate may be difficult to predict. Additionally, ascorbate reacts with superoxide and hydroxyl radicals (Cabelli and Bielski, 1983).

Reduced glutathione (GSH) also reduces the chromanoxyl radical to vitamin E. Niki et al. (1982) reported that GSH directly reduced vitamin E in a non-membrane system as determined by a decreased ESR signal from the vitamin E radical. However, when vitamin E is present in a membrane system, rapid reduction by GSH requires the presence of a membrane protein. This GSH-dependent vitamin E reductase was demonstrated in microsomal membranes by inhibition of lipid peroxidation at relatively low vitamin E concentrations when GSH was present (Reddy et al., 1982; Burk, 1983; McCay et al., 1986). The GSH-dependent vitamin E reductase has not been purified currently.

Interaction of lipid peroxyl radicals with reduced vitamin E leads to formation of lipid hydroperoxides. Hydroperoxides, however, are not inert in a lipid peroxidation system (Figure 2). Recall that hydroperoxides are formed during the propagation step in peroxidation and that ferrous ion can react with them to

produce ferric ion and presumably a lipid alkoxyl radical ($LO\cdot$) (Kappus, 1985). (Alkoxyl radicals can abstract a hydrogen from PUFA.) Because hydroperoxides are reactive with ferrous ion, complete termination of lipid peroxidation would seem to require conversion of the hydroperoxides to less reactive products unable to support lipid peroxidation. Several cytosolic systems have been proposed to convert hydroperoxides to such products. The cytosolic GSH-peroxidase system converts lipid hydroperoxides to lipid alcohols (Halliwell and Gutteridge, 1985) (Figure 1). (GSH-peroxidase is a selenium containing enzyme. It also converts hydrogen peroxide to water and molecular oxygen.) The reduction of lipid hydroperoxides by GSH-peroxidase, however, has been found to require phospholipase A2 for optimal activity (Sevanian et al., 1983; Tan et al., 1984; van Kuijk et al, 1985). (Phospholipase A2 cleaves the ester bond on the number 2 carbon of the glycerol backbone freeing the fatty acid.) Thus, hydroperoxidase activity was increased by cleaving the hydroperoxide from the phospholipid molecule. The increase in peroxidase activity was about 65000-fold higher on free fatty acids in the aqueous phase compared to esterified fatty acids in the lipid phase (van Kuijk et al., 1985). Ursini et al. (1982) reported a non-selenium containing GSH-dependent protein which reduced lipid hydroperoxides to alcohols while the fatty acid remained in the membrane. In addition to the action of peroxidases,

glutathione S-transferases have been shown to inhibit lipid peroxidation in microsomes (Burk et al., 1980; Bell et al., 1984) presumably by removal of lipid radicals.

The nature of the lipid substrate also influences peroxidation. As reviewed by Wills (1985), the rate of fatty acid peroxidation depends on the number of double bonds in the molecule. Thus, the highly unsaturated fatty acids containing 5 or 6 double bonds, as do a high portion of the omega-3 fatty acids, peroxidize more rapidly than those with fewer double bonds. Tien and Aust (1982c) found that differences in the rate and extent of lipid peroxidation observed in rat and rabbit liver microsomes could be attributed to the relative degrees of saturation of the microsomal lipids. Rabbit microsomes were found to contain higher amounts of linoleic acid (18:2), lower amounts of arachidonic acid (20:4), and undetectable amounts of docosahexenoic acid (22:6). The shift to more saturated fatty acids resulted in the lower rate of peroxidation observed in rabbit microsomes. Kornburst and Mavis (1980) reported the relative polyunsaturated fatty acid contents of microsomes from several rabbit organs. Of the organs examined, brain was highest, followed in decreasing content by lung, liver, kidney, testis, and heart. Rates of lipid peroxidation, however, did not follow this order, presumably because of differences in vitamin E contents among the organs.

Most of the work cited so far has been done in systems using material derived from liver. In recent years, however, there has been increasing interest in lung toxicoses. Examples of pulmonary toxicoses that may involve lipid peroxidation and/or partially reduced oxygen are paraquat toxicity, hyperoxic lung damage, adult respiratory distress syndrome (ARDS), and asbestosis. Paraquat (1,1'-dimethyl-4,4'-bipyridylium dichloride or methyl viologen) produces pulmonary damage after systemic or inhalation exposure. Paraquat induced pulmonary damage has been divided into destructive and proliferative phases. During the destructive phase, morphological changes are first observed in type I and type II alveolar epithelial cells. Within 48 hours there is damage to pulmonary capillaries, and there is also some early necrosis of bronchiolar cells. The destructive phase is associated with alveolar and interstitial edema and collapse of lung parenchema. The proliferative phase is characterized by migration of fibroblasts into alveolar and interstitial spaces followed by synthesis of excess collagen and eventual death (reviewed in Kehrer and Kacew, 1985).

Bus *et al*. (1974) proposed that paraquat toxicity was due to superoxide production. They showed that paraquat was reduced by cytochrome P450 reductase to form the paraquat radical which autoxidizes to produce superoxide. (NADPH was the source of reducing equivalents.) Paraquat

stimulated lipid peroxidation was inhibited by superoxide dismutase and by antibodies against cytochrome P450 reductase. Since the initial report, numerous authors have found that paraquat stimulated lipid peroxidation, however, other have reported inhibition of peroxidation. The contradictary results were reviewed by Trush *et al*. (1981) who showed that the different results were due to the concentration of paraquat used. The concentration of paraquat used in *in vitro* systems was critical in maintaining a certain redox state. Thus, paraquat may be acting analogously to many reducing agents which are known to be pro-oxidants at low concentrations but antioxidants at higher concentrations (Tien *et al*., 1982a). This phenomenon may be related to maintenance of pools of both ferric and ferrous ion.

Diquat, 1,1'-ethylene 2,2'-bipyridylium, is a herbicide structurally related to paraquat. Diquat, like paraquat produces superoxide via its reduction by cytochrome P450 reductase (Thomas and Aust, 1986), but it does not produce paraquat-like pulmonary toxicity. In diquat toxicity, type II alveolar epithelial cells are not damaged and non-lethal exposure results in complete recovery after a few days. Active uptake of paraquat by the type II cells has been proposed as the reason for the difference in toxicity (Witschi *et al*., 1977). This proposal is supported by a difference in uptake between wild-type and a paraquat-tolerant mutant of *Escherichia*

coli (Kao and Hassan, 1985). Thus, paraquat pulmonary toxicity depends on redox cycling of paraquat with production of superoxide, and on uptake of the chemical by pulmonary type II epithelial cells.

Exposure to high concentrations of oxygen in the atmosphere produces hyperoxic pulmonary damage that appears to involve pulmonary endothelial cells and type I epithelial cells. (Note: High oxygen concentrations exacerbate paraquat toxicity and also results in paraquat-like toxicity from diquat [Kehrer and Kacew, 1985].) Early morphologic changes include hydropic degeneration of endothelium with loss of tight junctions and formation of cellular blebs followed by pulmonary edema. Sloughing of type I epithelial cells and hyaline membrane formation follow. Later there is hyperplasia of type II cells and organization of fibrin in the hyaline membrane with intra-alveolar fibrosis (Fantone and Ward, 1985). Although the mechanism responsible for hyperoxic lung damage is not known, excess production of partially reduced oxygen species may be responsible. As stated earlier, cellular sources of partially reduced oxygen are the mitochondrial and microsomal electron transport systems and several cellular oxidase enzymes. Freeman et al. (1982) showed that rat lung homogenates had increased cyanide resistant respiration as indicated by oxygen uptake when exposed to high oxygen concentrations. However, the actual amount of cyanide resistant respiration was very low regardless of

the atmospheric oxygen concentration (2.2 umoles O_2 consumed/min/lung at 80% O_2). A 16% increase in oxygen comsumption rate was found at 80% versus 10% oxygen atmosphere. There was also a 118% increase in lipid peroxidation that could be inhibited 29% by superoxide dismutase and 15% by catalase under 80% oxygen. Cyanide resistant respiration was in terms of O_2 consumed per min. per lung and lipid peroxidation was in terms of nmoles of malondialdehyde per lung. (Rat lungs weigh approximately one gram.) In two subsequent papers by this group, they reported that most of the cyanide resistant respiration could be attributed to superoxide production and that hydrogen peroxide was derived entirely from superoxide. They also reported that microsomes were more important in the production of partially reduced oxygen than mitochondria. In a 100% O_2 atmosphere, mitochondria produced 2.9 nmoles H_2O_2/g of lung while microsomes produced 19.7 nmoles (Turrens et al., 1982a; Turrens et al., 1982b). Regardless of the trends in cyanide resistant respiration and lipid peroxidation, the reported values were quite low, leading to questions about their significance. However, intravenous administration of superoxide dismutase and catalase have been reported to lessen hyperoxic lung damage in rats (reviewed in Fantone and Ward, 1985).

Adult respiratory distress syndrome (ARDS) is characterized morphologically by diffuse alveolar damage in

much the same way as is hyperoxic lung damage, however, there is evidence that the etiology of ARDS involves phagocyte derived enzymes and oxygen radicals. (Fantone and Ward, 1985). Bronchoalveolar lavage fluids from ARDS patients contain higher than normal levels of neutrophil elastase activity and increased amounts of inactivated a_1-antiprotease. Inactivated antiprotease results from oxidation of methionine residues in the protein. The neutrophil sequestration that occurs in the lung microvasculature during ARDS may be complement mediated (Till and Ward, 1985). These investigators showed that complement activation in rats resulted in increased protein leakage from the circulation into lung tissue and that the leakage was partially inhibited by neutrophil depletion, superoxide dismutase, catalase, and lactoferrin. Presumably, lactoferrin inhibited lung damage by chelating free iron making it unavailable for reduction. Neutrophil derived proteases were shown not to be responsible for the endothelial damage that resulted from complement activated neutrophils.

Exposure to certain asbestos fibers results in asbestosis as well as a high incidence of lung cancer, mesotheliomas and gastrointestinal cancer (Robbins et al. 1984). (Asbestosis refers to the fibrotic lung disease resulting from asbestos fiber inhalation.) The mechanism(s) responsible for asbestos induced lesions is not know, but an alteration in cellular membranes via

oxygen radicals and lipid peroxidation has been proposed (Mossman and Landesman, 1983; Weitzman and Weitberg, 1985). The iron in the asbestos fibers catalyzed lipid peroxidation in these studies. In another *in vitro* system, Fontecave *et al*. (1987) showed that (crocidolite) asbestos stimulated NADPH-dependent lipid peroxidation in rat liver microsomes. This system was not inhibited by superoxide dismutase, catalase, mannitol or desferroxamine. (Desferroxamine is a very strong ferric ion chelator that in most cases results in inhibition of iron dependent processes such as lipid peroxidation.) The authors questioned involvement of oxygen radicals in their system. They felt that desferroxamine failed to inhibit because it could not remove the catalytic iron from the asbestos crystalline matrix. Work on cytotoxicity in hamster tracheal epithelial cells showed that superoxide dismutase protected cells from exposure to long asbestos (chrysotile and crocidolite) fibers. However, superoxide dismutase did not affect toxicity from shorter fibers (Mossman *et al*., 1986). (Long fibers are regarded as more toxic.)

Few specific details on partially reduced oxygen production by lung microsomes are known. Most of the available data on production of partially reduced oxygen are derived from studies using liver microsomes. Morehouse *et al*. (1984) investigated $O_2^{\cdot-}$ production by individual components of the microsomal electron transport chain in rat liver. They showed that in the absence of rapid

autoxidizing iron chelates such as EDTA, whole microsomes produced more $O_2^{\cdot-}$ than did purified cytochrome P450 reductase. The bulk of microsomal $O_2^{\cdot-}$ presumably originates via dissociation from cytochrome P450. There are several reasons for believing the majority of $O_2^{\cdot-}$ originates from cytochrome P450. Microsomal production of partially reduced oxygen increases in the presence of many substrates for cytochrome P450 (Hildebrandt and Roots, 1975; Nordblom and Coon, 1977). Cytochrome P450 inhibitors partially inhibit microsomal $O_2^{\cdot-}$ and H_2O_2 production (Estabrook and Werringloer, 1977; Roots et al., 1980). Different rates of superoxide and hydrogen peroxide production are found in microsomes from animals treated with different cytochrome P450 inducers (Hildebrandt and Roots, 1975).

Administration of inducers of cytochromes P450 to animals results in induction of specific cytochrome P450 isozymes that can be grouped according to the inducer used. Cytochrome P450c and cytochrome P450d are prototypically induced by 3-methylcholanthrene (Goldstein et al., 1982) and cytochrome P450b is prototypically induced by phenobarbital (Thomas et al., 1983). After treatment with 3-methylcholanthrene, microsomal contents of cytochromes P450c and P45d increased from 3 to 78% and 5 to 24% of the total cytochrome P450, respectively. Phenobarbital increased the amount of cytochrome P450b from 4 to 55% (Thomas et al., 1983). Morehouse (Ph. D. Thesis, Michigan

State Univ., 1986) showed that purified cytochrome P450d produced $O_2^{\cdot-}$ at a higher rate than did cytochrome P450c which in turn was higher than that of cytochrome P450b, thus, the difference in rates of partially reduced oxygen production in response to different cytochrome P450 inducers can be explained. The resting enzyme spin state of cytochrome P450d may account for the greater rate of $O_2^{\cdot-}$. Most cytochromes P450 are predominantly in the low spin state prior to binding of the substrate, but cytochrome P450d is primarily found in the high spin state. A high spin state favors transfer of an electron to the heme iron of the cytochrome from cytochrome P450 reductase (Bjorkhem, 1982).

(Spin state refers to the location of the electron in the heme iron. In ferric iron there are 5 electrons and 5 orbitals of slightly different energy levels that can contribute to the spin state of the atom. The 5 orbitals may either contain one electron each or 2 of them may contain paired electrons and a third an unpaired single electron. Since electrons which occupy the same orbital have opposite spins, the net spin of orbitals containing paired electrons is zero. The more unpaired electrons there are, the higher the net spin of the iron atom. Since the orbitals have different energies, high spin state iron has a higher net energy content than that of low spin state iron.)

Chemical modification of the cytochrome P450 protein

can also increase production of partially reduced oxygen. Parkinson et al. (1986) reported that the alkylating agent, 2-bromo-4'-niroacetophenone, resulted in an approximately 4 fold increase in superoxide production by purified cytochrome P450c. The protein was alkylated at a cysteine residue removed from both the heme and substrate binding site. The alkylated cytochrome was reduced by cytochrome P450 reductase at about a 30 fold higher rate. The heme was not converted to a high spin state. Thus, the rate of reduction by the reductase is more important than the resting spin state of the protein. The catalytic activity of alkylated cytochrome P450c was greatly reduced. Other cytochromes P450 were not affected by alkylation.

Chemical induction of cytochrome P450 isozymes is limited in rat lung microsomes compared to those from liver. Phenobarbital does not cause induction in lung microsomes. In liver 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a 3-methylycholanthrene type inducer, causes induction of cytochromes P450c and P450d. In rat lung, however, TCDD causes induction of only P450c (Goldstein and Linko, 1984). These investigators reported that cytochrome P450c increased from 4.8 to 523 pmoles/mg microsomal protein. This was an increase from 2 to 39% of the total cytochrome P450 content. Cytochrome P450d increased from 2 to 3% of the total cytochrome P450 content.

REFERENCES

Aust, S.D., C.E. Thomas, L.A. Morehouse, M. Saito, and J.R. Bucher. Active oxygen and toxicity. In: Biological Reactive Intermediates III (J.J. Kocsis, D.J. Jollow, C.M. Witmer, J.O. Nelson, and R. Snyder. eds.) pp. 513-526.Plenum Publishing, New York. (1986).

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

Bauernfeind, J. Tocopherols in foods. In: Vitamin E: A Comprehensive Treatise. (L.J. Machlin, ed.) pp.99-167. Marcel Dekker, Inc., New York. (1980).

Beinert, H. Micromethods for quantitative determination of iron and copper in biological material. In: Methods in Enzymology, Volume 54 (S. Fleischer and L. Packer, eds.) pp. 435-445. Academic Press, New York. (1978).

Bell, J.G., C.B. Cowey, and A. Youngson. Rainbow trout liver microsomal lipid peroxidation. The effect of purified glutathione peroxidase, glutathione S-transferase and other factors. Biochem. Biophys. Acta 795:91-99. (1984).

Bjorkhem, I. Rate limiting step in microsomal cytochrome P450 catalyzed hydroxylations. In: Hepatic Cytochrome P450 Monooxygenase System. (J.B. Schenkman and D. Kupfer, eds.) pp. 645-666. Pergamon Press, NY (1982).

Boguth, W., and H. Niemann. Electron spin resonance of chromanoxy free radicals from a-, d_2-, g-, d-tocopherol and tocol. Biochem. Biophys. Acta 248:121-130. (1971).

Braughler, J.M., L.A. Duncan, and R.L. Chase. The involvement of iron in lipid peroxidation: Importance of ferric to ferrous ratios in initiation. J. Biol. Chem. 261:10282-10289. (1986).

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

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

Burk, R.F., M.J. Trumble, and R.A. Lawrence. Rat hepatic cytosolic glutathione-dependent enzyme protection against lipid peroxidation in the NADPH-microsomal lipid peroxidation system. Biochem. Biophys. Acta 618:35-41. (1980).

Burk, R.F. Glutathione-dependent protection against lipid peroxidation by a microsomal glutathione-dependent labile factor. FEBS Lett. 159:24-28. (1983).

Burton, G.W., and K.U. Ingold. Autoxidation of biological molecules. 1. The antioxidant activity of vitamin E and related chain-breaking phenolic antioxidants in vitro. J. Am. Chem. Soc. 103:6472-6477. (1981).

Burton, G.W., T. Doba, E.J. Gabe, L. Hughes, F.L. Lee, L. Prasad, and K.U. Ingold. Autoxidation of biological molecules. 4. Maximizing the antixidant activity of phenols. J. Am. Chem. Soc. 107:7053-7065. (1985).

Burton, G.W., and K.U. Ingold. Vitamin E: Application of the principles of physical organic chemistry to the exploration of its structure and function. Acc. Chem. Res. 19:194-201. (1986).

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

Cabelli, D.E., and B.H.J. Bielski. Kinetics and mechanism for the oxidation of ascorbic acid/ascorbate by HO_2/O_2^- radicals. A pulse radiolysis and stopped-flow photolysis study. J. Phys. Chem. 87:1809-1812. (1983).

Comporti, M. Biology of disease: Lipid peroxidation and cellular damage in toxic liver injury. Lab. Invest. 53:599-623 (1985).

Csallany, A.S., H.H. Draper, and S.N. Shah. Conversion of d-a-tocopherol-C^{14} to tocopheryl-p-quinone in vivo. Arch. Biochem. Biophys. 98:142-145. (1962).

Czapski, G., and S. Goldstein. When do metal complexes protect the biological system from superoxide toxicity and when do they enhance it? Free Rad. Res. Comm. 1:157-161. (1986).

Draper, H.H. Role of vitamin E in plants, microbes, invertebrates, and fish. In: Vitamin E: A Comprehensive Treatise. (L.J. Machlin ed.) pp.391-395. Marcel Dekker, Inc. New York (1980).

Estabrook, R.W., and J. Werringloer. Active oxygen--fact or fancy. In: Microsomes and Drug Oxidations., (V. Ullrich, A. Hildebrandt, I. Roots, and R.W. Estabrook, eds.) pp. 748-757. Pergamon Press, NY (1977).

Fantone, J.C., and P.A. Ward. Oxygen-derived radicals and their metabolites: Relationship to tissue injury. In: Current Concepts. Upjohn Co. Kalamazoo, Mi 49001. (1985).

Fontecave, M., D. Mansuy, M. Jaouen, and H. Pezerat. The stimulatory effects of asbestos on NADPH-dependent lipid peroxidation in rat liver microsomes. Biochem. J. 214:561-565. (1987).

Freeman, B.A., and J. D. Crapo. Biology of disease: Free radicals and tissue injury. Lab. Invest. 47:412-426 (1982).

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

Gallo-Torres, H.E. Absorption. In: Vitamin E: A Comprehensive Treatise. (L.J. Machlin ed.) pp.170-192. Marcel Dekker, Inc. New York (1980).

Goldstein, J.A., and P. Linko. Diffential induction of two 2,3,7,8-tetrachlorodibenzo-p-dioxin-inducible forms of cytochrome P-450 in extrahepatic versus hepatic tissues. Molec. Pharmacol. 25:185-191. (1984).

Goldstein, J.A., P. Linko, M.I. Luster, and D.W. Sundheimer. Purification and characterization of a second form of hepatic cytochrome P-448 from rats treated with a pure polychlorinated biphenyl isomer. J.Biol. Chem. 257:2702-2707. (1982).

Goto, K., H. Tamura, and M. Nagayama. The mechanism of oxygenation of ferrous ion in neutral solutions. Inorg. Chem. 9:963-964. (1970).

Halliwell, B., and J. M. C. Gutteridge. Free Radicals in Biology and Medicine. Claredon Press. Oxford. (1985).

Halliwell, B., and J. M. C. Gutteridge. Oxygen free radicals and iron in relation to biology and medicine: Some problems and concepts. Arch. Biochem. Biophys. 246:501-514 (1986).

Harris, D.C., and P. Aisen. Facilitation of Fe(II) autoxidation by Fe(III) complexing agents. Biochem. Biophys. Acta 329:156-158. (1973).

Hildebrandt, A.G., and I. Roots. Reduced nicatinamide adenine dinucleotide phosphate (NADPH)-dependent formation and breakdown of hydrogen peroxide during mixed function oxidation reactions in liver microsomes. Arch. Biochem. Biophys. 171:385-397. (1975)

Ingold, K.U., G.W. Burton, D.O. Foster, M. Zuker, L. Hughes, S. Lacelle, E. Lusztyk, and M. Slaby. A new vitamin E analogue more active than a-tocopherol in the rat curative myopathy bioassay. FEBS Lett. 205:117-120. (1986).

Kao, S.M., and H.M. Hassan. Biochemical characterization of a paraquat-tolerant mutant of Escherichia coli. J. Biol. Chem. 260:10478-10481. (1985).

Kappus, H. Lipid peroxidation: Mechanisms, analysis, enzymology and biological relevance. In: Oxidative Stress. (H. Sies ed.) pp.273-310 Academic Press. London. (1985).

Kasparek, S. Chemistry of tocopherols and tocotrienols. In: Vitamin E: A Comprehensive Treatise. (L.J. Machlin ed.) pp.7-65. Marcel Dekker, Inc. New York (1980).

Kehrer, J.P., and S. Kacew. Sstemically applied chemicals that damage lung tissue. Toxicol. 35:251-293. (1985).

Kornburst, D.J., and R.D. Mavis. Relative susceptibilities of microsomes from lung, heart, liver, kidney, brain, and testes to lipid peroxidation: Correlation with vitamin E content. Lipids 15:315-322. (1979).

Koppenol, W.H., and J. Butler. Energetics of interconversion reactions of oxyradicals. Adv. Free Rad. Biol. Med. 1:91-131. (1985).

McCay, P.B., P. Pfeifer, and W.H. Stipe. Vitamin E protection of membrane lipids during electron transport functions. Ann. N.Y. Acad. Sci. 203:62-73. (1972).

McCay, P.B. Viamin E: Interactions with free radicals and ascorbate. Ann. Rev. Nutr. 5:323-340. (1985).

McCay, P.B., E.K. Lai, S.R. Powell, G. Breuggeman. Vitamin E functions as an electron shuttle for glutathione-dependent "free radical reductase" activity of biological membranes. Fed. Proc. 45(3):451. (1986).

Minotti, G., and S. D. Aust. The requirement for iron(III) in the initiation of lipid peroxidation by iron(II) and hydrogen peroxide. J. Biol. Chem. 262:1098-1104. (1987).

Minotti, G., and S. D. Aust. The role of iron in the initiation of lipid peroxidation. (1987). (in press)

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

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

Mossman, B.T., and J.M. Landesman. Importance of oxygen free radicals in asbestos-induced injury to airway epithelial cells. Chest 83:50S. (1983).

Mossman, B.T., J.P. Marsh, and M.A. Shantos. Alteration of superoxide dismutase activity in tracheal epithelial cells by asbestos and inhibition of cytotoxicity by antioxidants. Lab. Invest. 54:204-212. (1986).

Niki, E., J. Tsuchiya, R. Tanimura, and Y. Kamiya. Regeneration of vitamin E from a-chromanoxyl radical by glutathione and vitamin C. Chem. Lett. 1982: 789-792. (1982).

Niki, E., M. Takahashi, and E. Komuro. Antioxidant activity of vitamin E in liposomal membranes. Chem. Lett. 1986:1573-1576. (1986).

Nordblom, G.D., and M.J. Coon. Hydrogen peroxide formation and stoichiometry of hydroxylation reactions catalyzed by highly purified liver microsomal cytochrome P450. Arch. Biochem. Biophys. 180:343-347. (1977).

Parkinson, A., P.E. Thomas, D.E. Ryan, L.D. Gorsky, J.E. Shively, J.M. Sayer, D.M. Jerina, and W. Levin. Mechanism of inactivation of rat liver microsomal cytochrome P-450c by 2-bromo-4'-niroacetophenone. J. Biol. Chem. 261:11487-11495. (1986).

Pascoe, G.A., and D.J. Reed. Relationship between cellular calcium and vitamin E metabolism during protection against cell injury. Arch. Biochem. Biophys. 253:287-296. (1987).

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

Robbins, S.L., R.S. Cotran and V. Kumar. Pathologic Basis of Disease. W.B. Saunders Co. Philadelphia, PA 19105. pp 460-461. (1984).

Roots, I., G. Laschinsky, A.G. Hildebrandt, G. Heinemeyer, and S. Nigam. Inhibition of hexobarbital-induced microsomal H_2O_2 production by metyrapone. In: Microsomes, Drug Oxidations, and Chemical Carcinogenisis. (M.J. Coon, A.H. Conney, R.W. Estabrook, H.V. Gelboin, J.R. Gillette, and P.J. O'Brien. eds.) pp. 375-378. Academic Press, NY (1980).

Scarpa, M., A. Rigo, M. Maiorino, F. Ursini, and C. Gregolin. Formation of a-tocopherol radical and recycling of a-tocopherol by ascorbate during peroxidation of phosphatidylcholine liposomes: An electron paramagnetic resonance study. Biochem. Biophys. Acta 801:215-219. (1984).

Sevanian, A., S.F. Muakkassah-Kelly, and S. Montestruque. The influence of phospholipase A2 and glutathione peroxidase on the elimination of membrane lipid peroxides. Arch. Biochem. Biophys. 223:441-452. (1983).

Tan, K.H., D.J. Meyer, J. Belin, and B. Ketterer. Inhibition of microsomal lipid peroxidation by glutathione and glutathione transferases B and AA. Role of endogenous phospholipase A2. Biochem. J. 220:243-252. (1984).

Tappel, A.L. Will antioxidant nutrients slow aging processes? Geriatrics 23:97-105. (1968).

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

Thomas, C.E., and S.D. Aust. Free radicals and environmental toxins. Ann. Emergen. Med. 15:1075-1083. (1986).

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

Thomas, P.E., L.M. Reik, D.E. Ryan, and W. Levin. Induction of two immunochemically related rat liver cytochrome P-450 isozymes, cytochromes P-450c and P-450d, by structurally diverse xenobiotics. J. Biol. Chem. 258:4590-4598. 91983).

Tien, M., J.R. Bucher, and S.D. Aust. Thiol-dependent lipid peroxidation. Biochem. Biophys. Res. Comm. 107:279-285. (1982a).

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

Tien, M., and S.D. Aust. Rabbit liver microsomal lipid peroxidation. The effect of lipid on the rate of peroxidation. Biochem. Biophys. Acta 712:1-9. (1982c).

Till, G.O., and P.A. Ward. Oxygen radicals in complement and neutrophil-mediated acute lung injury. J. Free Rad. Biol. Med. 1:163-168. (1985).

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

Turrens, J.F., B.A. Freeman, and J.D. Crapo. Hyperoxia increases H_2O_2 release by lung mitochondria and microsomes. Arch. Biochem. Biophys. 217: 411-421. (1982a).

Turrens, J.F., B.A. Freeman, J.G. Levitt, and J.D. Crapo. The effect of hyperoxia on superoxide production by lung submitochondrial particles. Arch. Biochem. Biophys. 217: 401-410. (1982b).

Tyler, D.D. Role of superoxide radicals in the lipid peroxidation of intracellular membranes. FEBS Lett. 51:180-183. (1975).

Ursini, F., M.M. Valente, L. Ferri, and G. Gregolin. Purification from pig liver of a protein which protects liposomes and biomembranes from peroxidative degradation and exhibits glutathione peroxidase activity on phosphatidylcholine hydroperoxides. Biochem. Biophys. Acta 710:197-211. (1982).

van Kuijk, F.J.G.M., G.J. Handelman, E.A. Dratz. Consecutive action of phospholipase A2 and glutathione peroxidase is required for reduction of phospholipid hydroperoxides and provides a convenient method to determine peroxide values in membranes. J.Free Rad. Biol. Med. 1:421-427. (1985).

Weitzman, S.A., and A.B. Weitberg. Asbestos-catalyzed lipid peroxidation and its inhibition by desferroxamine. Biochem. J. 225:259-262. (1985).

Wills, E.D. The role of dietary components in oxidative stress in tissues. In: Oxidative Stress. (H. Sies ed.) pp. 217-233. Academic Press. London. (1985).

Witschi, H., S. Kacew, K.I. Hirai and M.G. Cote. In vivo oxidation of reduced nicotinamide-adenine dinucleotide phosphate by paraquat and diquat in rat lung. Chem.-Biol. Interactions 19:143-160. (1977).

Chapter I

Importance of the Polyunsaturated Fatty Acid to Vitamin E Ratio in the Resistance of Rat Lung Microsomes to Lipid Peroxidation.

ABSTRACT

Rat lung microsomes and liposomes made from isolated lung microsomal lipids were much more resistant to lipid peroxidation that those from liver in both enzymatic and non-enzymatic systems. The polyunsaturated fatty acid (PUFA) content of isolated lung microsomal lipids was 28% of total fatty acids while that of liver was 54%. The vitamin E (a-tocopherol) content of isolated lung microsomal lipids was 2.13 nmoles/umole lipid phosphate and that of liver was 0.43. Individually, neither the lower PUFA content nor higher vitamin E levels could account for the resistance of lung microsomal lipids to peroxidation. Distearoyl-L-a-phosphatidylcholine and/or a-tocopherol were added to liver microsomal lipids to achieve different PUFA to vitamin E ratios at PUFA contents of 28% or 54% and the resulting liposomes were subjected to an NADPH-dependent lipid peroxidation system. The NADPH-dependent peroxidation system contained cytochrome P450 reductase, EDTA-Fe^{+3}, and ADP-Fe^{+3}. Liposomes having PUFA to vitamin E ratios less than approximately 250 nmoles PUFA/nmole vitamin E were resistant to peroxidation whereas lipid peroxidation, as evidenced by malondialdehyde production,

occurred in liposomes having higher ratios. When lipid peroxidation occurred, 40-60% of the liposomal vitamin E was irreversibly oxidized. Irreversible oxidation did not occur in the absence of lipid peroxidation. These studies indicated the low PUFA to vitamin E ratio in lung microsomes and isolated microsomal lipids was sufficient to account for the observed resistance to lipid peroxidation.

INTRODUCTION

Lipid peroxidation has been associated with cellular damage under a wide variety of circumstances (Comporti, 1985). Several factors determine the rate of lipid peroxidation. These include the concentration and nature of initiator to which the substrate lipid is subjected (Trush et al., 1981; Tien et al., 1982; Morehouse et al., 1984; Winterbourn and Sutton, 1986), the composition of the lipid (Tien and Aust, 1982), and the presence of antioxidants (Tappel, 1972; McCay, 1985). The primary determinant related to lipid composition is the relative amount of polyunsaturated fatty acids (PUFA) (Tien and Aust, 1982). Fatty acids with greater unsaturation and lipids containing higher amounts of PUFA are more susceptible to peroxidation. An important biological antioxidant system involves vitamin E (Tappel, 1972; McCay, 1985).

Dietary manipulation of tissue vitamin E levels have been shown to dramatically affect in vivo and in vitro lipid peroxidation (Tappel, 1972; Tappel, 1980; Buckingham, 1985). Exhaled pentane, a product of lipid peroxidation, increased in vitamin E deficient animals (Tappel, 1980;

Buckingham, 1985). Red blood cells from these animals were also more susceptible to hypotonic lysis (Tappel, 1980; Buckingham, 1985). These results support other studies that indicated *in vitro* lipid peroxidation can be completely inhibited by addition of a-tocopherol to lipid membranes (Gutteridge, 1978; Fukuzawa *et al*. 1985). More specifically, Fukuzawa *et al*. (1985) showed that lipid peroxidation did not occur above a critical level of added a-tocopherol while it readily occurred below this level.

Much of our understanding of lipid peroxidation is derived from *in vitro* systems employing rat liver microsomes. Recently, however, lung microsomal lipid peroxidation has received attention primarily due to concern about the effect of exposure to oxygen and pulmonary toxicants. Compared to liver, lung microsomal lipids have been shown to peroxidize at a very low rate and a lag period was observed before peroxidation occurred (Kornburst and Mavis, 1980; Trush *et al*., 1981).

Resistance to lipid peroxidation may be of considerable advantage to the lung, thus it is important to understand the mechanisms. As suggested by others (Horwitt, 1962; Kornburst and Mavis, 1980), we propose two factors are responsible for this resistance, the relative amount of PUFA and the vitamin E content of rat lung microsomes. The results of this study demonstrate that the total amount of vitamin E in the membranes is not the sole determinant of resistance to lipid peroxidation but, that

the ratio of PUFA to vitamin E is important. These findings appear to explain the resistance of lung microsomal lipid to lipid peroxidation.

MATERIALS AND METHODS

NADPH, ADP, dl-a-tocopherol, distearoyl-L-a-phosphatidylcholine, paraquat, 2-thiobarbituric acid, bromelain (E.C. 3.4.22.4) and cytochrome c (Type VI) were purchased from Sigma Chemical Company (St. Louis, MO). EDTA was obtained from Mallinckrodt Chemical Company (Paris, KY). Ferric chloride was purchased from Baker Chemical Company, (Phillipsburg, NJ). L-ascorbic acid was purchased from Fischer Scientific Company (Fairlawn, NJ). Other chemicals were of analytical grade or better and were used without further purification. All buffers and reagents were passed through Chelex 100 ion exchange resin (Bio-Rad Laboratories, Richmond, CA) to remove contaminating transition metal ions.

Rat liver and lung microsomes were isolated from Sprague-Dawley rats by the method of Pederson et al. (1973). Total microsomal lipids were extracted from freshly isolated microsomes by the procedure of Folch et al. (1957), taking care to prevent exposure to atmospheric oxygen. All solvents were purged with argon and all steps were done under argon at 4°C to minimize autoxidation of unsaturated lipids. Extracted lipid was stored under argon

in argon-saturated $CHCL_3$:CH_3OH (2:1) at -20°C in sealed glass tubes wrapped with aluminum foil. Lipid phosphate determinations were performed by the method of Bartlett (1959). Liposomes were prepared anaerobically under argon by indirect sonication of the extracted microsomal lipid in argon-saturated 50 mM NaCl (Pederson et al., 1973). In certain cases, distearoyl-L-a-phosphatidylcholine and/or a-tocopherol in $CHCL_3$:CH_3OH (2:1) were mixed with lipids prior to removal of solvent and sonication.

Lipid peroxidation was done using both microsomal and liposomal systems. Microsomal lipid peroxidation reaction mixtures contained microsomes (0.6 mg protein/ml), ADP-Fe^{+3} (500 uM ADP, 100 uM $FeCl_3$), and NADPH (0.4 mM) in 50 mM NaCl. Liposomal reaction mixtures contained liposomes (0.25 umoles lipid phosphate/ml), ADP-Fe^{+3} (500 uM ADP, 100 uM $FeCl_3$), EDTA-Fe^{+3} (110 uM EDTA, 100 uM $FeCl_3$), protease-solubilized NADPH-cytochrome P450 reductase (0.1 units/ml), and NADPH (0.4 mM) in 50 mM NaCl. Reactions were carried out at pH 7.0 at 37°C in a shaking water bath and initiated by addition of NADPH. Lipid peroxidation was assayed by thiobarbituric acid (TBA) reactive material. The molar extinction value for malondialdehyde (MDA) (1.54×10^5 M^{-1}, cm^{-1}) was used (Buege and Aust, 1978) and the data expressed as nmole MDA per minute per mg protein for microsomes or per umole lipid phosphate for liposomes. Butylated hydroxytoluene was not included in the TBA reagent such that the assay would include all product that

would produce TBA reactive substances (Buege and Aust, 1978).

Protease-solubilized NADPH-cytochrome P450 reductase was isolated from microsomes of male Sprague-Dawley rats previously treated with 0.1% phenobarbital in their drinking water for 10 days with some modification of the procedure of Pederson et al. (1973). Fractions from the Sephadex G-100 column (Pharmacia Fine Chemicals, Piscataway, NJ) containing NADPH-cytochrome c reductase activity were pooled and subjected to affinity chromatography on 2',5'-ADP agarose (PL Biochemicals, Milwaukee, WI). The purified enzyme was assayed in 50 mM NaCl with activity expressed as one unit equaling 1 umole cytochrome c reduced/min/ml.

Analyses for fatty acids were by gas-liquid chromatographic analysis of methyl esters prepared by the method of Morrison and Smith (1964). Chromatography was performed on a Varian Model 3700 gas chromatograph equipped with a flame ionization detector. The glass column (6 ft x 1/4 inch outer diameter) was packed with 10% DEGS-PS on 80/100 Supelcoport (Supelco, Bellefonte, PA). The percentage PUFA was calculated by dividing the sum of peak area corresponding to linoleic acid (18:2), arachidonic acid (20:4), and docosahexenoic acid (22:6) by the total assignable peak area x 100. Fatty acid methyl ester standards were purchased from Nu-Chek Prep (Elysian, MN).

Vitamin E determinations were done on 1 ml incubation

samples in triplicate according to Desai (1984) using 2 ml of absolute ethanol and 2 ml of saturated NaCl containing 7.5% L-ascorbic acid, followed by addition of 0.3 ml saturated KOH and saponification at 60°C for 30 min. Then vitamin E was extracted with 3 ml of hexane. Analyses were done by reverse phase high performance liquid chromatography and fluorescence detection with excitation at 290 nm and emission at 330 nm (Shimadzu Fluorescence Spectrophotometer, Model RF530S, Kyoto, Japan). The column (250 mm x 4.6 mm, internal diameter) was packed with Econosphere C18, 5 micron (Alltech/Applied Science, Deerfield, IL/State College, PA). The mobile phase was glass distilled methanol containing 2.5% water. The retention time for vitamin E was about 8 min.

RESULTS

Table 1 compares rates of lipid peroxidation in lung and liver microsomes. Enzymatic lipid peroxidation in liver microsomes required NADPH and ADP-Fe^{+3}. Substitution of EDTA-Fe^{+3} for ADP-Fe^{+3} did not support lipid peroxidation. However, addition of EDTA-Fe^{+3} to an NADPH and ADP-Fe^{+3} containing system increased the rate of peroxidation. A similar addition of paraquat, which undergoes cyclic reduction and autoxidation to produce O_2^- and initiate lipid peroxidation (Bus et al., 1974), also stimulated peroxidation. No lipid peroxidation was detected in lung microsomes under any of these conditions.

The use of a non-enzymatic lipid peroxidation system, ascorbate and ADP-Fe^{+3}, allowed for differentiation between resistance to lipid peroxidation based on differences in microsomal enzymes and that due to inherent properties of the membranes. This comparison was conducted as we, in agreement with others (Buege and Aust, 1975; Kornburst and Mavis, 1980), found cytochrome P450 and NADPH cytochrome P450 reductase levels to be considerably lower in lung microsomes (data not shown). Ascorbate-dependent lipid peroxidation data (Table 1) showed lung microsomes were

Table 1. Rates of lipid peroxidation in rat lung and liver microsomes.[a]

		Lung	Liver
Enzymatic			
	NADPH	0.01	0.1
	+ EDTA-Fe^{+3}	0.05	0.1
	+ ADP-Fe^{+3}	0.05	3.12
	+ EDTA-Fe^{+3} ADP-Fe^{+3}	0.05	6.95
	+ ADP-Fe^{+3} Paraquat	0.05	3.93
Non-enzymatic			
	Ascorbate		
	+ADP-Fe^{+3}	0	6.72

Enzymatic lipid peroxidation incubations (3 ml) contained microsomes (0.6 mg protein/ml) and NADPH (0.4 mM) in 50 mM NaCl, pH 7.0. EDTA-Fe^{+3} (110 uM EDTA, 100 uM $FeCl_3$), paraquat (150 uM) were added either alone or in combination. Non-enzymatic lipid peroxidation incubations (3 ml) contained microsomes (0.6 mg protein/ml), ascorbate (50 uM), and ADP-Fe^{+3} (500 uM ADP, 100 uM $FeCl_3$) in 50 mM NaCl, pH 7.0.

[a]nmole malondialdehyde min^{-1} mg $protein^{-1}$

still resistant to lipid peroxidation under conditions where lipid peroxidation occurred in liver microsomes.

Liposomes derived from lung microsomal lipid were also resistant to lipid peroxidation in a reconstituted system employing isolated NADPH-cytochrome P450 reductase. Table 2 contains the rates of lipid peroxidation, the percentage PUFA of total fatty acids, the vitamin E content and a calculated ratio of PUFA to vitamin E. The percentage of PUFA was much lower in lung lipids and the vitamin E content was about 5 times higher than in liver lipids. When these differences are expressed in terms of the PUFA to vitamin E ratio, there was a 9.4 fold difference between lung and liver.

The relative percentage of the various fatty acids in liposomes prepared from isolated lung and liver microsomal lipids before and after peroxidation are shown in Table 3. Fatty acid determinations following lipid peroxidation were done to determine the relative amount of individual polyunsaturated fatty acids lost and to confirm the data obtained using the TBA assay. Fatty acid composition from lung liposomes did not change over a 90 min incubation time while that from liver liposomes was changed after 10 minutes (Table 3). Liver linoleic acid (18:2), arachidonic acid (20:4), and docosahexenoic acid (22:6) as percentages of palmitic acid (16:0) decreased 8, 33, and 24%, respectively, over the 10 minute incubation period. These

Table 2. Lipid peroxidation rates, percentage polyunsaturated fatty acids, and vitamin E levels in rat lung and liver microsomal lipids.

	Lung	Liver
Lipid peroxidation rate[a]	0.01	7.15
Percentage polyunsaturated fatty acids	28	54
Vitamin E content[b]	2.13	0.43
Polyunsaturate/vitamin E[c]	131	1235

Peroxidation reaction mixtures (3 ml) contained liposomes prepared as in Materials and Methods (0.25 umole lipid phosphate/ml), protease solubilized cytochrome P450 reductase (0.1 unit/ml), EDTA-Fe^{+3} (110 uM EDTA, 100 uM $FeCl_3$), ADP-Fe^{+3} (500 uM ADP, 100 uM $FeCl_3$) and NADPH (0.4 mM) in 50 mM NaCl, pH 7.0. Percentages polyunsaturated fatty acids (PUFA) (w/w) was determined by adding GC peak areas corresponding to linoleic acid (18:2), arachidonic acid (20:4), and docosahexenoic acid (22:6) and dividing by the total sum of fatty acid peak areas x 100. Vitamin E content was determined as given in Materials and Methods.

[a]nmoles MDA/umole lipid PO_4/min.
[b]nmole/umole lipid PO_4.
[c]nmoles PUFA/nmoles vitamin E/umole lipid PO_4.

Table 3. Liposomal fatty acid contents before and after lipid peroxidation.

Fatty acid	Lung time of incubation[a] 0	Lung time of incubation[a] 90	Liver time of incubation[a] 0	Liver time of incubation[a] 10
16:0	100 (35%)[b]	100	100 (21%)	100
16:1	6	8	0	0
18:0	40	41	149	151
18:1	45	45	51	52
18:2	33	33	81	73
20:4	35	36	99	66
22:6	9	10	58	34

Fatty acid contents were determined by GC as given in Materials and Methods. Lipid peroxidation mixtures (22 ml) contained liposomes prepared as in Materials and Methods (0.25 umole lipid phosphate/ml), protease solubilized cytochrome P450 reductase (0.1 unit/ml), EDTA-Fe^{+3} (110 uM EDTA, 100 uM $FeCl_3$), ADP-Fe^{+3} (500 uM ADP, 100 uM $FeCl_3$) and NADPH (0.4 mM) in 50 mM NaCl, pH 7.0. Values expressed as % of palmitic acid (16:0) (w/w).

[a]min.
[b]The values in parentheses represent the percentage of palmitic acid (16:0) in each sample.

decreases represent respective losses of 10, 33, 41% of the original amount of 18:2, 20:4, and 22:6.

To further investigate the role of PUFA in resistance to lipid peroxidation and to evaluate the role of vitamin E, liposomes were made from liver microsomal lipids mimicking those from the lung with respect to percentage PUFA and vitamin E content. This was done by addition of vitamin E or lipid containing saturated fatty acids. Neither the addition of distearoyl-L-a-phosphatidylcholine nor vitamin E to isolated liver microsomal lipids to equal the amount present in lung lipids (percentage PUFA or nmoles vitamin E/umole lipid phosphate) resulted in prevention of lipid peroxidation. Addition of saturated phospholipid resulted in a lower rate of peroxidation but did not abolish it. When vitamin E was added, peroxidation proceeded essentially as it had in non-supplemented lipids (Figure 3).

These results indicated that alone, neither the PUFA nor the vitamin E content of lung microsomal lipids could account for the observed resistance to lipid peroxidation. Thus, attention was turned to the role of an interaction between the two. Liposomes were made from liver microsomal lipid and vitamin E and/or distearoyl-L-a-phosphatidylcholine to provide liposomes with either 28% or 54% PUFA and various concentrations of vitamin E. (Twenty-eight percent polyunsaturates was the amount present in lung microsomal lipids and 54% was the amount in liver.

Figure 3.

Effect of adding vitamin E or saturated phospholipid to liver liposomes to achieve levels present in lung microsomal lipids.

Incubations (5 ml) contained contained liposomes prepared as in Materials and Methods (0.25 umole lipid phosphate/ml), protease solubilized cytochrome P450 reductase (0.1 unit/ml),EDTA-Fe^{+3} (110 uM EDTA, 100 uM $FeCl_3$), ADP-Fe^{+3} (500 uM ADP, 100 uM $FeCl_3$) and NADPH (0.4 mM) in 50 mM NaCl, pH 7.0. Alpha-tocopherol (1.7 nmol/umol lipid phosphate) was added to lipids as given in Materials and Methods. Malondialdehyde (MDA) formation was monitored by TBA reactive material at 535 nm as given in Materials and Methods.

■ liposomes

□ liposomes with added a-tocopherol

○ liposomes with added saturated lipid.

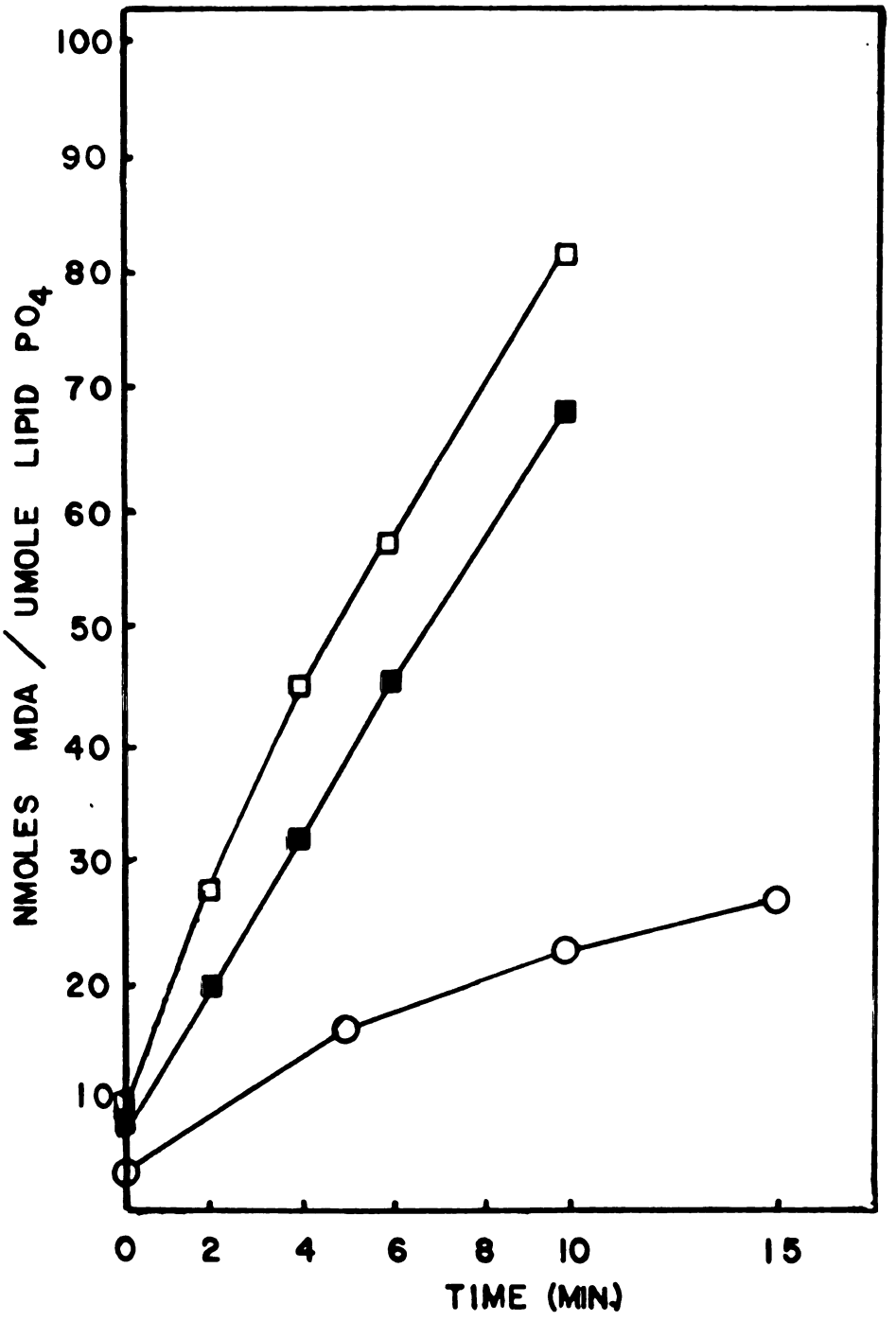

NMOLES MDA / UMOLE LIPID PO4
100
90
80
70
60
50
40
30
20
10
0
2
4
6
8
10
15
TIME (MIN)

Figure 3

Lung microsomal lipid could not be used in this determination because it could not be modified sufficiently to peroxidize.) At 28% polyunsaturates, vitamin E was added to create ratios of PUFA to vitamin E ranging from 225 to 2273. At 54% PUFA the ratio varied from 102 to 1250. The effects of these varying ratios were assessed by quantifying MDA production and vitamin E recovery after incubation in the reconstituted lipid peroxidation system. At both percentages, there were sharp demarcations between ratios where lipid peroxidation occurred and where it did not (Figures 4 and 5). At 28% PUFA, MDA production was less than 8 nmoles/umole lipid phosphate in 15 minutes and vitamin E recoveries were greater than 90% when the polyunsaturate to vitamin E ratio was 290 or less. At ratios of 410 or greater roughly 25 nmoles of malondialdehyde/umole lipid phosphate were produced in 15 minutes (Figure 4). In 54% PUFA, MDA levels were 10 nmoles/umole lipid phosphate or less and vitamin E recoveries were nearly 100% when the polyunsaturate to vitamin E ratio was 233 or less. At ratios of 263 or more, vitamin E recoveries were between 40 and 50% and MDA production levels exceeded 30 nmoles/umole lipid phosphate (Figure 5).

Figure 4.

The effect of vaying the polyunsaturate fatty acid (PUFA) to vitamin E ratio on malondialdehyde (MDA) production and vitamin E recovery after lipid peroxidation in liposomes containing 28% PUFA. Non-a-tocopherol fortified liposomes contained a PUFA to vitamin E ratio of 2273.

nmoles MDA/umole lipid phosphate after 15 min of incubation.

percentage vitamin E recovaery after 15 min of incubation.

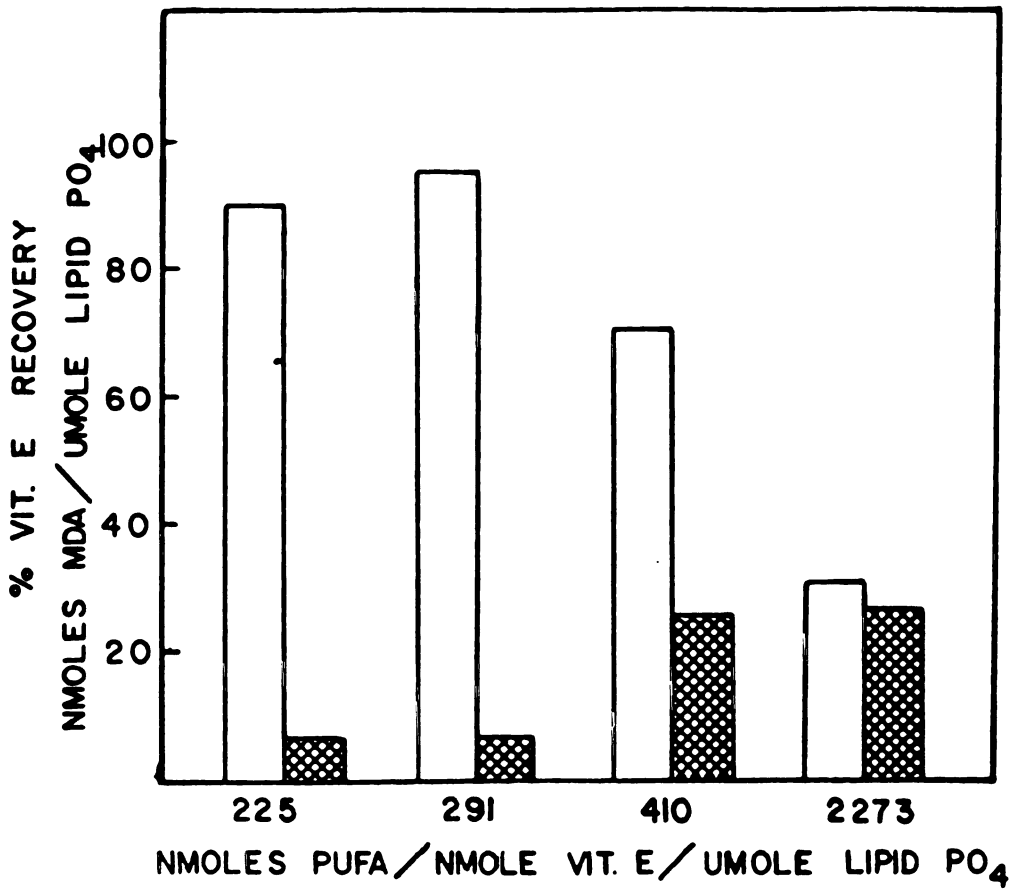
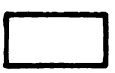

% VIT. E RECOVERY
NMOLES MDA / UMOLE LIPID PO4
100
80
60
40
20
225
291
410
2273
NMOLES PUFA / NMOLE VIT. E / UMOLE LIPID PO4

Figure 4

Figure 5.

The effect of vaying the polyunsaturate fatty acid (PUFA) to vitamin E ratio on malondialdehyde (MDA) production and vitamin E recovery after lipid peroxidation in liposomes containing 54% PUFA. Non-a-tocopherol fortified liposomes contained a PUFA to vitamin E ratio of 1250.

nmoles MDA/umole lipid phosphate after 15 min of incubation.



percentage vitamin E recovaery after 15 min of incubation.

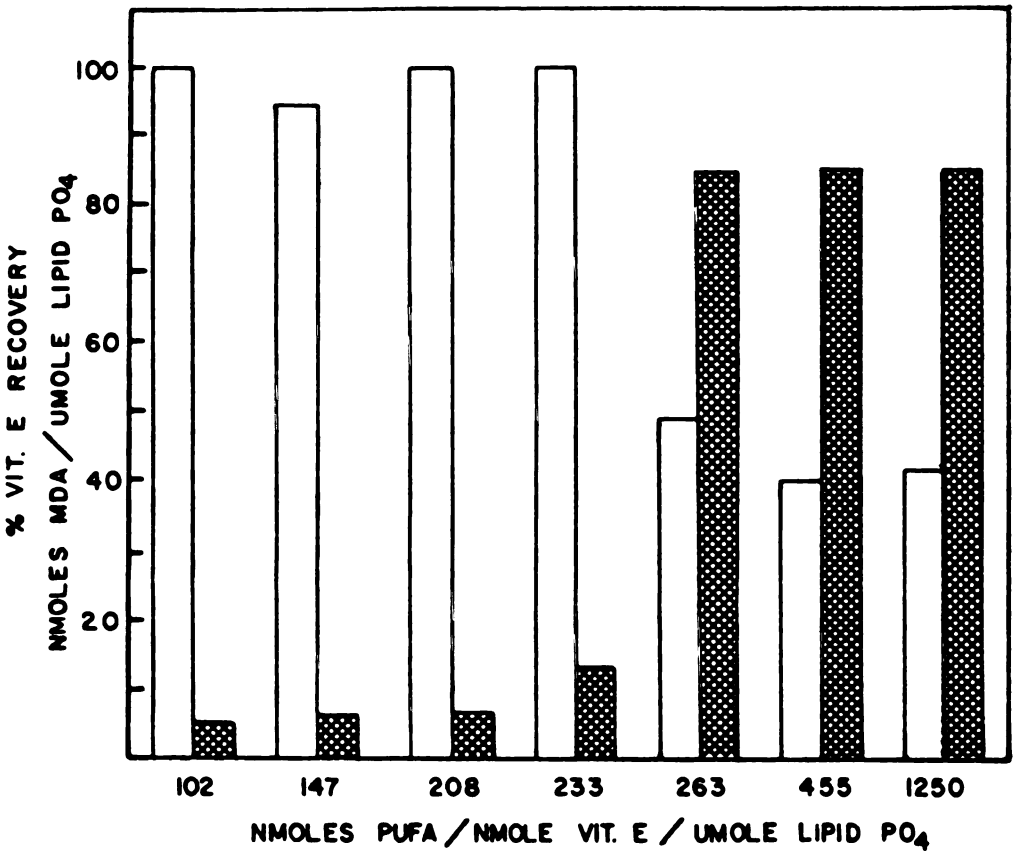

% VIT. E RECOVERY
NMOLES MDA / UMOLE LIPID PO4
100
80
60
40
20
102
147
208
233
263
455
1250
NMOLES PUFA / NMOLE VIT. E / UMOLE LIPID PO4

Figure 5

DISCUSSION

The resistance of rat lung microsomes to enzymatic and non-enzymatic lipid peroxidation as compared to liver microsomes is well documented (Kornburst and Mavis, 1980; Trush et al., 1981). This is most often attributed to the relatively high vitamin E content of lung microsomal membranes (Kornburst and Mavis, 1980). As a lipid soluble antioxidant, vitamin E is considered to be essential for the maintenance and integrity of cellular membranes. Vitamin E is thought to function as a chain-breaking terminator of the self-propagating peroxidative process by reducing lipid peroxyl radicals to less reactive species (Tappel, 1980). Our initial studies, however, indicated that other factors, in addition to vitamin E content, may play a major role in controlling lipid peroxidation in rat lung microsomes and phospholipids prepared from them. The relative susceptibility of phospholipid membranes to peroxidation was found to depend also on the PUFA content; the PUFA to vitamin E ratio accurately predicted whether or not peroxidation occurred. These results differ slightly from those obtained by Kornburst and Mavis (1980) who found that the ratio was important in determining the rate and

extent of peroxidation, not its occurrence. In their work, using an ascorbate and iron dependent, non-enzymatic system, microsomes from many rat tissues including lung were shown to peroxidize. We, on the other hand, were unable to demonstrate peroxidation in rat lung microsomes using an NADPH-enzyme dependent or an ascorbate-iron dependent system.

The polyunsaturate to vitamin E ratio was found to be critical in determining the occurrence of lipid peroxidation but had little effect on its rate. Addition of vitamin E to liver lipid resulted in complete inhibition of lipid peroxidation when the ratio of polyunsaturates to vitamin E was below a critical value. At ratios above this value, lipid peroxidation occurred at rates which were independent of vitamin E content. These results are in agreement with those of Fukuzawa et al. (1985) who, working with egg yolk lecithin (at a constant polyunsaturate content), found that peroxidation did not occur above a critical vitamin E level. In our work, rates of lipid peroxidation, when it occurred, were found to vary with PUFA content and hence, could be varied by adding different amounts of saturated lipid to liver microsomal lipids. The dependence of rates of lipid peroxidation on polyunsaturate content agrees with the results of Tien and Aust (1982) who compared lipid peroxidation rates in rabbit and rat liver microsomes. Our data indicate the original ratio below which lipid peroxidation does not occur is approximately

230 in liver phospholipids and 290 in liver phospholipids to which saturated fatty acid phospholipid had been added to mimic lung phospholipids in percentage PUFA. Therefore, lung microsomal lipids with a polyunsaturate to vitamin E ratio of 131, have a much lower ratio than is necessary to inhibit lipid peroxidation.

Our results, in which we were unable to demonstrate lipid peroxidation in rat lung microsomes or lipids derived from the microsomes, were similar to results obtained by Trush et al. (1981). Using a NADPH generating system as a source of reducing equivalents, they reported nearly complete resistance of rat lung microsomes to peroxidation. They further showed that inclusion of paraquat in the peroxidation system or use of lung microsomes from vitamin E deficient rats resulted in lipid peroxidation. When we included paraquat there was not an increase in lipid peroxidation, perhaps because NADPH was used as the reductant. The effect of paraquat was reported to be dependent on a high steady-state concentration of NADPH. Based on their data, the concentration of NADPH used in our studies should have produced a marginal effect, but NADPH was added only at the initiation of the reaction and thus, did not represent steady-state concentration.

The vitamin E assay procedure used in this study included ascorbate as a reductant to recover any reversibly oxidized vitamin E. Thus, any decrease in vitamin E recovery represented irreversible oxidation. Accordingly,

nearly 100% of the vitamin E was recovered in the absence of lipid peroxidation. However, when lipid peroxidation occurred, vitamin E was much lower and recoveries were inversely related to PUFA content. Fukuzawa et al. (1985) using a vitamin E determination method without a reductant, showed a slow rate of vitamin E loss in the absence of lipid peroxidation in membranes containing polyunsaturated fatty acids. They also reported, and we have confirmed, that vitamin E levels remain constant in membranes not containing polyunsaturated fatty acids.

REFERENCES

Bartlett, G.R. Phosphorus assay in column chromatography. J. Biol. Chem. 234:497-471. (1959).

Buckingham, K.W. Effect of dietary polyunsaturated/saturated fatty acid ratio and dietary vitamin E on lipid peroxidation in the rat. J. Nutr. 115:1425-1435. (1985).

Buege, J.A., and S.D. Aust. Comparative studies of rat liver and lung NADPH cytochrome c reductase. Biochem. Biophys. Acta 385:371-379. (1975).

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

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

Comporti, M. Biology of disease: Lipid peroxidation and cellular damage in toxic liver injury. Lab. Invest. 53:599-623. (1985).

Desai, I.D. Vitamin E analysis methods for animal tissues. In: Methods in Enzymology, Volume 105 (S.P. Colowick, N.O. Kaplan, and L. Packer, eds.) pp. 138-147, Academic Press, New York (1984).

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

Fukuzawa, K., S. Takase, and H. Tsukatani. The effect of concentration on antioxidant effectiveness of a-tocopherol in lipid peroxidation induced by superoxide free radicals. Arch. Biochem. Biophys. 240:117-120. (1985).

Gutteridge, J.M.C. The membrane effects of vitamin E, cholesterol, and their acetates on peroxidative susceptibility. Res. Comm. Chem. Pathol. Pharm. 22:563-572. (1978).

Horwitt, M.K. Interrelations between vitamin E and polyunsaturated fatty acids in adult men. Vit. Horm. 30:541-558. (1962).

Kornburst, D.J., and R.D. Mavis. Relative susceptibility of microsomes from lung, heart, liver, kidney, brain, and testes to lipid peroxidation: Correlation with vitamin E contents. Lipids 15:315-322. (1980).

McCay, P.B. Vitamin E: Interactions with free radicals and ascorbate. Ann. Rev. Nutr. 5:323-340. (1985).

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

Morrison, W.R., and L.M. Smith. Preparation of fatty acid methyl esters and dimethylacetals from lipid with boronfluoride-methanol. J. Lipid Res. 4:600-615. (1964).

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

Tappel, A.L. Vitamin E and free radical peroxidation of lipids. Ann. N.Y. Acad. Sci. 203:12-28. (1972).

Tappel, A.L. Vitamin E and selenium protection from in vivo lipid peroxidation. Ann. N.Y. Acad. Sci. 355:18-31. (1980).

Tien, M., and S.D. Aust. Rabbit liver microsomal lipid peroxidation: The effect of lipid on the the rate of peroxidation. Biochem. Biophys. Acta 712:1-9. (1982).

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

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

Winterbourn, C.C., and H.C. Sutton. Iron and xanthine oxidase catalyze formation of an oxidant species distinguishable from $OH^{\cdot}$: Comparison with Haber-Weiss reaction. Arch. Biochem. Biophys. 244:27-34. (1986).

Chapter II

The Effect of Iron and Reduced Glutathione on Vitamin E-Dependent Inhibition of Lipid Peroxidation in Rat Liver Microsomes.

ABSTRACT

Addition of reduced glutathione (GSH) to an NADPH-dependent lipid peroxidation system containing rat liver microsomes lowered the rate of lipid peroxidation in proportion to the microsomal vitamin E concentration. The lipid peroxidation system contained rat liver microsomes, ADP-Fe^{+3}, and NADPH. Vitamin E was added to microsomes to achieve values greater than 0.14 nmol/mg protein. Vitamin E concentrations greater than approximately 1.0 nmol/mg protein completely inhibited peroxidation regardless of the presence of GSH. In microsomes containing 0.14 nmol vitamin E/mg protein, the addition of GSH resulted in a 42% decrease in the rate of peroxidation. Ninety percent inhibition was obtained using microsomes containing approximately 0.25 nmol of vitamin E/mg protein. This level of inhibition corresponded to a lag phase observed before rapid peroxidation began. The duration of the lag phase and the amount of malondialdehyde produced during the lag phase were functions of the microsomal vitamin E concentration, iron concentration and the ratio of the iron chelator (ADP) to iron. Lower iron concentrations and higher ADP:Fe^{+3} ratios resulted in shorter lag phases with

greater rates of MDA production. The higher the ADP:Fe^{+3} ratio the slower the rate of iron reduction by GSH. In lipid peroxidation incubations containing different ADP:Fe^{+3} ratios, lipid hydroperoxide concentrations were inversely proportional to the lengths of the lag phases. These results suggest that GSH affected lipid peroxidation in two ways; GSH maintained vitamin E in the reduced form, and it interacted with iron to maintain low lipid hydroperoxide concentrations. Both functions were necessary for inhibition of microsomal lipid peroxidation. The rate of accumulation of lipid hydroperoxides determined the length of the lag phase, and the end of the lag phase coincided with a lipid hydroperoxide concentration such that the vitamin E concentration was insufficient to prevent the propagation phase of lipid peroxidation.

INTRODUCTION

Vitamin E has been proposed to inhibit propagation of lipid peroxidation via donation of a hydrogen atom to a lipid peroxyl radical to form a lipid hydroperoxide and reversibly oxidized vitamin E (a chromanoxyl radical) (McCay, 1985). Theoretical calculations for the inhibition of lipid peroxidation by vitamin E by Niki et al. (1986) were based on competing reaction rates of lipid peroxyl radicals ($LO_2\cdot$) with polyunsaturated fatty acids (LH) (reaction 7) or with vitamin E (IH) (reaction 8). In these reactions, $LO_2\cdot$ either abstracts a hydrogen from LH to propagate lipid peroxidation or from IH which is a termination reaction.

$$LO_2\cdot + LH \longrightarrow LOOH + L\cdot \quad (7)$$

$$LO_2\cdot + IH \longrightarrow LOOH + I\cdot \quad (8)$$

The efficiency of inhibition of peroxidation according to this model depends on relative rates of reactions 7 and 8, which in turn depend on the relative concentrations of polyunsaturated fatty acids and vitamin E. In addition, the limited mobility of vitamin E in a membrane system may be an important factor determining the rate of reaction (8). Hence, in a membrane system such as in microsomes,

inhibition of peroxidation could be expected to require more vitamin E than a homogeneous solution.

Since vitamin E is consumed during inhibition of peroxidation, the relative rates of reactions (7) and (8) change in favor of reaction (7). Therefore, the vitamin E required for *in vitro* inhibition must take consumption into account. *In vivo* cellular processes, on the other hand, presumably maintain vitamin E in the reduced form, and thus, inhibition of *in vivo* lipid peroxidation by vitamin E does not have to account for its consumption. Reddy *et al.* (1982) reported that reduced glutathione (GSH) lowered the concentration of vitamin E required to inhibit microsomal lipid peroxidation. They proposed that GSH maintained vitamin E levels by donating a hydrogen to the chromanoxyl radical, and they suggested involvement of a membrane protein in this mechanism. The function of the membrane protein was shown to be specific for GSH (Reddy *et al.*, 1982; Burk, 1983; McCay *et al.*, 1986). This microsomal protein will be referred to here as GSH-dependent vitamin E reductase. The inhibitory effect of the vitamin E and GSH interaction was shown to be proportional to GSH concentration, but with the exception that vitamin E was required, no information was given on the effect of vitamin E concentration (Burk, 1983; Haenen and Bast, 1983).

GSH may affect lipid peroxidation by mechanisms other than via an interaction with vitamin E. Like other thiols, it can directly reduce iron. Hence, it may be a pro-

oxidant or an antioxidant depending on the system employed. Several authors have demonstrated a pro-oxidant effect on microsomal or liposomal lipid peroxidation. Tien et al. (1982) showed that thiol concentration determined whether there was a pro-oxidant or an antioxidant effect. With GSH, however, they found only a pro-oxidant effect. In contrast, Franco and Jenkins (1986) found GSH-dependent inhibition of lipid peroxidation using lung microsomes from rats fed a vitamin E deficient diet. Other thiols similarly inhibited peroxidation. The inhibitory effect of GSH was unaltered by heating, hence participation of vitamin E and GSH-dependent vitamin E reductase was unlikely.

In this study we investigated inhibition of microsomal lipid peroxidation in the presence of GSH as a function of vitamin E concentration. We were interested in the conditions at which the vitamin E concentration present in liver microsomes was sufficient to inhibit peroxidation. Before we could investigate the interaction of GSH and vitamin E, we had to ascertain the effect of GSH on our system in the absence of vitamin E. We found that GSH inhibited peroxidation via maintenance of reduced vitamin E concentrations and via an interaction with iron that was related to lipid hydroperoxide concentrations.

MATERIALS AND METHODS

ADP, dl-a-tocopherol, 2-thiobarbituric acid, and GSH were purchased from Sigma Chemical Company (St. Louis, MO). Ferric chloride was purchased from Baker Chemical Company (Phillipsburg, NJ). L-Ascorbic acid was purchased from Fischer Scientific Company (Fairlawn, NJ). NADPH and the vitamin E deficient rat diet were obtained from U.S. Biochemical Company (Cleveland, OH). The standard laboratory ration (Wayne Rodent Blox) was purchased from Wayne Pet Foods (Chicago, IL). All buffers and reagents used in lipid peroxidation incubations and lipid hydroperoxide determinations were passed through Chelex 100 ion exchange resin (Bio-Rad Laboratories, Richmond, CA) to remove contaminating transition metal ions.

Chelated iron (Fe^{+3}) solutions were made by dissolving 6.8 mg $FeCl_3 \cdot 6H_2O$ in 0.5 ml of water and lowering the pH by adding 1 N HCl until the solution became pale yellow. Depending on the ADP:iron ratio desired, the appropriate amount of ADP was dissolved in 4.5 ml of water and the pH adjusted to between 4.4 and 4.7 with 1 N NaOH. The iron solution was then added to the ADP solution 20 ul at a time while maintaining a constant pH. Finally, the pH of the

solution was adjusted to 7.0. ADP-Fee^{+3} solutions were stored at 4°C for 3 days before use.

Rat liver microsomes were isolated from Sprague-Dawley rats by the method of Pederson et al. (1973) and stored in argon-saturated 50 mM Tris HCl, pH 7.4 containing 50% glycerol (v/v). Microsomes could be stored up to 1 week without loss of GSH-dependent vitamin E reductase activity. Microsomal lipid peroxidation reaction mixtures contained microsomes (0.6 mg protein/ml), ADP-Fe^{+3}, and NADPH (0.4 mM) in 50 mM NaCl. ADP-Fe^{+3} was used at 500 uM ADP and 100 uM $FeCl_3$ unless stated otherwise. Reactions were carried out at pH 7.0 and 37°C in a shaking water bath and initiated by addition of NADPH. When included, GSH from a 200 mM, pH 7.0, stock solution was added at the same time as NADPH to yield a final concentration of 5 mM. If added earlier, GSH-dependent lipid peroxidation began. Lipid peroxidation was assayed by thiobarbituric acid (TBA) reactive material. The molar extinction value for malondialdehyde (1.56×10^5 M^{-1} cm^{-1}) was used (Buege and Aust, 1978) and the data expressed as nmol malondialdehyde (MDA)/mg protein.

Vitamin E was added to microsomes as follows. First, an aliquot of dl-a-tocopherol in argon-saturated $CHCl_3$:CH_3OH (2:1) was evaporated to dryness under a stream of argon. Then, microsomes in glycerol-Tris buffer were added. The tubes were stoppered, inverted several times, wrapped with aluminum foil, left at room temperature for 10

min, and then stored at 4°C overnight. Any vitamin E not associated with the microsomal membranes was removed by diluting the microsomes 5-fold with argon-saturated glycerol-Tris buffer followed by resedimentation. Nearly 100% of the added vitamin E was found associated with the microsomes.

Total vitamin E content of the microsomes was assayed by the method of Desai (1984) except that saponification was not done. This method uses ascorbic acid, and thus, includes the reversibly oxidized chromanoxyl radical as well as vitamin E in the reduced form. Total vitamin E concentrations were reported per mg protein unless stated otherwise. Total vitamin E analyses were done in triplicate and the mean concentration and standard deviation calculated. The reduced form of vitamin E was measured by the method of Fukuzawa et al. (1985). GSH, when added directly to the assay, did not increase vitamin E content. Thus, conversion of chromanoxyl radicals to vitamin E occurred only in the lipid peroxidation incubations and not during the vitamin E assays. Vitamin E (reduced form) analyses were done in quadruplicate and mean concentrations and standard deviations calculated. All vitamin E concentrations were determined by reverse phase high pressure liquid chromatography with fluorescence detection using a column (250 mm x 4.6 mm, internal diameter) packed with Econosphere C18, 5 micron (Alltech/Applied Science, Deerfield, IL/College Station PA)

as previously outlined (Chapter I).

Lipid hydroperoxide content of microsomes was done by the iodometric method of Buege and Aust (1978). In this method, triplicate 1 ml aliquots of lipid peroxidation reaction mixtures were added to 5 ml argon-saturated $CHCl_3$:CH_3OH (2:1). After centrifugation, 3 ml of the chloroform layer were removed, placed in the dark, and dried under a stream of argon at 45°C. (The rest of the assay procedure was carried out in the dark or under indirect yellow light.) After the solvent had evaporated but still under a stream of argon, 1 ml of argon-saturated glacial acetic acid:chloroform (3:2) and 50 ul of KI solution (7.2 M in argon-saturated H_2O) were added. Taking care to exclude atmospheric air, tubes were stoppered, thoroughly mixed, and incubated at room temperature for 5 min. Then 3 ml of cadmium acetate solution (20 mM in H_2O) were added and tubes removed from the dark. Lipid hydroperoxide concentrations were determined by absorbance at 353 nm using the molar extinction coefficient of cumene hydroperoxide (1.73×10^4 M^{-1} cm^{-1}).

RESULTS

Before using GSH to maintain vitamin E concentrations, the effect of GSH on microsomal lipid peroxidation without participation of vitamin E had to be determined. The effect of 5 mM GSH on lipid peroxidation in microsomes containing very low vitamin E concentrations is shown in Figure 6. In this experiment, GSH was added to a peroxidation reaction containing liver microsomes from rats fed a vitamin E deficient diet for 8 weeks. These microsomes contained about 3% of the vitamin E (0.0045 nmol/mg protein) found in liver microsomes from rats fed the standard laboratory ration. Addition of GSH decreased the amount of MDA produced after 5 and 15 min of incubation by 20% and 5.8%, respectively. The MDA produced decreased from 45 to 36 and from 69 to 65 nmol/mg protein after 5 min and 15 min, respectively. The rate of peroxidation after 5 min of incubation decreased from 9 to 7.2 nmol MDA/min/mg protein. Even though the microsomes contained a small amount of vitamin E, participation of vitamin E was ruled out because a similar decrease in MDA production was observed using microsomes that had been stored for approximately 1 month, and thus, had lost their GSH-

Figure 6.

The Effect of GSH on Malondialdehyde Production in Liver Microsomes from Rats Fed a Vitamin E Deficient Diet.

Incubation reactions contained microsomes (0.6 mg protein/ml), ADP-Fe^{+3} (500 uM ADP, 100 uM $FeCl_3$), and NADPH (0.4 mM) in 50 mM NaCl, pH 7.0. Reactions were initiated with NADPH. GSH (5 mM) was added at the same time as NADPH. Microsomes contained 0.0045 nmol vitamin E/mg protein.

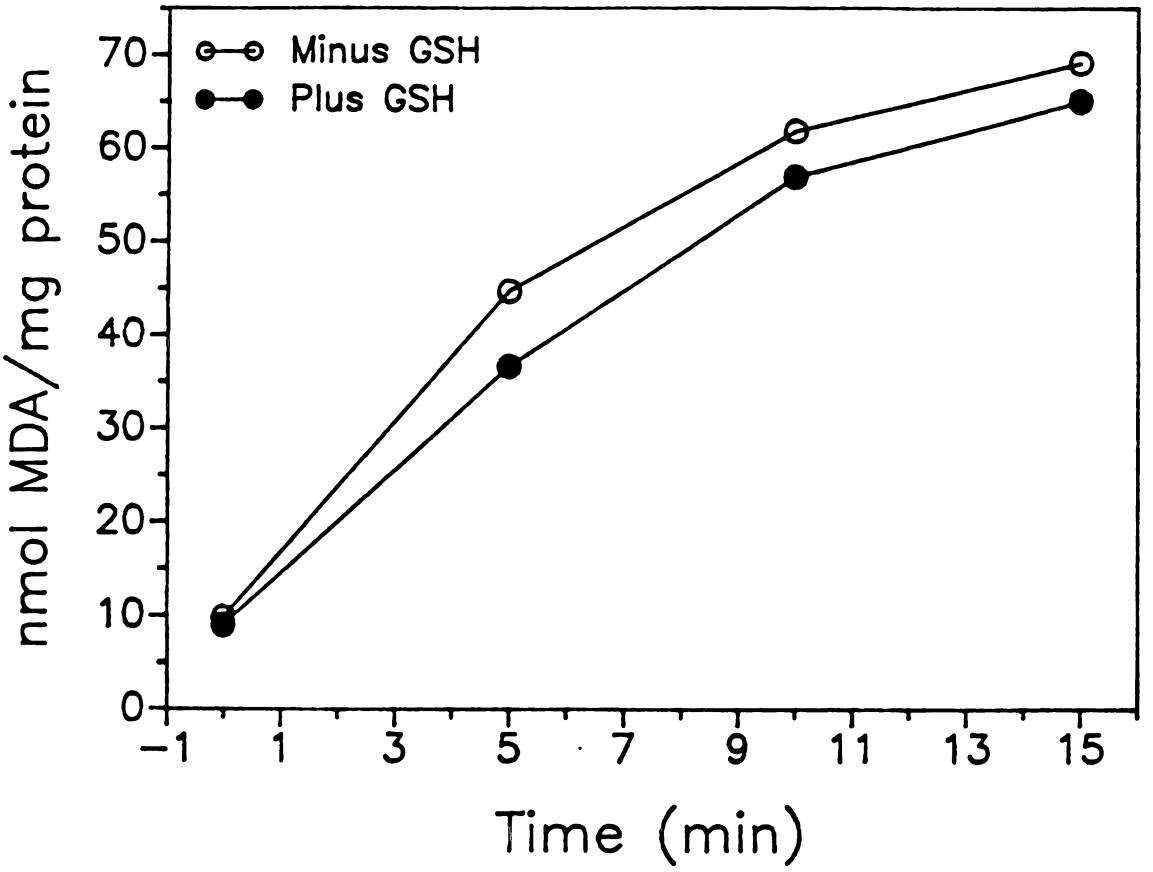

Minus GSH
Plus GSH
nmol MDA/mg protein
70
60
50
40
30
20
10
0
−1
1
3
5
7
9
11
13
15
Time (min)

Figure 6

dependent vitamin E reductase activity and most of their vitamin E (data not shown). Thus, addition of GSH resulted in partial inhibition of peroxidation without involvement of vitamin E. Yet, this direct antioxidant activity was not sufficient to preclude using GSH in further studies to maintain vitamin E concentrations.

The effect of increasing microsomal vitamin E concentrations on the rate of lipid peroxidation in the presence or absence of 5 mM GSH is shown in Figure 7. The rates were calculated from data obtained after 5 min of incubation. In the absence of GSH, rates of lipid peroxidation remained fairly constant with vitamin E concentrations up to approximately 1 nmol/mg protein. Above 1 nmol/mg protein, the rates declined. In the presence of GSH, however, the rates declined rapidly reaching approximately 90% inhibition at vitamin E concentrations of about 0.2 nmol/mg protein. A plot of MDA production at different vitamin E concentrations versus time of incubation that were used to construct Figure 7 is shown in Figure 8A. In microsomes containing 0.14 and 0.36 nmol vitamin E/mg protein, MDA production had a vitamin E concentration-dependent lag phase. And at each vitamin E concentration tested, a different amount of MDA was produced. MDA production during the lag phase was attributed to the relative rates at which reactions (7) and (8) were occurring and to limited mobility of vitamin E within the membrane. Thus, MDA production during the lag

Figure 7.

The Effect of Microsomal Vitamin E Concentration and GSH on Rates of Malondialdehyde Production.

Incubation reactions contained microsomes (0.6 mg protein/ml), ADP-Fe^{+3} (500 uM ADP, 100 uM $FeCl_3$), and NADPH (0.4 mM) in 50 mM NaCl, pH 7.0. Reactions were initiated with NADPH. GSH (5 mM) was added at the same time as NADPH. Vitamin E was added to microsomes as outlined in Materials and Methods. Vitamin E contents were reported as total vitamin E in incubations. Total vitamin E determinations were done as outlined in Materials and Methods.

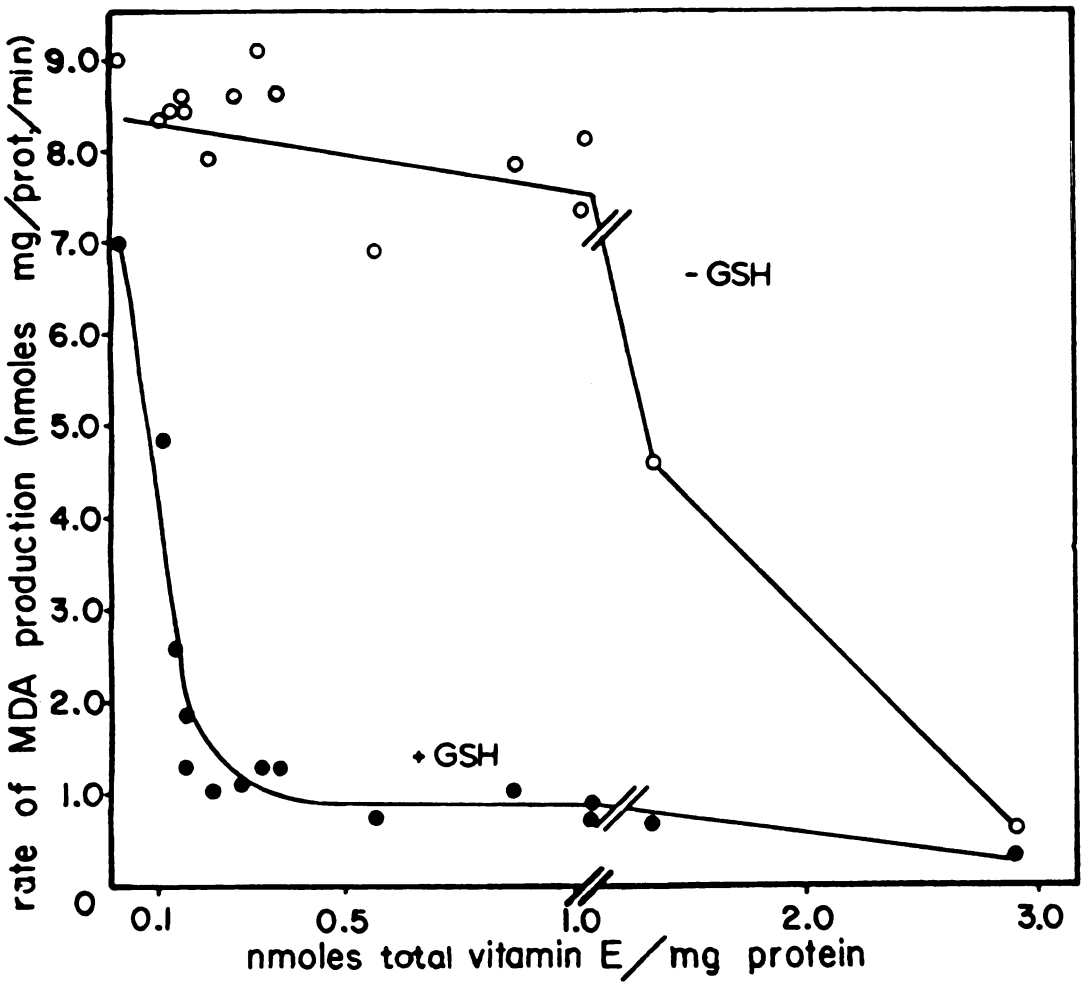

rate of MDA production (nmoles mg/prot./min)
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
- GSH
+ GSH
0.1
0.5
1.0
2.0
3.0
nmoles total vitamin E / mg protein

Figure 7

Figure 8.

The Effect of ADP-Fe^{+3} Concentration on Malondialdehyde Production in Microsomes Containing Different Vitamin E concentrations.

Incubation reactions contained microsomes (0.6 mg protein/ml), ADP-Fe^{+3}, and NADPH (0.4 mM) in 50 mM NaCl, pH 7.0. Reactions were initiated with NADPH. GSH (5 mM) was added at the same time as NADPH. A). Reaction incubation contained 100 uM ADP-Fe^{+3} (500 uM ADP, 100 uM $FeCl_3$). B). Reaction incubation contained 50 uM ADP-Fe^{+3} (250 uM ADP, 50 uM $FeCl_3$). C). Reaction incubation contained 20 uM ADP-Fe^{+3} (100 uM ADP, 20 uM $FeCl_3$). The ADP:Fe^{+3} ratios were 5:1 in all incubations. Vitamin E contents were reported as total vitamin E in incubations. Total vitamin E determinations were done as outlined in Materials and Methods.

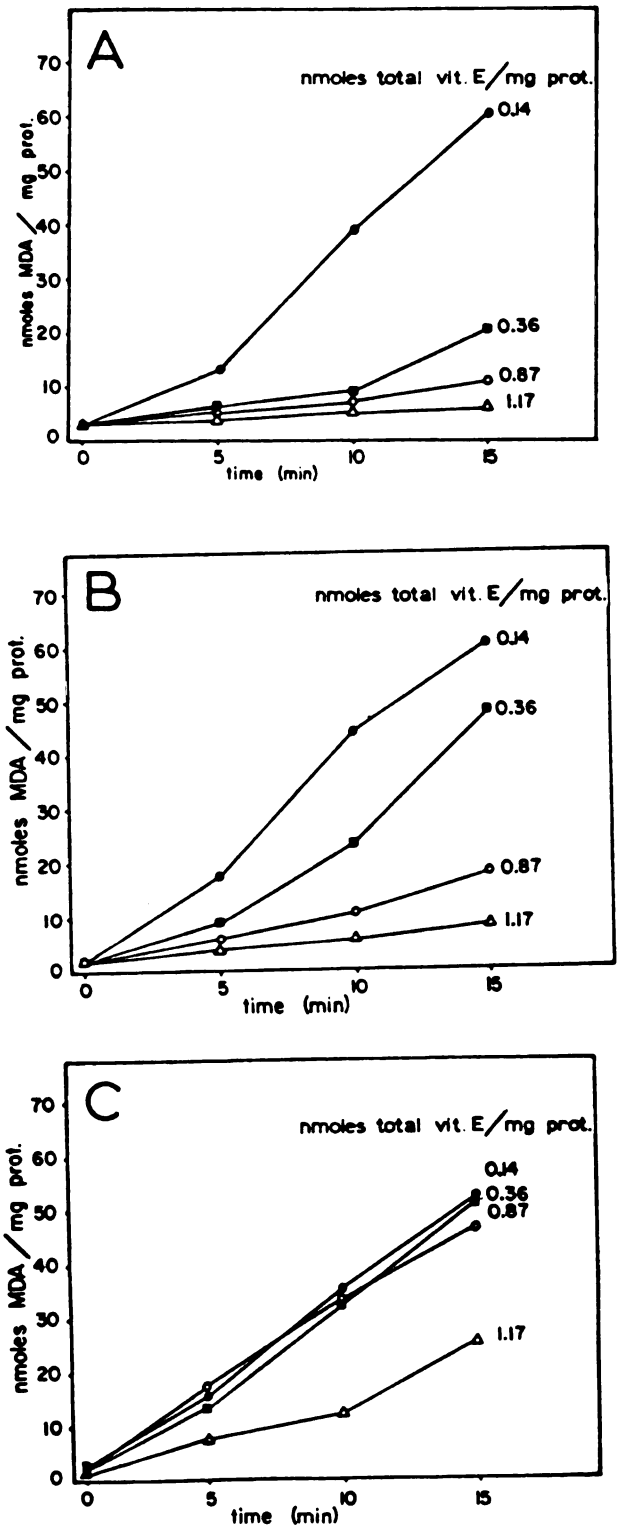

A
nmoles total vit. E/mg prot.
0.14
0.36
0.87
1.17
nmoles MDA / mg prot.
time (min)
B
nmoles total vit. E/mg prot.
0.14
0.36
0.87
1.17
nmoles MDA / mg prot.
time (min)
C
nmoles total vit. E/mg prot.
0.14
0.36
0.87
1.17
nmoles MDA / mg prot.
time (min)

Figure 8

phase may have resulted from an inability of vitamin E to reach the proximity of a lipid radical before it reacted with another polyunsaturated fatty acid.

To increase the efficacy of vitamin E to inhibit peroxidation, the rate of lipid peroxidation was lowered by decreasing the iron concentration in our reaction mixtures. The results of decreasing the ferric ion concentration in the reaction mixtures from 100 uM to 50 uM to 20 uM are shown in Figures 8A, B and C, respectively. Lowering the iron concentration did not increase the effectiveness of vitamin E to inhibit MDA production as was expected. Instead, vitamin E was progressively less effective with lower iron concentrations.

Microsomes containing 0.87 nmol vitamin E per mg protein that were used in the experiment at different iron concentrations (Figure 8) were also used to evaluate the effect of the presence and absence of GSH. Figure 9 shows the effect of different iron concentrations, with and without GSH, on MDA production. In the presence of GSH, lowering the iron concentration resulted in increased MDA production. In the absence of GSH, MDA production and hence, the rate of lipid peroxidation, was unaffected by decreasing the iron concentration from 100 uM to 50 uM. Reduction of the iron concentration to 20 uM, however, lowered the amount of MDA produced. Thus, the effect on lipid peroxidation in the presence of GSH of lowering the

Figure 9.

The Effect of ADP-Fe^{+3} Concentration and GSH on Malondialdehyde Production In Microsomes.

Incubation reactions contained microsomes (0.6 mg protein/ml), ADP-Fe^{+3}, and NADPH (0.4 mM) in 50 mM NaCl, pH 7.0. Reactions were initiated with NADPH. GSH (5 mM) was added at the same time as NADPH. ADP:Fe^{+3} ratios were 5:1. Microsomal total vitamin E concentration was 0.87 nmol/mg protein. Total vitamin E determinations were done as outlined in Materials and Methods.

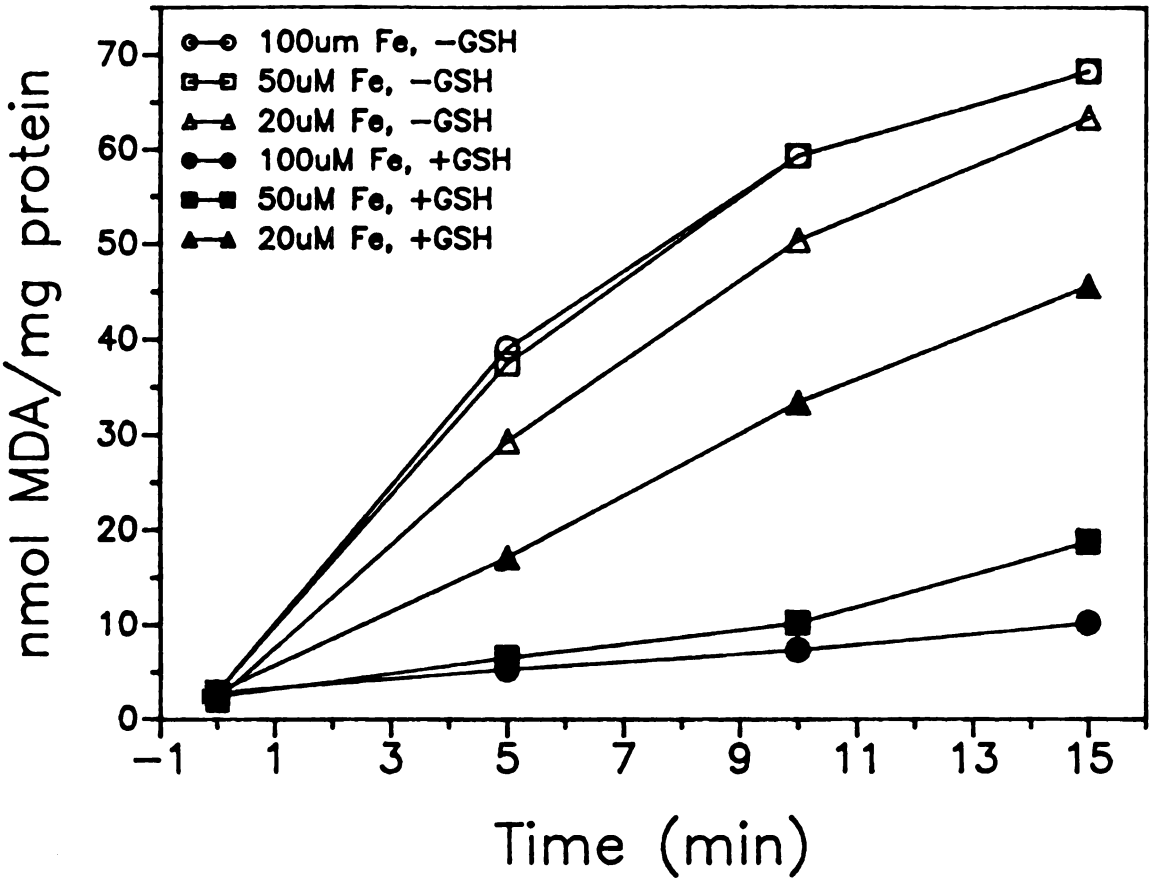

100um Fe, −GSH
50uM Fe, −GSH
20uM Fe, −GSH
100uM Fe, +GSH
50uM Fe, +GSH
20uM Fe, +GSH
nmol MDA/mg protein
0
10
20
30
40
50
60
70
−1
1
3
5
7
9
11
13
15
Time (min)

Figure 9

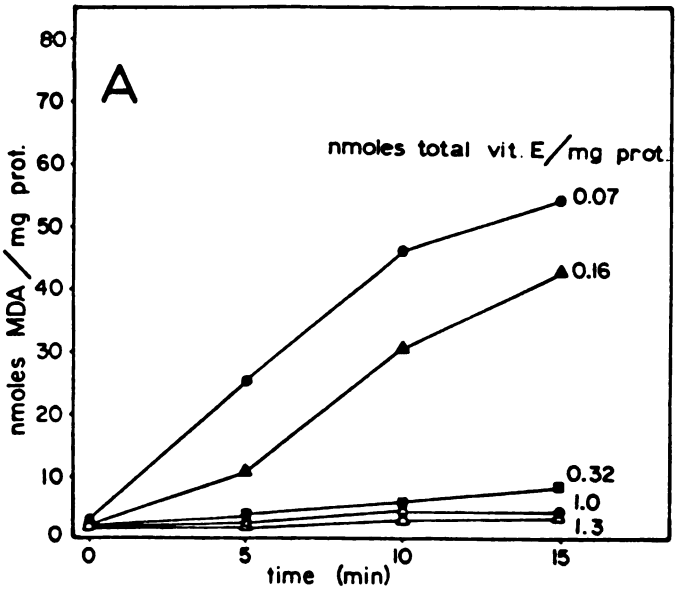
iron concentration from 100 and 50 uM iron was not related to the ability of iron to catalyze lipid peroxidation.

To show that vitamin E and GSH-dependent inhibition of lipid peroxidation involved an interaction between iron and GSH, ADP chelated iron was added to lipid peroxidation mixtures at either 5:1 or 15:1 ADP:Fe^{+3} ratios. Iron concentrations were held at 50 uM. Previous studies in the laboratory had shown that GSH-dependent reduction of ferric iron chelated by ADP depended on the ADP:Fe^{+3} ratio and was greater at low ADP:Fe^{+3} ratios (Minotti and Aust, unpublished). The 5:1 ratio (Figure 10A) required a lower vitamin E concentration to inhibit peroxidation than did the 15:1 ratio (Figure 10B). Nearly complete inhibition was observed at different vitamin E concentrations with the 2 ratios suggesting that there was an iron-GSH effect on lipid peroxidation. Whether the iron-GSH effect was involved in maintenance of vitamin E levels was investigated using 5:1, 10:1 and 15:1 ADP:Fe^{+3} ratios in the incubation reactions. Involvement of vitamin E was suspected because inhibition of lipid peroxidation in the presence of GSH was shown to be vitamin E concentration dependent (Figure 7). Except for the data generated at 15 min using the 15:1 ratio, vitamin E concentrations were within the standard deviations of each other regardless of the ADP:Fe^{+3} ratio (Figure 11). Differences were found in MDA production using different ADP:Fe^{+3} ratios just as had been observed earlier at different vitamin E concentrations

Figure 10.

The Effect of ADP:Fe^{+3} Ratios on Malondialdehyde Production in Liver Microsomes Containing Different Vitamin E Concentrations.

Incubation reactions contained microsomes (0.6 mg protein/ml), ADP-Fe^{+3} (50 uM $FeCl_3$), and NADPH (0.4 mM) in 50 mM NaCl, pH 7.0. Reactions were initiated with NADPH. GSH (5 mM) was added at the same time as NADPH. A). Incubation contained 250 uM ADP (ADP:Fe^{+3} was 5:1). B). Incubation contained 750 uM ADP (ADP:Fe^{+3} was 15:1). Vitamin E contents were reported as total vitamin E in incubations. Total vitamin E determinations were done as outlined in Materials and Methods.


A
nmoles total vit. E/mg prot.
0.07
0.16
0.32
1.0
1.3
nmoles MDA/mg prot.
time (min)
0
5
10
15
20
30
40
50
60
70
80

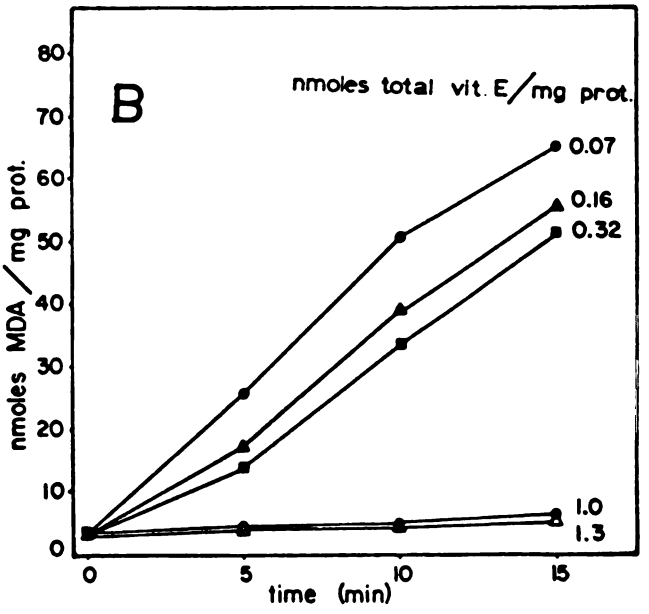

B
nmoles total vit. E/mg prot.
0.07
0.16
0.32
1.0
1.3
nmoles MDA/mg prot.
time (min)
0
5
10
15
20
30
40
50
60
70
80

Figure 10

Figure 11.

The Effect of Different ADP:Fe^{+3} Ratios on Malondialdehyde Production and Vitamin E Concentration in Microsomes.

Incubation reactions contained microsomes (0.6 mg protein/ml), ADP-Fe^{+3} (50 uM $FeCl_3$), and NADPH (0.4 mM) in 50 mM NaCl, pH 7.0. Reactions were initiated with NADPH. GSH (5 mM) was added at the same time as NADPH. The ADP:Fe^{+3} ratios were 5:1, 10:1, and 15:1). Vitamin E determinations were done in quadruplicate. Error bars indicate standard deviations.

●, ▲, ■ represents malondialdehyde production.

○, △, □ represents vitamin E concentration.

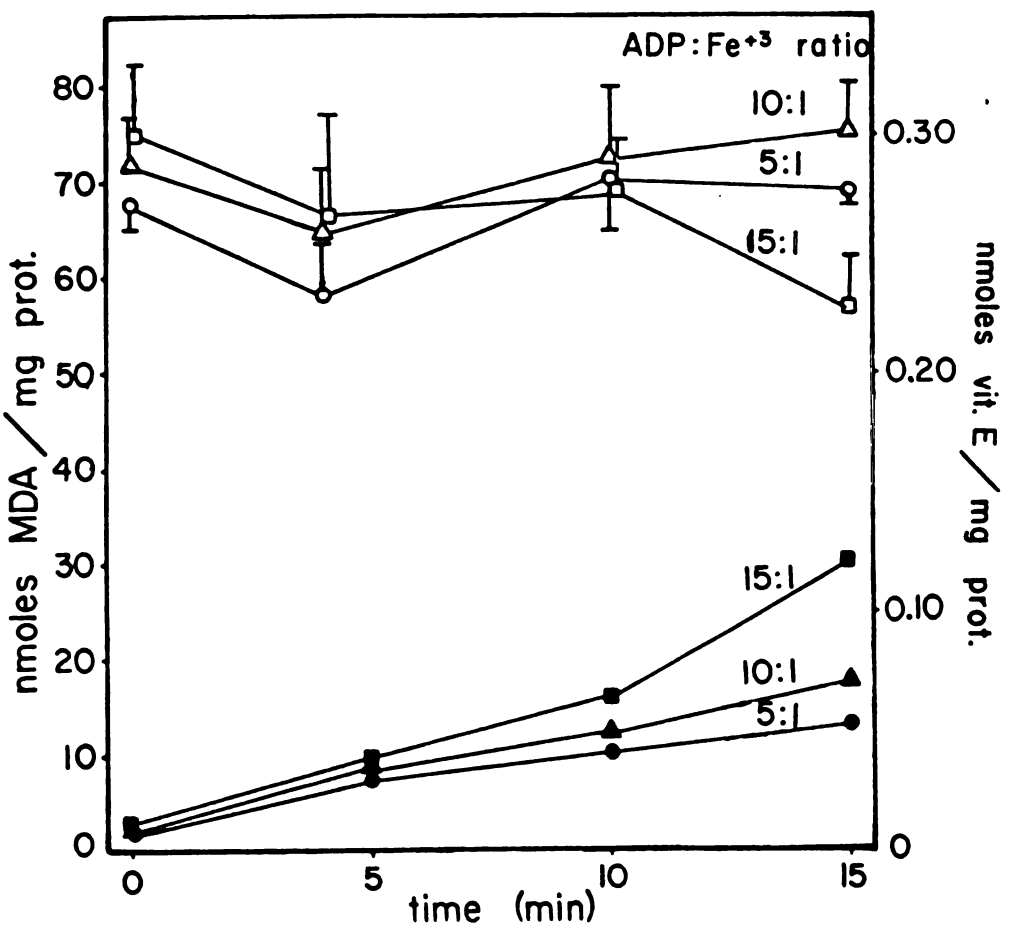

ADP:Fe+3 ratio
10:1
5:1
15:1
15:1
10:1
5:1
nmoles MDA / mg prot.
nmoles vit. E / mg prot.
time (min)
0
5
10
15
0
10
20
30
40
50
60
70
80
0
0.10
0.20
0.30

Figure 11

(Figure 8A) or different iron concentrations (Figure 9). The data obtained after 15 min of incubation using the 15:1 ADP:Fe^{+3} ratio demonstrated that vitamin E concentrations remained constant until the end of the lag phase regardless of MDA production, and indicated that the differences in inhibition of lipid peroxidation could not be attributed to differences in vitamin E concentrations. Thus, the interaction between GSH and iron did not involve vitamin E.

Since the proposed mechanism of vitamin E action results in the production of lipid hydroperoxides, lipid hydroperoxide concentrations were measured to determine if the interaction between GSH and iron was related to the concentration of hydroperoxides. Figure 12 shows the amounts of MDA and lipid hydroperoxides produced in incubation reactions using microsomes which contained sufficient vitamin E to inhibition lipid peroxidation in the presence of GSH. In the presence of GSH, lipid hydroperoxide production was roughly 2-fold higher than MDA production. In contrast, hydroperoxide production was about 4-fold higher than that of MDA in the absence of GSH. While these results demonstrated a relative difference between MDA and hydroperoxide concentrations depending on the presence of GSH, a direct influence of GSH on hydroperoxide concentrations was not established because the relative differences in MDA and hydroperoxide production could have been related to the extent of lipid peroxidation.

Figure 12.

The Effect of GSH on Malondialdehyde and Lipid Hydroperoxide Production in Microsomes with Added Vitamin E.

Incubation reactions contained microsomes (0.6 mg protein/ml), ADP-Fe^{+3} (250 uM ADP, 50 uM $FeCl_3$), and NADPH (0.4 mM) in 50 mM NaCl, pH 7.0. Reactions were initiated with NADPH. GSH (5 mM) was added at the same time as NADPH. Lipid hydroperoxide determinations were done in triplicate. Error bars indicate standard deviations. Incubation represented by open symbols (○, △) did not contain GSH. Incubation represented by solid symbols (●, ▲) contained GSH.

○, ● **represents malondialdehyde production.**

△, ▲ **represents lipid hydroperoxide production.**

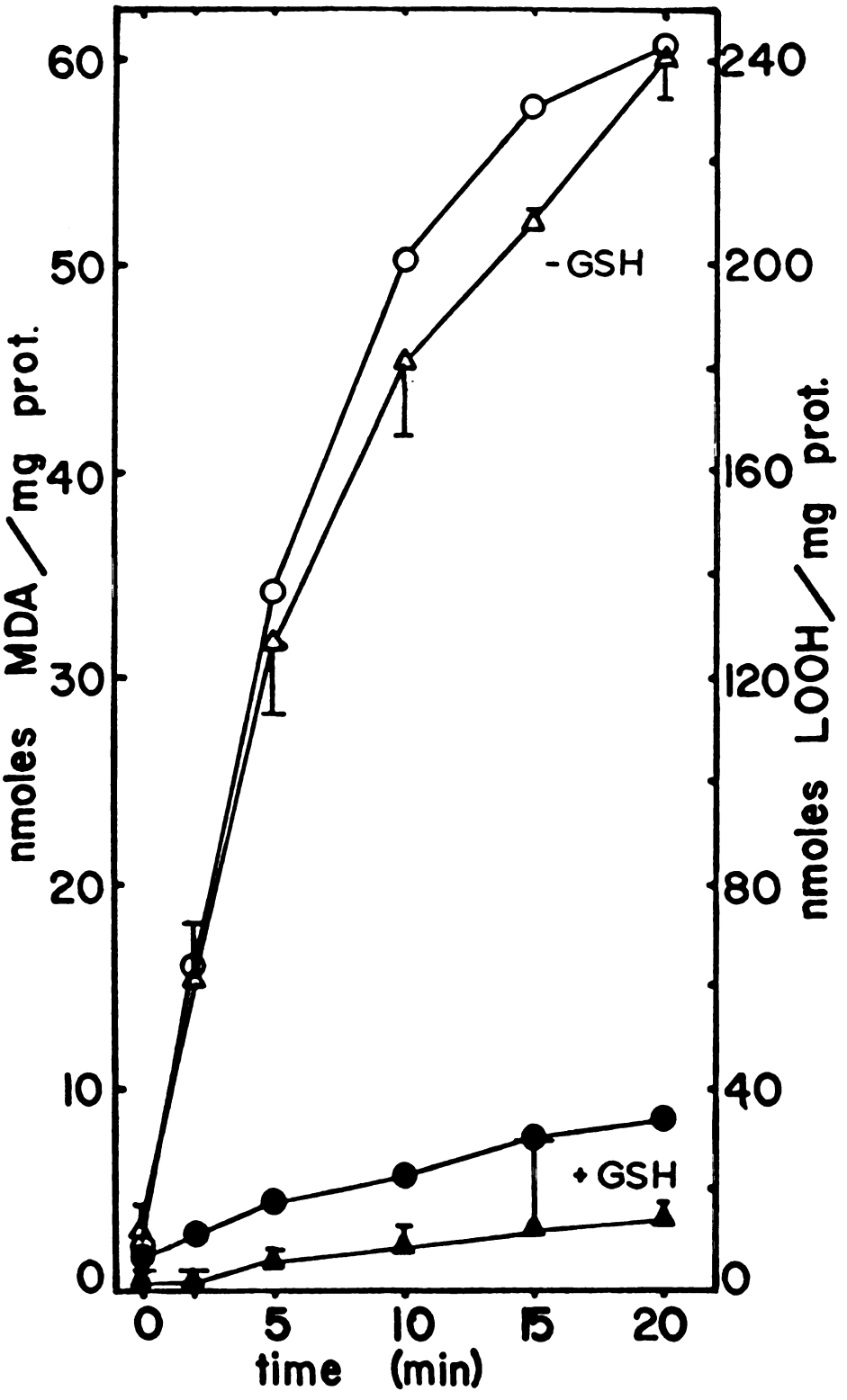

60
50
40
30
20
10
0
nmoles MDA / mg prot.
240
200
160
120
80
40
0
nmoles LOOH / mg prot.
-GSH
+GSH
0
5
10
15
20
time (min)

Figure 12

Microsomes that had been stored for 1 month were used to minimize vitamin E-dependent effects on lipid peroxidation and maximize hydroperoxide production. Storage diminishes microsomal vitamin E content and results in loss of GSH-dependent vitamin E reductase activity. Prolonged storage also results in increased levels of preformed lipid hydroperoxides and increased rates of lipid peroxidation. Figure 13 shows the effect of GSH on lipid hydroperoxide concentrations in incubation reactions undergoing rapid peroxidation. In these incubation reactions lipid hydroperoxide concentrations increased with increasing ADP:Fe^{+3} ratios. Hydroperoxide concentrations ranged from 1.5-fold to 2.2-fold higher than MDA production at ADP:Fe^{+3} ratios of 5:1 and 15:1, respectively. The lipid hydroperoxide concentrations at the different ADP:Fe^{+3} ratios in this experiment were inversely proportional to the length of the lag phases that had been observed earlier at these ADP:Fe^{+3} ratios (Figure 10). Thus, the interaction between GSH and iron affected lipid hydroperoxide concentrations which consequently affected the accumulation of lipid hydroperoxides and the length of time before commencement of lipid hydroperoxide-dependent lipid peroxidation.

Figure 13.

The Effect of GSH and Different ADP:Fe^{+3} Ratios on Malondialdehyde and Lipid Hydroperoxide Production in Aged Microsomes without Added Vitamin E.

Incubation reactions contained microsomes (0.6 mg protein/ml), ADP-Fe^{+3} (50 uM $FeCl_3$), and NADPH (0.4 mM) in 50 mM NaCl, pH 7.0. Reactions were initiated with NADPH. All incubations contained GSH (5 mM) which was added at the same time as NADPH. A). Incubation contained ADP:Fe^{+3} ratios of 5:1, 10:1, or 15:1. Lipid hydroperoxide determinations were done in triplicate. Error bars represent standard deviations.

□, ○, △ represent malondialdehyde production.

■, ●, ▲ represent lipid hydroperoxide production.

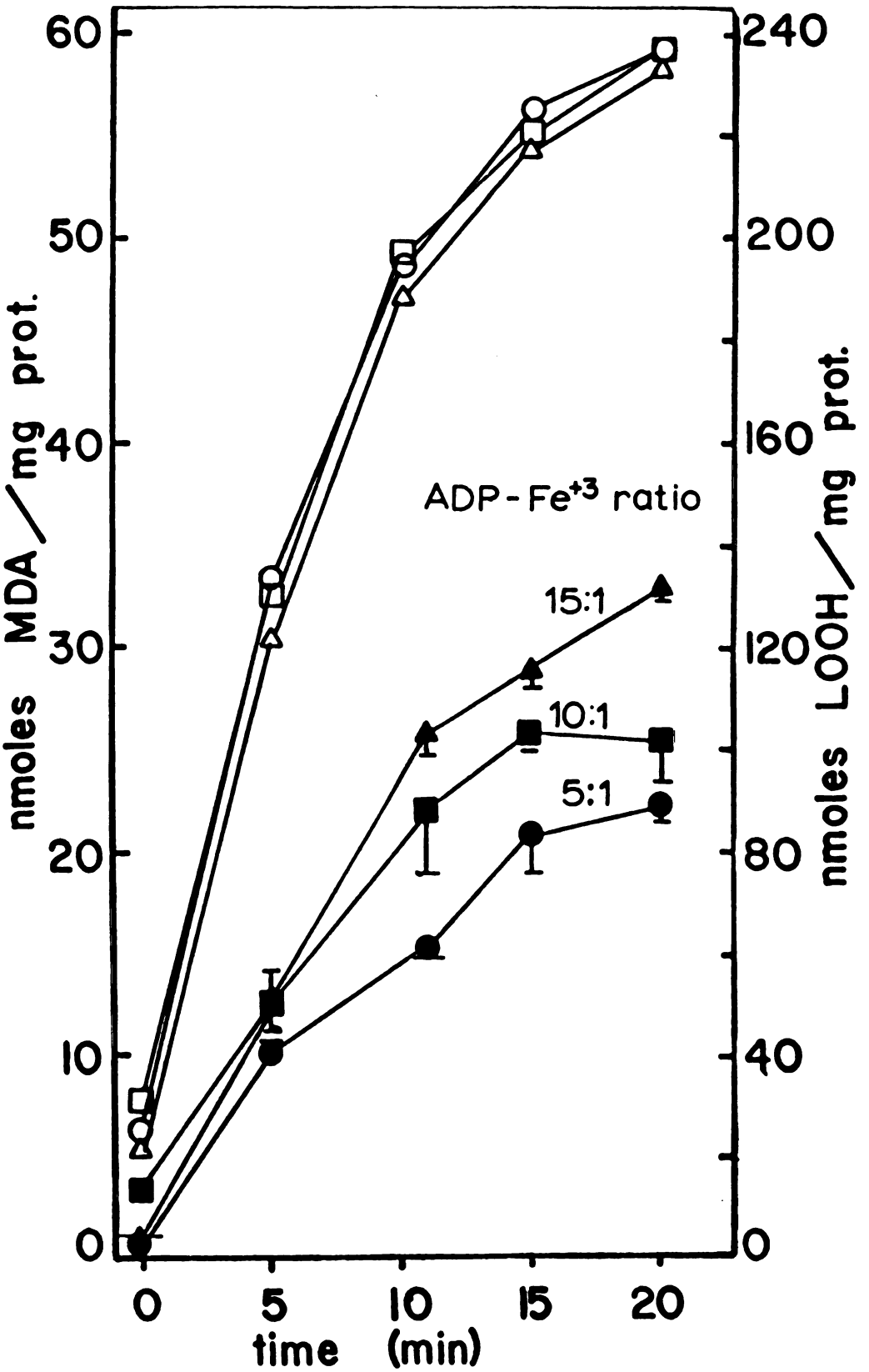

60
50
40
30
20
10
0
240
200
160
120
80
40
0
nmoles MDA / mg prot.
nmoles LOOH / mg prot.
ADP-Fe+3 ratio
15:1
10:1
5:1
0
5
10
15
20
time (min)

Figure 13

DISCUSSION

In reports dealing with the effect of GSH on microsomal lipid peroxidation, complete or nearly complete inhibition of lipid peroxidation was found to occur at the vitamin E concentration present in microsomes upon isolation (Reddy et al., 1982; Burk, 1983; McCay et al., 1986). In contrast, the amount of vitamin E in microsomes isolated from rats fed the standard laboratory diet decreased the rate of MDA production only 42% (Figure 7). Of the 42% inhibition, 20% could be attributed to GSH without the participation of vitamin E (Figure 6). Thus, the amount of vitamin E in microsomes at the time of isolation was responsible for only about a 20% decrease in the rate of peroxidation.

An increase from 42% inhibition to approximately 90% required twice as much in microsomal vitamin E. In an effort to increase the amount of inhibition from 42% to 90% without addition of vitamin E, the rate of lipid peroxidation was decreased by lowering the iron concentration in the peroxidation reactions. However, lower iron concentration resulted in less inhibition at each vitamin E concentration tested. Similarly, using

higher ADP:Fe^{+3} ratios resulted in less inhibition of peroxidation. Since the rate of iron reduction by GSH depends on the ADP:Fe^{+3} ratio, the effect of iron on inhibition of peroxidation was proportional to iron reduction by GSH. Thus, the difference in the amount of vitamin E required to cause nearly complete inhibition of peroxidation in this study compared to earlier reports, may be due to the effect of iron chelation and concentration.

The inhibition of lipid peroxidation by GSH and vitamin E was characterized by a lag phase of duration proportional to the microsomal vitamin E concentration. During the lag phase, vitamin E concentrations remained constant providing evidence for the efficient transfer of electrons (or hydrogen atoms) from GSH to chromanoxyl radicals with regeneration of vitamin E. Inhibition of peroxidation characterized by a lag phase was similar to results of Burk (1983) and Haenen and Bast (1983) who also reported that GSH caused a lag phase in microsomal lipid peroxidation, except that they found the lengths of the lag phases to be proportional to GSH concentration. Based on this work with different vitamin E concentrations and the work of Burk (1983) and Haenen and Bast (1983) with different GSH concentrations, inhibition of microsomal lipid peroxidation depends on the concentration of both vitamin E and GSH.

At high vitamin E concentrations (in excess of 1.0 nmol/mg protein), lipid peroxidation was inhibited

regardless of GSH. Thus, vitamin E-dependent inhibition of peroxidation in microsomes is similar to that of liposomal lipid peroxidation systems (Fuzukawa et al., 1985; Liebler et al., 1986). In the liposomal systems, peroxidation was inhibited when vitamin E concentrations exceeded a critical value. As was shown in Figure 7 with vitamin E addition to microsomes (in the absence of GSH), lower concentrations of vitamin E in lipsomes did not affect peroxidation (Chapter I, Figure 3). Similarities between vitamin E-dependent inhibition of peroxidation in microsomes and liposomes also indicate that the method of adding vitamin E to microsomes resulted in its incorporation into the microsomal membrane.

Recent publications on inhibition of lipid peroxidation by vitamin E cite lipid hydroperoxide formation from lipid peroxyl radicals as the reaction responsible for the inhibition of peroxidation (reviewed in McCay, 1985). In the GSH-containing system, an additional reaction seemed to be necessary for inhibition to occur. This additional reaction was the conversion of lipid hydroperoxides to products that were non-reactive in lipid peroxidation. By comparing the relative amounts of lipid hyroperoxides and MDA in the presence and absence of GSH, the effect of GSH on lipid hydroperoxide concentrations without participation of vitamin E was demonstrated. In the presence of GSH and without participation of vitamin E, hydroperoxide concentrations were 1.5 to 2.2-fold higher than those of MDA (Figure 13), while in the absence of GSH

hydroperoxide concentrations were 4-fold higher than MDA (Figure 12). Because lipid hydroperoxide concentrations in the incubation reactions could be expected to influence the rate of lipid peroxidation, the different amount of MDA produced during the lag phase at different iron concentrations (Figure 8) and different ADP:Fe^{+3} ratios (Figure 11) can be explained by differences in lipid hydroperoxide concentrations. The difference in relative amounts of lipid hydroperoxides may also explain the partial inhibition of peroxidation observed at very low vitamin E concentrations (Figure 6).

If conversion of lipid hydroperoxides to non-reactive products did not occur and vitamin E-dependent hydroperoxide formation from lipid peroxyl radicals were the only termination reaction to take place, then as long as the initiation process continued there would be a gradual increase in the number of radicals (or potential radicals) in the system. Reaction of peroxyl radicals with vitamin E according to the proposed mechanism of vitamin E action does not remove radicals from the system. Radicals remain because lipid hydroperoxides react with ferrous or ferric iron to ultimately produce another lipid peroxyl radical (Figure 2 in the Review of Literature; Kappus, 1985). Thus, as long as rates of reactions (7) and (8) favor hydroperoxide production by vitamin E rather than by another polyunsaturated fatty acid, the quantities of lipid hydroperoxides would increase without propagation of lipid

peroxidation. The net result would be an increasing requirement for vitamin E in order to prevent the rate of reaction (7) from exceeding that of reaction (8). Gradual accumulation of lipid hydroperoxides provides a possible explanation for the vitamin E concentration-dependent lag phases which were observed in the presence of GSH. Lipid hydroperoxides may have accumulated until a concentration was reached at which vitamin E could no longer prevent propagation from occurring. At this time hydroperoxide-dependent lipid peroxidation would begin.

REFERENCES

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

Burk, R.F. Protection by GSH against lipid peroxidation induced by ascorbate and iron in rat liver microsomes. Biochem. Pharmacol. 31:601-602. (1982).

Burk, R.F. Glutathione-dependent protection by rat liver microsomal protein against lipid peroxidation. Biochem. Biophys. Acta 757:21-28. (1983).

Desai, I.D. Vitamin E analysis methods for animal tissues. In: Methods in Enzymology, Volume 105 (S.P. Colowick, N.O. Kaplan and L. Packer, eds.) pp. 138-147, Academic Press, New York (1984).

Franco, D.P., and S.G. Jenkins. Rat lung microsomal lipid peroxidation: effects of vitamin E and reduced glutathione. J. Appl. Phys. 61:785-790. (1986).

Fukuzawa, K., S. Takase and H. Tsukatani. The effect of concentration on the antioxidant effectiveness of a-tocopherol in lipid peroxidation induced by superoxide free radicals. Arch. Biochem. Biophys. 240:117-120. (1985).

Haenen, G.R.M.M., and A. Bast. Protection against lipid peroxidation by a microsomal glutathione-dependent labile factor. FEBS Lett. 159:24-28. (1983).

Kappus, H. Lipid peroxidation: Mechanisms, analysis, enzymology and biological relevance. In Oxidative Stress (H. Sies, ed.) pp. 273-310. Academic Press, London. (1985).

Liebler, D.C., D.S. Kling, and D.J. Reed. Antioxidant protection of phospholipid bilayers by a-tocopherol: Control of a-tocopherol status and lipid peroxidation by ascorbic acid and glutathione. J. Biol. Chem. 261:12114-12119. (1986).

McCay, P.B. Vitamin E: Interactions with free radicals and ascorbate. Ann. Rev. Nutr. 5:323-340. (1985).

McCay, P.B., E.K. Lai, S.R. Powell, and G. Breuggeman. Vitamin E functions as an electron shuttle for glutathione-dependent "free radical reductase" activity in biological membranes. Fed. Proc. 45(3):451. (1986).

Niki, E., M. Takahashi, and E. Komuro. Antioxidant activity of vitamin E in liposomal membranes. Chem. Lett. 1986:1573-1576. (1986).

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

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

Tien, M., J. R. Bucher, and S.D. Aust. Thiol-dependent lipid peroxidation. Biochem. Biophys. Res. Comm. 107:279-285. (1982).

Chapter III

The Relationship of $O_2^{\cdot-}$ and H_2O_2 Production to Rat Lung and Liver Microsomal Lipid Peroxidation.

ABSTRACT

The rate of superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) generation and the parameters that affect their production or detection were measured in rat lung and liver microsomes. Cytochrome P450 concentration, cytochrome P450 reductase activity, ferritin concentration, and catalase activity were measured. Isolated lung microsomes were heavily contaminated with hemoglobin which prevented cytochrome P450 determinations. Various procedures were tested for removing hemoglobin, catalase, and ferritin from lung microsomes. Of the procedures tested, column chromatography was the best because it allowed quantification of the lung cytochrome P450 whereas the others did not. Treated lung microsomes contained 0.027 nmol cytochrome P450/mg protein while liver microsomes contained 0.76 nmol/mg protein. Lung microsomes produced 1.0 nmol $O_2^{\cdot-}$ and 0.51 nmol H_2O_2/min/mg protein while liver microsomes produced 3.0 nmol $O_2^{\cdot-}$ and 7.6 nmol H_2O_2/min/mg protein. The low $O_2^{\cdot-}$ and H_2O_2 production rates in lung microsomes were corroborated by measuring microsomal NADPH oxidation rates. These data suggested that the observed rates of $O_2^{\cdot-}$ and H_2O_2 production in lung microsomes were

too low to support significant lipid peroxidation. Lung microsomes from rats fed the standard laboratory diet were resistant to lipid peroxidation which was explained by their low polyunsaturated fatty acid and high vitamin E content. Lung microsomes from rats fed a vitamin E deficient diet for 8 weeks were susceptible to peroxidation because of a 97% lower vitamin E content. However, lung microsomes from rats fed the vitamin E deficient diet amended with alpha-tocopherol acetate peroxidized, even though their polyunsaturated fatty acid and vitamin E content were equal to those from peroxidation resistant lung microsomes. Data are presented which indicate that arachidonic acid content of microsomal membranes may be especially important in determining resistance to lipid peroxidation.

INTRODUCTION

Production of superoxide ($O_2^{\cdot-}$) or hydrogen peroxide (H_2O_2) in excess of the protective capacity of cellular defenses may lead to toxicity. Examples of pulmonary toxicoses that may be the result of excess production of $O_2^{\cdot-}$ or H_2O_2 are hyperoxic lung damage, the result of breathing air containing a higher than normal oxygen concentration, and paraquat toxicity (reviewed in Kehrer and Kacew, 1985). In support of this mechanism of toxicity for hyperoxic lung damage, Freeman and Crapo (1981) and Freeman *et al.* (1982) showed as indicated by cyanide resistant oxygen uptake that increased atmospheric oxygen concentrations increased $O_2^{\cdot-}$ and H_2O_2 production in rat lung slices and rat lung homogenates. Sources of $O_2^{\cdot-}$ and H_2O_2 within cells are the mitochondrial and microsomal electron transport systems, and several enzymes such as glucose oxidase. Among these, microsomes were the major source of H_2O_2, exceeding mitochondrial production by about 10-fold (Turrens *et al.*, 1982). Within microsomes the sources of $O_2^{\cdot-}$ and H_2O_2 are cytochrome P450 and cytochrome P450 reductase (Freeman and Crapo, 1982). Thus, $O_2^{\cdot-}$ and H_2O_2 production could be expected to be proportional to the

concentrations of these electron transport components. Indirect evidence for involvement of $O_2^{\cdot}$ and H_2O_2 in hyperoxic lung damage associated with death of the animal was provided by Gonder et al. (1985) who measured cytochrome P450 contents following exposure to high oxygen concentrations in atmospheric air. They showed that induction of synthesis of liver cytochrome P450 in mice during exposure to 100% oxygen was inversely related to survival following exposure. Mice whose cytochrome P450 concentrations were not increased from exposure had less severe pulmonary lesions and a higher survival rate than mice whose cytochrome P450 concentrations increased following the exposure.

Paraquat is a selective pulmonary toxin because of active uptake by lung tissue (reviewed in Kehrer and Kacew, 1985). It produces $O_2^{\cdot}$ and H_2O_2 by redox cycling (Halliwell and Gutteridge, 1985). Paraquat is chemically reduced by cytochrome P450 reductase with generation of a paraquat radical (Bus et al., 1974). Subsequent autoxidation of the radical produces $O_2^{\cdot}$ (Farrington et al., 1973). Iron, depending on its chelator, can affect the production of $O_2^{\cdot}$ via the same mechanism as paraquat. Morehouse et al. (1984) found that reduction of the iron, in chelates such as EDTA-Fe^{+3}, by cytochrome P450 reductase resulted in increased $O_2^{\cdot}$ production. Superoxide production was unaffected by chelates such as ADP-Fe^{+3} which are not reduced by the reductase enzyme and/or which,

when reduced, autoxidize slowly.

One proposed mechanism of toxicity from $O_2^{\cdot-}$ and H_2O_2 is peroxidative damage to cellular lipids. Freeman et al. (1982) reported increased concentrations of lipid peroxidation products in rat lung homogenates following in vivo exposure to high concentrations of atmospheric oxygen, and following in vitro exposure of lung homogenates to 100% oxygen. Thus, any insult which results in increased $O_2^{\cdot-}$ and H_2O_2 production could be expected to lead to increased lipid peroxidation. However, such is not true with paraquat. In some reports paraquat promoted in vitro lipid peroxidation while in others it was inhibitory. This effect may have been dependent on the paraquat concentration (Trush et al., 1981).

Paraquat may affect cellular free iron concentrations in addition to increasing cellular levels of $O_2^{\cdot-}$ and H_2O_2. Under some circumstances, free iron concentrations may be more significant in lipid peroxidation than $O_2^{\cdot-}$ and H_2O_2 concentrations. The majority of intracellular non-heme iron is sequestered in ferritin and hence the concentration of free iron available for catalysis of lipid peroxidation is very low (Halliwell and Gutteridge, 1985). Regardless of $O_2^{\cdot-}$ and H_2O_2 concentrations, lipid peroxidation does not occur in the absence of free iron (Halliwell and Gutteridge, 1985). Thomas et al. (1985) showed that $O_2^{\cdot-}$ released iron from ferritin in vitro and Thomas and Aust (1985) showed that paraquat stimulated this release. After

in vivo exposure of rats to diquat, a hepatotoxin chemically related to paraquat, hepatic ferritin iron content was decreased. The decrease in ferritin iron was accompanied by an increase in cellular free iron (Reif, Thomas, Beals and Aust, unpublished).

Although pulmonary toxicity from high oxygen concentrations and paraquat probably involves $O_2^{\cdot-}$ and H_2O_2, the importance of these oxygen species in lipid peroxidation is less certain. To further investigate the relevance of $O_2^{\cdot-}$ and H_2O_2 production to lipid peroxidation $O_2^{\cdot-}$, H_2O_2, and the factors important in generation or detection were quantified in rat lung microsomes. The production of these partially reduced oxygen species was evaluated for their contribution to lipid peroxidation. It was found that partially reduced oxygen production by the microsomes did not influence NADPH-dependent peroxidation. Data are also presented which indicate that arachidonic acid content of microsomal membranes may be especially important in determining resistance to lipid peroxidation.

MATERALS AND METHODS

NADPH, ADP, cytochrome c (Type VI), superoxide dismutase, horseradish peroxidase, 2-thiobarbituric acid, paraquat, and butylated hydroxytoluene were purchased from Sigma Chemical Co. (St. Louis, MO). EDTA was obtained from Mallinckrodt Chemical Co. (Paris, KY). Hydrogen peroxide was purchased from Aldrich Chemical Co. (Milwaukee, WI). Ferric chloride was purchased from Baker Chemical Co. (Phillipsburg, NJ). L-Ascorbic acid and dithionite (sodium hydrosulfate) were obtained from Fischer Scientific Co. (Fairlawn, NJ). Desferroxamine B (mesylate salt) was purchased from Ciba Pharmaceutical Co. (Summit, NJ). Catalase was purchased from Cooper Biomedical Co. (Malvern, PA). All buffers and reagents used in lipid peroxidation and measurements of $O_2^{\cdot-}$ and H_2O_2 were passed through Chelex 100 ion exchange resin (Bio-Rad Laboratories, Richmond, CA) to remove contaminating transition metal ions.

Microsomes were obtained from 225 g male Sprague-Dawley rats (Charles River, Portage, MI) fed either a standard laboratory diet (Rodent Blox; Wayne Pet Foods, Chicago, IL), or a vitamin E deficient diet (Vitamin E Test Diet; US Biochemical Co., Cleveland, OH) with or without

150 IU of alpha-tocopherol acetate/kg diet (US Biochemical Co.) for 8 weeks (Reddy et al., 1982). The latter diets were stored at -40°C to prevent oxidative degradation. Rats fed the vitamin E deficient diet with or without alpha-tocopherol acetate supplementation appeared healthy at the end of 8 weeks. Rats were killed by CO_2 anesthesia and decapitation. The vitamin E deficient diet resulted in rat liver microsomes containing about 3% the amount of vitamin E present in liver microsomes from rats fed the standard laboratory diet.

Liver and lung microsomes were isolated by the method of Pederson et al. (1973). This procedure is referred to in this report as the standard isolation method. Each lung lobe was separately excised and the airways and connective tissue were trimmed. The lobes were individually minced, pooled, and homogenized with a Potter-Elvehjem teflon-glass tissue grinder in 5 volumes of argon-purged 1.15% KCl, pH 7.4 containing 0.2% nicotinamide. The homogenate was centrifuged at 7500 x g for 20 min and, the supernatant filtered through cheesecloth and centrifuged again at 16,000 x g for 20 min. The supernatant was then filtered through glass wool. Microsomes were sedimented by ultracentrifugation at 105,000 x g for 90 min and stored at -20°C in argon-purged 50 mM Tris, pH 7.4 containing 50% glycerol (v/v).

Microsomes from in situ perfused lungs were obtained in the following manner. The thoracic cavity of CO_2

anesthetized rats was opened and saline (0.9%) introduced into the circulatory system through a 20 gauge needle inserted into the right atrium of the heart. Blood was drained from the carcass by cutting the vessels in the neck and a femoral vessel. Perfusion continued until the lungs became pale. Then they were excised and the microsomes isolated as described.

Ultrafiltration chromatography of microsomes was done by the procedure of Thomas and Aust (1985). The Tris-glycerol buffer was removed from stored microsomes by ultracentrifugation, resedimentation, and resuspension in 0.02 M Tris-HCl, pH 7.4 containing 0.15 M KCl. Microsomes that had not been stored were resuspended directly in Tris-KCl following ultracentrifugation. Microsomes then were chromatographed on a 30 x 3 cm Sepharose CL-2B column (Pharmacia Fine Chemical, Piscataway, NJ) equilibrated with Tris-KCl. Column fractions containing microsomes (in the void volume) were pooled and the microsomes resedimented by ultracentrifugation. All solutions were thoroughly purged with argon and all steps were done at 4°C to minimize autoxidation of unsaturated lipids.

Liver microsomal cytochrome P450 content was measured by the method of Omura and Sato (1964). In this procedure a few crystals of dithionite (sodium hydrosulfite) were added to microsomal solutions to reduce the heme iron in cytochrome P450. Then the sample cuvette was bubbled with carbon monoxide for 45 sec. The difference spectrum of the

solution before and after carbon monoxide bubbling was used to quantify the amount of cytochrome P450 present. An extinction coefficient of 91 mM^{-1} cm^{-1} at 450 nm was used. The amount of cytochrome P450 in lung microsomes was measured by the method of Matsubara et al. (1974). In this procedure the microsomal solution was first bubbled with carbon monoxide. A difference spectrum between dithionite and non-dithionite containing cuvettes was obtained as above and the same extinction coefficient was used. The method of Matsubara et al. (1974) yielded the same value for liver cytochrome P450 concentration as did the method of Omura and Sato (1964). The chemical basis for the success of reversing carbon monoxide binding and reduction is that cytochrome P450 contains ferric iron while hemoglobin contains ferrous iron. Carbon monoxide binds to ferrous but not ferric iron. Thus, reduction of all iron to the ferrous form by dithionite followed by carbon monoxide binding yields the total amount of heme iron present in the system. Carbon monoxide binding followed by reduction with dithionite yields only the heme iron which is in the ferric form.

Cytochrome P450 reductase activity was quantified by reduction of cytochrome c (Pederson et al., 1973). All spectrophotometric assays except those for lipid peroxidation were done using a Cary Model 219 spectrophotometer.

Microsomal $O_2^{\cdot-}$ production was measured by monitoring

the reduction of acetylated cytochrome c in 50 mM NaCl (Morehouse et al., 1984). Acetylated cytochrome c was used because it is readily reduced by $O_2^{\cdot-}$ but is less rapidly reduced directly by cytochrome P450 reductase than is the unacetylated protein. However, the amount of reduction of the acetylated protein by the reductase still is significant compared to the amount of reduction by $O_2^{\cdot-}$. Therefore, the acetylated cytochrome c reduction was monitored in the presence and in the absence of superoxide dismutase (approximately 115 U/ml). Superoxide production was determined as the amount of acetylated cytochrome c reduction that was inhibited by superoxide dismutase. Incubation reactions also contained catalase (about 1600 U/ml) to prevent reoxidation of acetylated cytochrome c by H_2O_2. Desferroxamine (1 mM) was included to prevent iron from artifactually increasing $O_2^{\cdot-}$ production.

Hydrogen peroxide production was quantified using a modification of the procedure of Hildebrandt and Roots (1975). Microsomes (0.6 mg protein/ml) were incubated with NADPH (0.4 mM) in the presence of sodium azide (3 mM) and desferroxamine (1 mM) at 37°C in a shaking water bath. Azide was added to prevent decomposition of H_2O_2 by microsomal catalase. Desferroxamine was used to inhibit non-heme iron catalyzed decomposition of H_2O_2. Aliquots (0.5 ml) of the incubation mixture were quenched in 3% trichloroacetic acid and placed on ice for 20 min. Tubes then were centrifuged at low speed for 10 min to sediment

particulate material. Supernatant fluid (0.66 ml) was removed and neutralized with 0.06 ml of 2 M triethanolamine. Hydrogen peroxide was quantified using horseradish peroxidase (1 U/ml) and ortho-dianisidine (0.5 mM). The oxidation of dianisidine was monitored at 460 nm.

Microsomal ferritin was quantified in microsomes by the ELISA method of Thomas and Aust (1985). Catalase was quantified spectrophotometrically by measuring the decrease in absorbance at 240 nm corresponding to a decrease in H_2O_2 concentration (Beers and Sizer, 1952). NADPH oxidation was monitored by adding NADPH to microsomes and monitoring the decrease in absorbance at 340 nm.

Lipid peroxidation, vitamin E determinations, fatty acid determinations, and calculations of polyunsaturated fatty acid to vitamin E ratios were the same as outlined in Chapter I. Lipid peroxidation was measured as malondialdehyde (MDA) production using a Coleman II spectrophotometer.

RESULTS

Since $O_2^{\cdot-}$ and H_2O_2 originate from cytochrome P450 and cytochrome P450 reductase in microsomes (Morehouse *et al.*, 1984), the cytochrome P450 concentration and the cytochrome P450 reductase activity in lung microsomes was measured. Microsomes isolated from liver tissue were included as controls. Quantifications in lungs were complicated by contaminants such as catalase, superoxide dismutase, and ferritin. In addition, lung microsomes contain high levels of hemoglobin which precluded accurate spectrophotometric quantification of cytochrome P450. To improve lung microsomal cytochrome P450 measurements, clean up treatments by column chromatography on Sepharose CL-2B and *in situ* perfusion of the lungs with saline were tried in conjunction with the standard isolation method described. Of the treatments, column chromatography was the best because it removed enough hemoglobin to allow detection of cytochrome P450, and it lowered the levels of other contaminants such as catalase and ferritin (Table 4). Lowered ferritin concentration was desired as the iron in ferritin is converted to its reduced form by $O_2^{\cdot-}$ thereby artifactually lowering the observed $O_2^{\cdot-}$ production rate.

Table 4. Activities and Concentrations of Selected Variables in Lung and Liver Microsomes Harvested from Rats Fed the Standard Laboratory Diet.

	Lung microsomes[a]			Liver microsomes[a]	
Variable	SIM	CC	P	SIM	CC
Cytochrome P450 reductase[b]	0.064	0.047	0.064	0.19	0.22
Cytochrome P450[c]	--[d]	0.027	--	0.62	0.76
H_2O_2 production[e]	0.98	0.51	0.83	3.8	7.6
$O_2^{\cdot}$ production[e]	2.0	1.0	1.4	1.7	3.0
NADPH oxidation[e]	2.6	1.5	2.1	7.1	5.1
Ferritin[f]	2.2	ND[g]	--	7.0	0.4
Catalase[b]	17	6.2	45	150	38

[a]SIM = standard isolation method, CC = treated by column chromatography, P = perfused *in situ*.

[b]Activity in umol/min/mg protein.

[c]Concentration in nmol/mg protein.

[d]Not determined.

[e]Rate in nmol/min/mg protein.

[f]Concentration in ug/mg protein.

[g]Below limit of detection.

The *in situ* lung perfusion treatment also resulted in lower hemoglobin contamination as evidenced by the slight decrease in H_2O_2 and $O_2^{\cdot}$ production and NADPH oxidation values compared to the standard isolation method, but catalase contamination was increased (Table 4).

Comparing lung to liver microsomes, cytochrome P450 reductase activity was 4 times lower and the cytochrome P450 concentration was 28 times lower in chromatographed lung microsomes versus chromatographed liver microsomes (Table 4). Superoxide and H_2O_2 generation rates also were lower in lung than in liver as expected since most microsomal $O_2^{\cdot}$ and H_2O_2 is derived from cytochrome P450. The value for H_2O_2 production by lung microsomes was especially low (0.51 nmol/min/mg protein) and represented the lower limit of the assay. In lung, column chromatography lowered the rates of $O_2^{\cdot}$ and H_2O_2 production while in liver chromatography increased the production rates.

The low rates of $O_2^{\cdot}$ and H_2O_2 production by lung microsomes (Table 4) made it necessary to demonstrate the accuracy of the measurements. To do this, NADPH oxidation was measured. The rates of microsomal NADPH oxidation in the presence or absence of various iron chelates and paraquat, are shown in Table 5. The reaction mixture was the same as that used to measure $O_2^{\cdot}$ and H_2O_2 production, thus these results can be

Table 5. NADPH Oxidation in Perfused and Non-perfused Lung Microsomes and in Liver Microsomes Harvested from Rats Fed the Standard Laboratory Diet.[a]

addition	Lung microsomes[b] SIM	CC	P	Liver microsomes[c]
NADPH	2.6	1.5	2.1	5.1
+ EDTA-Fe^{+3}	3.8	3.1	4.8	9.2
+ ADP-Fe^{+3}	1.9	0.0	2.6	6.3
+ ADP-Fe^{+3} and EDTA-Fe^{+3}	2.9	2.7	4.1	9.7
+ Paraquat	11	8.3	14	31
+ EDTA-Fe^{+3} and paraquat	9.1	7.6	10	21
+ ADP-Fe^{+3} and paraquat	10	6.7	8.3	28
+ ADP-Fe^{+3} and EDTA-Fe^{+3} and paraquat	9.5	7.6	11	35

[a]Values are nmol/min/mg protein.

[b]SIM = standard isolation method, CC = treated by column chromatography, P = perfused in situ.

[c]treated by column chromatography.

compared with those in Table 4. The rate of NADPH oxidation was very low in the chromatographed lung microsomes (1.54 nmol/min/mg protein, Table 5) and supported the low rates observed for $O_2^{\cdot-}$ and H_2O_2 production in lung microsomes seen previously. Based on this rate of NADPH oxidation in the chromatographed lung microsomes, an equal molar amount of H_2O_2 and twice as much $O_2^{\cdot-}$ could have been produced. This is because oxidation of NADPH yields 2 electrons per molecule and oxygen reduction to H_2O_2 requires 2 electrons per molecule while $O_2^{\cdot-}$ production only requires one per molecule. Thus, rates of H_2O_2 and $O_2^{\cdot-}$ production are very low in lung microsomes.

Acceleration of the rate of NADPH oxidation was attempted by various additions to the reaction mixture. Additions of EDTA-Fe^{+3} or paraquat or both accelerated NADPH oxidation, and the trends in oxidation were similar in lung and liver microsomes (Table 5). Since both EDTA-Fe^{+3} and paraquat can act directly as electron acceptors for the cytochrome P450 reductase, and both autoxidize rapidly (Morehouse et al., 1984), the rates of $O_2^{\cdot-}$ and H_2O_2 production were presumably higher with these additions.

The rates of lipid peroxidation in lung and liver microsomes from rats fed the standard laboratory diet or vitamin E deficient diets with and without vitamin E supplementation as alpha-tocopherol acetate were examined. In rats fed the standard laboratory diet, lung microsomes

were resistant to peroxidation (regardless of the presumed rates of $O_2^{\cdot}$ and H_2O_2 production) as low lipid peroxidation rates ($\leq$ 0.05 nmol/min/mg protein) were observed with all additions (Table 6). In the liver microsomal lipid peroxidation incubation reactions from these rats, addition of ADP-Fe^{+3} resulted in accelerated peroxidation over NADPH alone. This rate was further accelerated by ADP-Fe^{+3} with EDTA-Fe^{+3} or with paraquat (Table 6). Using ferritin as the source of iron resulted in a low rate of peroxidation that was stimulated by the addition of paraquat probably by increasing iron release from ferritin. In rats fed the vitamin E deficient diet with and without alpha-tocopherol acetate, the observed rates of peroxidation in lung microsomes showed that the system was capable of peroxidation reactions (Table 6). In vitamin E deficient lung microsomes, inclusion of EDTA-Fe^{+3} with ADP-Fe^{+3} in the peroxidation mixture increased the rate of peroxidation over ADP-Fe^{+3} alone. This effect was not observed in lung microsomes from vitamin E supplemented rats. The rates of peroxidation in the presence of NADPH plus ADP-Fe^{+3} in liver microsomes from rats fed the two vitamin E deficient diets were approximately equal to those from rats fed the standard laboratory diet.

Due to the fact that lung microsomes from rats fed the standard laboratory diet were resistant to peroxidation (Table 6), lung microsomes from rats fed the vitamin E deficient diet were used to examine the contribution of

Table 6. NADPH-dependent Lipid Peroxidation Lung and Liver Microsomes Harvested from Rats Fed either a Standard Laboratory or a Vitamin E Deficient Diet with or without Vitamin E Supplementation.[a]

	Lung microsomes			Liver microsomes		
		Diet[b]			Diet	
addition	Std.	-E	+E	Std.	-E	+E
NADPH	0.01	--[c]	--	0.10	--	--
+ EDTA-Fe^{+3}	0.05	--	--	0.10	--	--
+ ADP-Fe^{+3}	0.05	0.38	0.14	3.12	3.25	3.85
+ ADP-Fe^{+3} and EDTA-Fe^{+3}	0.05	0.79	0.11	6.95	--	--
+ ADP-Fe^{+3} and paraquat	0.04	--	--	3.95	--	--
+ ADP-Fe^{+3} and ferritin	0.0	--	--	0.87	--	--
+ ADP and ferritin and paraquat	0.0	--	--	1.08	--	--

[a]Values are nmol/min/mg protein at 15 min incubation. Microsomes from rats fed the vitamin E deficient diets were not chromatographed while the others were.

[b]Std. = standard laboratory rat diet, -E = diet deficient in vitamin E, +E = diet deficient in vitamin E but supplemented with 150 IU alpha-tocopherol acetate/kg diet.

[c]Not determined.

H_2O_2 and $O_2^{\cdot-}$ to lipid peroxidation. It was found that the rates of H_2O_2 production in microsomes from rats fed the two vitamin E test diets were similar to those obtained from rats fed the standard laboratory diet, whereas the $O_2^{\cdot-}$ production rates in microsomes from rats fed the vitamin E test diet were about 2 times lower than those from rats fed the standard diet (Table 7). This difference, however, was judged not to be biologically significant. Thus, differences in peroxidation among lung microsomes from rats fed the different diets could not be attributed to the rate of H_2O_2 and $O_2^{\cdot-}$ production.

The fact that peroxidation was observed in lung microsomes from rats fed the vitamin E deficient diet to which alpha-tocopherol acetate had been added was unexpected. In previous work (Chapter I) resistance to peroxidation was attributed to relatively low polyunsaturated fatty acid and high vitamin E content in lung microsomes. Profiles of the fatty acid content of lung microsomes from rats fed the vitamin E deficient diet, with or without added vitamin E, were nearly identical (Table 8). Differences in the fatty acid composition of the standard laboratory diet and the vitamin E deficient diets were observed but the percentages of polyunsaturated fatty acid contents were approximately the same.

Since the fatty acid composition of microsomal membranes was influenced by dietary fatty acid intake, the fatty acid composition of the diets was analysed. The

Table 7. Superoxide and Hydrogen Peroxide Production in Lung and Liver Microsomes Harvested from Rats Fed a Standard Laboratory Diet or a Vitamin E Deficient Diet with and without Vitamin E Supplementation.[a]

	Lung microsomes		
		Diet[b]	
Variable	Std.	-Vit.E	+Vit.E
Superoxide	0.49	0.19	0.25
Hydrogen peroxide	0.98	1.37	1.07

[a]Values are nmol/min/mg protein at 5 min incubation. Microsomes were not chromatographed.

[b]Std. = standard laboratory diet, -Vit.E = diet deficient in vitamin E, +Vit.E = diet deficient in vitamin E but supplemented with 150 IU alpha-tocopherol acetate/kg diet.

Table 8. Identification and Quantification of Fatty Acids in Lung Microsomes Harvested from Rats Fed either a Standard Laboratory or a Vitamin E Deficient Diet with and without Vitamin E Supplementation.[a]

	Diet[b]		
Fatty acid	Std.	+Vit.E	-Vit.E
16:0	100 (35)[c]	100 (35.1)	100 (35.3)
16:1	6	3	3
18:0	40	33	35
18:1	45	46	44
18:2	33	29	24
20:4	35 (12.3)	45 (15.9)	49 (17.9)
22:6	9	11	8
24:0	--	16	16
24:1	--	3	3
% PUFA[d]	28.0	29.8	29.0

aValues are expressed as percentages of palmitic acid (16:0).

[b]Std. = standard laboratory rat diet, -Vit.E = diet deficient in vitamin E, +Vit.E = diet deficient in vitamin E but supplemented with 150 IU alpha-tocopherol acetate/kg diet.

[c]Numbers in parentheses are percentages of the total fatty acid content of the microsomes.

[d]Polyunsaturated fatty acid, 18:2 + 20:4 + 22:6 / total fatty acid content x 100.

vitamin E deficient diet contained only 4 detectable fatty acids while the standard laboratory diet contained a whole range of acids (Table 9). This was expected since the fatty acids in the vitamin E deficient diet originated from corn oil while those in the standard laboratory diet originated from its mixture of grains, yeast, animal liver and fish meal.

Since the polyunsaturated fatty acid content of the lung microsomes from rats fed the vitamin E deficient diet was similar to that from rats fed the standard diet, the vitamin E content was expected to explain the occurrence of peroxidation in lung microsomes from rat fed the vitamin E supplemented diet. However, as shown in Table 10, vitamin E content and hence, the polyunsaturated fatty acid to vitamin E ratios were similar between microsomes from rats fed the standard diet and the diet without vitamin E. Neither the vitamin E content nor the polyunsaturated fatty acid:vitamin E ratios could account for the differences in the rates of peroxidation observed in lung microsomes from rats fed the standard laboratory diet or those fed the alpha-tocopherol acetate supplemented vitamin E deficient diet. The only difference believed to be biologically significant was the higher concentration of arachidonic acid (20:4) in the microsomes (see Table 8) from the rats fed the vitamin E deficient diet.

Table 9. Identification and Quantification of Fatty Acids in the Standard Laboratory and the Vitamin E Deficient Diet.[a]

	Diet[b]	
Fatty acid	Std.	-Vit.E
14:0	1.2	--[c]
16:0	16.8	13.8
16:1	1.4	--
18:0	4.0	--
18:1	20.4	29.1
18:2	46.7	55.4
18:3	4.5	1.7
20:4	2.2	--
22:6	2.8	--

[a]Values are percentages of total fatty acid content.

[b]Std. = standard laboratory rat diet, -Vit.E = diet deficient in vitamin E.

[c]Below limit of detection.

Table 10. Vitamin E Concentrations and Polyunsaturated fatty acid to vitamin E Ratios in Lung and Liver Microsomes from Rats Fed the Standard Laboratory Diet or a Vitamin E Deficient Diet with and without Vitamin E Supplementation.[a]

	Lung		Liver	
Diet	Vit.E[b]	PUFA:E[c]	Vit.E	PUFA:E
Standard laboratory	1.13	191	0.14	1,929
Vitamin E deficient	0.04	4866	0.0042	32,265
Vitamin E supplemented	1.20	194	0.44	368

[a]Vitamin E supplemented as 150 IU alpha-tocopherol acetate/kg diet.

[b]Vitamin E, nmol/mg protein.

[c]Polyunsaturated fatty acid:vitamin E, nmol/nmol vitamin E/mg protein.

DISCUSSION

In this study lung microsomal components that are believed to relate or contribute to toxicoses associated with $O_2^{\cdot-}$ and H_2O_2 were quantified. The values for lung microsomal cytochrome P450 content and cytochrome P450 reductase activity (Table 4) agreed with previously published data. Both Matsubara *et al*. (1974) and Capdevila *et al*. (1975) measured cytochrome P450 levels in rat lung microsomes. They found 0.033 and 0.05 nmol/mg protein, respectively, while 0.027 nmol/mg protein was attained in this study. Buege and Aust (1975) reported that NADPH-cytochrome P450 reductase activity in rat lung microsomes was 0.067 nmol cytochrome c reduced/min/mg protein. In this report NADPH-cytochrome P450 reductase activity was found to be 0.064 nmol/min/mg protein in microsomes isolated by our standard procedure and 0.047 nmol/min/mg protein for processed (chromatographed) microsomes.

Lung microsomal isolation and characterization of enzymatic activities proved much more difficult than from liver. Among the difficulties were the greater amount of connective tissue, the lower organ weight, and the relatively small population of cells that contained

significant quantities of endoplasmic reticulum (reviewed in Burke and Orrenius, 1979). In addition, rat lung microsomes contained a large quantity of hemoglobin that made quantification of cytochrome P450 difficult.

The high hemoglobin content of lung microsomes isolated by the standard method also could have lowered the amounts of H_2O_2 produced because of the potential release of iron from hemoglobin by H_2O_2 in the reaction mixture (Kanner and Harel, 1984; Sadrzadeh et al., 1987). However, when the microsomes were further treated by chromatography or by in situ perfusion of lungs to reduce the hemoglobin content, the observed rates of H_2O_2 production were similar (Table 4). This provided indirect evidence that the low amount of H_2O_2 detected in the lung microsomal system used was real.

The production of both $O_2^{\cdot}$ and H_2O_2 were lower in rat lung microsomes than in the liver microsomes which were included as positive controls (Table 4). These values, however, were nearly identical to those reported by Turrens et al. (1982) for porcine lung microsomes. In porcine lung microsomes, increasing the atmospheric O_2 content to 100% partial pressure, increased H_2O_2 production less than 2 fold. Such a modest increase in H_2O_2 production made it difficult to attribute hyperoxic lung damage resulting from exposure to 100% oxygen to oxygen radical catalyzed lipid peroxidation. The rates H_2O_2 and $O_2^{\cdot}$ production (Table 4) and the rates of lipid peroxidation (Table 6) in lung

microsomes were similar, hence, again it was difficult to directly attribute lipid peroxidation (measured as MDA production) to H_2O_2 and $O_2^{\cdot-}$ production. In order to directly attribute MDA production to production of partially reduced oxygen species, rates of H_2O_2 and $O_2^{\cdot-}$ production would have to be much higher. May and McCay (1968) showed that MDA was a minor product of lipid peroxidation, representing only about 15% of the total loss of polyunsaturated fatty acids. Morehouse et al. (1984) indirectly investigated the requirement for $O_2^{\cdot-}$ for the occurrence of lipid peroxidation. They measured the $O_2^{\cdot-}$ production rate in an NADPH-dependent peroxidation system using rat liver microsomes and duplicated the rate with an $O_2^{\cdot-}$-dependent peroxidation system. They showed that $O_2^{\cdot-}$ was of little importance in NADPH-dependent lipid peroxidation as the rate of peroxidation in this system was much greater than in the $O_2^{\cdot-}$-dependent system. The results of these workers was interpreted as support for data presented here which indicated that the rates of $O_2^{\cdot-}$ and H_2O_2 production in lung microsomes were too low to contribute to lipid peroxidation.

If membrane damage is considered to be a function of the free iron concentration, then any process or molecule that makes iron more accessible should promote lipid peroxidation. Superoxide has been shown to release iron from ferritin (Thomas et al., 1985), and H_2O_2 can release iron from hemoglobin (Kanner and Harel, 1984; Sadrzadeh et

al., 1987). In both cases the iron was capable of participating in lipid peroxidation. Thus, the significance of $O_2^{\cdot-}$ and H_2O_2 production in lipid peroxidation may lie in the ability of these compounds to modulate the free iron pool. In the present study the effects of NADPH plus various additions on lipid peroxidation in lung microsomes harvested from rats fed the standard laboratory diet or one deficient in vitamin E with or without supplementation of alpha-tocopherol acetate was examined. No difference was found in the amount of lipid peroxidation with any of the additions to microsomes from rats fed the standard ration (Table 6) suggesting that regardless of the iron or $O_2^{\cdot-}$ and H_2O_2 present, lung microsomal membranes were able to withstand the challenge. In microsomes from rats fed the vitamin E deficient diet (without vitamin E supplementation), lung microsomal lipid peroxidation was increased by ADP-Fe^{+3} and EDTA-Fe^{+3} additions to NADPH (Table 6). Similar results were observed with liver microsomes indicating that peroxidation occurred by the same mechanism in both organs.

In Chapter I it was shown that the polyunsaturated fatty acid to vitamin E ratio influenced the occurrence of lipid peroxidation but not its rate. This result was directly opposite that of Kornburst and Mavis (1980) who found that vitamin E content (and therefore, the polyunsaturated fatty acid to vitamin E ratio) influenced the rate of peroxidation not its occurrence. In the

present study lung microsomes isolated from rats fed a vitamin E deficient diet with or without supplementation of alpha-tocopherol acetate peroxidized at rates inversely proportional to their vitamin E content (Tables 6 and 10). In other words, in spite of a polyunsaturated fatty acid to vitamin E ratio supposedly inhibitory to lipid peroxidation, lung microsomes from rats fed the vitamin E deficient diet with addition of vitamin E peroxidized. When examined further, a difference was found in the polyunsaturated fatty acid profile between the microsomes which peroxidized and those which did not. The difference was in their relative arachidonic acid (20:4) content (Table 8). Lung microsomes from rats fed the vitamin E deficient diet contained 16 to 18% arachidonic acid while those from rats fed the standard laboratory diet contained about 12% arachidonic acid. Jordan and Schenkman (1982) showed that the amount of arachidonic acid in liver microsomal membranes directly correlated with malondialdehyde formation during lipid peroxidation. Varying concentrations of other fatty acids did not give direct correlations. The higher arachidonic acid content in the lung microsomes from rats fed the vitamin E deficient diets may be the result of the relatively high amount of linoleic acid (18:2) in this diet compared to the standard laboratory diet (Table 9). Hammer and Wills (1978) showed that high linoleic acid in a corn oil based diet resulted in higher levels of arachidonic acid in liver

microsomal lipids than occurred when rats were fed a standard laboratory diet. The role of arachidonic acid in the occurrence of lipid peroxidation in lung microsomes needs further investigation.

REFERENCES

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

Buege, J.A., and S.D. Aust. Comparative studies of rat liver and lung NADPH-cytochrome c reductase. Biochem. Biophys. Acta 385:371-379. (1975).

Burke, M.D., and S. Orrenius. Isolation and comparison of endoplasmic reticulum membranes and their mixed function oxidase activities from mammalian extrahepatic tissues. Pharmac. Ther. 7:549-599. (1979).

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

Capdevila, J., S.W. Jakobsson, B. Jernstrom, O. Helia, and S. Orrenius. Characterization of a rat lung microsomal fraction obtained by Sepharose 2B ultrafiltration. Cancer Res. 35:2820-2829. (1975).

Fantone, J.C., and P.A. Ward. Oxygen-derived radicals and their metabolites: Relationship to tissue injury. Current Concepts Upjohn Co. Kalamazoo, MI 49001. (1985).

Farrington, J.A., M. Ebert, E.J. Land, and K. Fletcher. Bipyridylium quaternary salts and related compounds. V. Pulse radiolysis studies of the reaction of paraquat radical with oxygen. Implications for the mode of action of bipyridyl herbicides. Biochem. Biophys. Acta 314:372-381. (1973).

Freeman, B.A., and J.D. Crapo. Hyperoxia increases oxygen radical production in rat lungs and lung mitochondria. J. Biol. Chem. 256:10986-10992. (1981).

Freeman, B.A., and J.D. Crapo. Biology of disease: Free radicals and tissue injury. Lab. Invest. 47:412-426. (1982).

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

Gonder, J.C., R.A. Proctor, and J.A. Will. Genetic differences in oxygen toxicity are correlated with cytochrome P-450 inducibility. Proc. Natl. Acad. Sci. 82:6315-6319. (1985).

Halliwell, B., and J.M.C. Gutteridge. Free Radicals in Biology and Medicine. Clarendon Press. Oxford. (1985).

Hammer, C.T., and E.D. Wills. The role of lipid components of the diet in the regulation of the fatty acid composition of rat liver endoplasmic reticulum and lipid peroxidation. Biochem J. 174:585-593. (1978).

Hildebrandt, A.G., and I. Roots. Reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent formation and breakdown of hydrogen peroxide during mixed function oxidation reactions in liver microsomes. Arch. Biochem. Biophys. 171:385-397. (1975).

Jordan, R.A., and J.B. Schenkman. Relationship between malondialdehyde production and arachidonate consumption during NADPH-supported microsomal lipid peroxidation. Biochem. Pharmacol. 31:1393-1400. (1982).

Kanner, J., and S. Harel. Initiation of membranal lipid peroxidation by activated metmyoglobin and methemoglobin. Arch. Biochem. Biophys. 237:314-321. (1984).

Kehrer, J.P., and S. Kacew. Systematically applied chemicals that damage lung tissue. Toxicology 35:251-293. (1985).

Kornburst, D.J., and R.D. Mavis. Relative susceptibility of microsomes from lung, heart, liver, kidney, brain, and testis to lipid peroxidation: Correlation with vitamin E content. Lipids 15:315-322. (1980).

Matsubara, T., R.A. Prough, M.D. Burke, and R.W. Estabrook. The preparation of microsomal fractions of rodent respiratory tract and their characterization. Cancer Res. 34:2196-2203. (1974).

May, H.E., and P.B. McCay. Reduced triphosphopyridine nucleotide oxidase-catalyzed alterations of membrane phospholipids. J. Biol. Chem. 243:2288-2295. (1968).

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

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

Pederson, T.C., J.A. Buege, and S.D. Aust. Microsomal electron transport, the role of reduced nicotinamide adenine dinucleotide phosphate-cytochrome c reductase in liver microsomal lipid peroxidation. J. Biol. Chem. 226:497-509. (1973).

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

Sadrzadeh, S.M.H., D.K. Anderson, S.S. Panter, P.E. Hallaway, and J.W. Eaton. Hemoglobin potentiates central nervous system damage. J. Clin. Invest. (1987). in press.

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

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

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

Turrens, J.F., B.A. Freeman, and J.D. Crapo. Hyperoxia increases H_2O_2 release by lung mitochondria and microsomes. Arch. Biochem. Biophys. 217:411-421. (1982).

SUMMARY

In vitro lipid peroxidation requires a suitable lipid substrate as well as an electron transport system capable of catalyzing peroxidation. In this dissertation, it was shown that rat lung microsomes were resistance to lipid peroxidation. Data were discussed indicating that resistance to peroxidation could be attributed to high lung microsomal vitamin E and low polyunsaturated fatty acid content. It was further shown using lung microsomes from rats fed a vitamin E deficient diet that the electron transport system, composed of NADPH, NADPH-cytochrome P450 reductase, cytochrome P450, and ADP-Fe^{+3}, was capable of catalyzing lung microsomal peroxidation. By adding vitamin E to liposomes with different polyunsaturated fatty acid contents, it was demonstrated that inhibition of peroxidation could be predicted by the polyunsaturated fatty acid to vitamin E ratio.

Vitamin E-dependent inhibition of lipid peroxidation in liver microsomes required a 7-fold increase in vitamin E concentration over that found in microsomes. Less vitamin E was required to inhibit microsomal lipid peroxidation in the presence of glutathione due to maintence of vitamin E

concentrations. Inhibition of peroxidation in the presence of glutathione, however, did not exceed 90% until the vitamin E concentration was sufficient to inhibit peroxidation regardless of glutathione. In addition to maintaining vitamin E concentrations, glutathione was found to interact with ADP chelated iron to maintain low lipid hydroperoxide concentrations in the peroxidation reaction mixtures.

The influence of superoxide and hydrogen peroxide production on lipid peroxidation was evaluated using lung microsomes from rats fed a vitamin E deficient diet which readily peroxidized. Both superoxide and hydrogen peroxide production rates were very low and did not differ regardless of the peroxidizability of the lung microsomes. Hence, these partially reduced oxygen species were not important in lipid peroxidation of rat lung microsomes.

VITA

Robert Arthur Leedle was born in Walworth county, Wisconsin on May 6, 1952. He attended grade school in Genoa City and high school in Lake Geneva, WI. Following graduation from high school in 1970, he attended the University of Wisconsin-Madison for 3 years, then transferred to the University of Illinois at Urbana-Champaign. From this university he received a Bachelor of Science degree in Microbiology (1975), a Master of Science degree in Dairy Science-Microbiology (1977), and a Doctor of Veterinary Medicine degree (1982). In January of 1983, Bob pursued his research interests at Michigan State University as an NIH postdoctoral fellow through the Department of Pathology and the Center for Environmental Toxicology. He is married.